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RNA Interference Based Therapy and Its Method of Delivery

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ABSTRACT

RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing that involves the generation of small interfering RNA (siRNA), micro RNA (miRNA), short hairpin RNA (shRNA) molecules from double stranded RNA (dsRNA) that are capable of binding to host mRNA molecules and inhibiting their translation and enhancing the degradation of the mRNA, so that leads to inhibition of gene expression of defective gene by suppression of the protein synthesis. This robust silencing effect of RNAi makes it a valuable research tool both in cell culture and in living organisms, in various diseases like cancer, metabolic syndrome, viral disease (HIV-1, Hepatitis B), neural and neuromuscular diseases (Muscular dystrophy, Spinal muscular atrophy, Alzheimer disease), Poly(Q), Parkinson, Asthma and Diabetes. Although introducing siRNA into cells in vivo remains a significant obstacle, as it is imperative for siRNA to reach the cytoplasm of the targeted cells because naked RNAs cannot penetrate cellular lipid membranes by themselves to become effective and induce silencing. So to overcome this challenges of delivery viral vectors like adenovirus and lentivirus and nonviral vectors like naked RNA, lipid based delivery vectors, cell penetrating peptides, polymer, inorganic molecules, chemical conjugates.

Key words: dsRNA, siRNA, miRNA, shRNA

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INTRODUCTION

RNA interference (RNAi), discovered by Mello and Fire in the early 1990s[1]. RNAi is a posttranscriptional mechanism of gene silencing highly conserved in eukaryotes. Today various methods are available for the RNA interference like small interfering RNA (siRNA), micro RNA (miRNA), small hairpin RNA (shRNA). The delivery of these various exogenous RNA by the viral as well as nonviral vectors. RNAi controls gene expression by interaction of complementary sequences between a target mRNA and a single stranded molecule of RNA: siRNA or miRNA. The siRNA binds the target mRNA with a perfect complementarily. The interaction between the small RNA and the target results in gene silencing when the siRNA or the miRNA is bound to the RNA-induced silencing complex RISC. Thus, RISC is guided to the target mRNA by the small RNA. Binding to the target with perfect complementarily induces RISC-mediated cleavage of the target RNA[2]. Originally demonstrated in the nematode worm Caenorhabditis elegans using long double-stranded RNA [1]. RNAi can be used to modulate various aspects of the function a gene, including its overall expression, the generation of splice variants and the timing of its expression. Although RNAi is unlikely to entirely replace knockout (KO) technology, it should serve as a complementary approach and significantly widen the repertoire of genetic perturbations available to model and understand disease gene variants[3]. Short hairpin RNA (shRNA) has a higher gene silencing potency than siRNA although synthetic siRNA have been widely used in vitro to effect gene silencing; the use of RNAi in vivo was greatly facilitated by technical developments that allow the use of DNA-encoded shRNA [4].

The development of genome-wide genetic analyses in mammalian cells was aided by advances in the application of RNA interference (RNAi) to suppress expression of cellular genes[5]. In mammalian cells, silencing of cellular genes can be achieved by transfection of synthesized small-interfering RNA molecules (siRNAs) or plasmid-based short hairpin RNAs (shRNAs) that are subsequently processed to siRNAs inside the cell[6]. The duration of the silencing effect correlates with the doubling time of a cell line since cell division dilutes the siRNA concentration present in the cells over time[7]. RNAi mediated by small interfering RNAs (siRNAs) is a powerful technology allowing the silencing of genes with great specificity and potency. It is a highly conserved natural mechanism described for plants, fungi, worms, insects, and mammals[8]. RNAi provides a common mechanism by which endogenous or exogenously encoded RNAs target mRNA transcripts for degradation or attenuated translation and thereby modulate gene expression. The requisite cell machinery, conserved throughout eukaryotic cells

including hepatocytes [9]. This robust silencing effect of RNAi makes it a valuable research tool both in cell culture and in living organisms, although introducing siRNA into cells in vivo remains a significant obstacle. RNAi shows many advantages over other therapeutic methods as very specific, without the toxic effects often observed during chemotherapy and the sequence-independent toxic effects of antisense therapy, and siRNA are more resistant to nuclease degradation than antisense oligonucleotides and therefore, exhibiting longer therapeutic effects than antisense therapy[10]. RNAi can be achieved artificially by three major ways: (i) introducing long double stranded RNA (dsRNA) which is cleaved into small interfering RNA (siRNA) by the enzyme Dicer in the cytoplasm, leading to the degradation of target mRNA; (ii) introducing plasmid DNA encodes for short hairpin RNA (shRNA) which is processed by Dicer into siRNA; and (iii) introducing small interfering RNA (siRNA) directly to initiate the mRNA degradation process as shown in figure 1. Amongst the types of nucleic acids that are involved in the RNAi, siRNA is the most popular candidate being studied in RNAi therapy. Introduction of long dsRNA (typically consist of 500 to 1000 base pairs) is known to induce interferon (IFN) response in mammalian cells rendering it unsuitable for RNAi therapy, whereas siRNA (typically consist of 21–23 base pairs) can avoid the INF response. From the delivery perspective, siRNA has advantage over the plasmid DNA encoding shRNA[11].

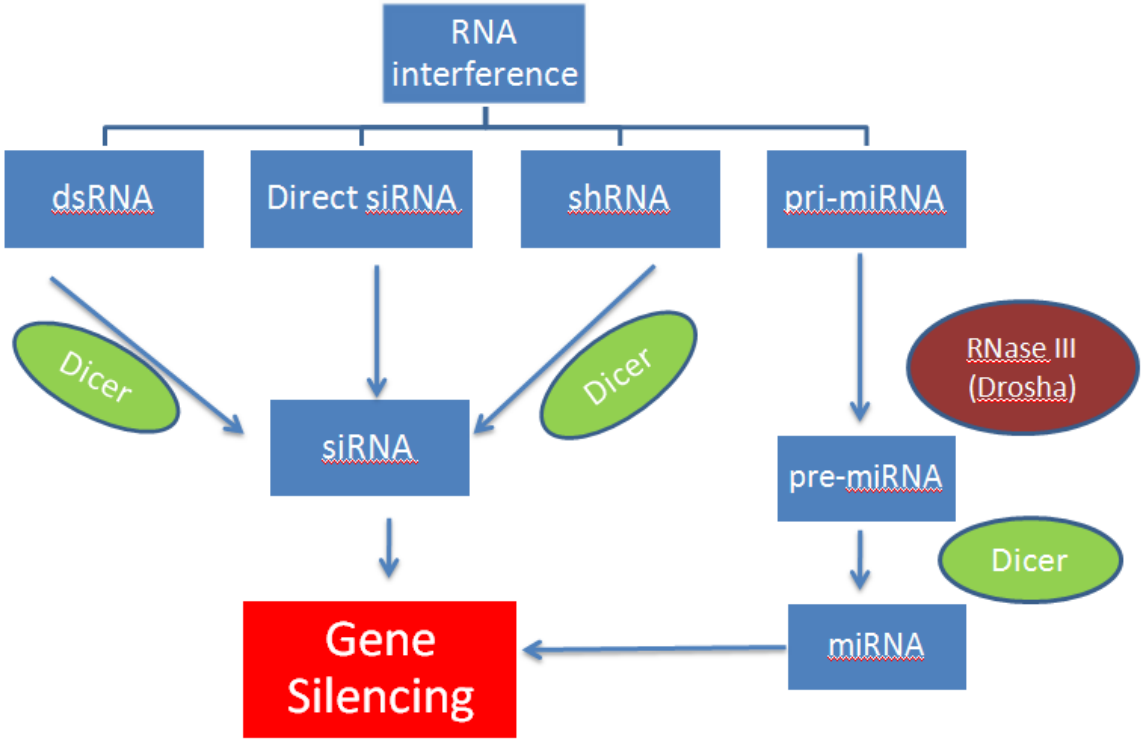

Figure 1: Gene Silencing by different types of RNA

Types of RNAi methods

1. Chemically synthesized siRNA: siRNA of 19–23 bp can be synthesized chemically in vitro. Advances in solid phase synthesis technique make it possible to produce siRNA with high purity and precise-controlled sequence. Both sense and antisense strands are synthesized separately and annealed to form double stranded siRNA duplex.
2. Vector based shRNA: shRNA is an RNA sequence that makes a tight hairpin turn that can be used to silence gene expression via RNAi. Vector based shRNA utilizes endogenous cellular machinery to function. After entering the cell nucleus, vector based shRNA with stem loop structure is expressed to produce pri-shRNA in the nuclei, which is then processed into pre-shRNA by an enzyme named Drosha. Pre-shRNA is exported to the cytoplasm by exportin-5, where it is further processed by Dicer (an RNAse III enzyme) to produce functional siRNA[12].

The mechanism of gene silencing by RNAi:

The term RNAi was first coined to describe the sequence-specific, post-transcriptional inhibition of gene expression induced by the introduction of a cognate double-stranded RNA (dsRNA) molecule into the nematode worm Caenorhabditis elegans. RNAi, triggered by dsRNA, was subsequently identified in a broad range of invertebrate species including Drosophila. This effect, known as Post Transcriptional Gene silencing (PTGS) is also triggered by cytoplasmically replicating RNA viruses in plants. Studies, predominately in plants, fungi, C. elegans, Drosophila and most recently in mammalian cells have now shown that dsRNA is a key trigger of gene silencing processes in these, and probably all species, and that these mechanisms involve the formation of a ribonucleic protein complex termed the RNA-induced silencing complex (RISC)[13]. Depending on the organism, RNAi is triggered by various types of molecule, including long dsRNAs, plasmid-based short hairpin RNAs (shRNAs) or endogenous hairpin micro RNAs (miRNAs). These are processed by the ribonuclease-III activity of the evolutionarily conserved Dicer enzyme to generate 21–22-nt siRNAs[14]. These siRNAs are then incorporated into a protein complex, known as the RNA-induced silencing complex (RISC), which in turn uses an ATP-dependent RNA-helicase activity to unwind the duplex siRNA into single-stranded siRNA[15]. The antisense strand of the duplex siRNA guides the RISC to the homologous mRNA, where the RISC-associated endoribonuclease cleaves the target mRNA at a position ~10 nucleotides from 5` end of the antisense siRNA sequence[16], which results in the silencing of the target gene as shown in figure 2 [17]. Gene knockdown by RNAi is post-transcriptional and so does not invoke any compensatory transcription, as is the case for gene deletion. RNAi is specific and, in most

cases, sensitive to even a single nucleotide mismatch. The short hairpin RNA (shRNA) encoding transgenes can be placed under the control of an inducible promoter, such as a tissue specific promoter. RNAi is also dose dependent, so the degree of knockdown can be controlled, although this is limited in C. elegans where RNAi amplification through RNA-dependent RNA polymerase activity is evoked[18].

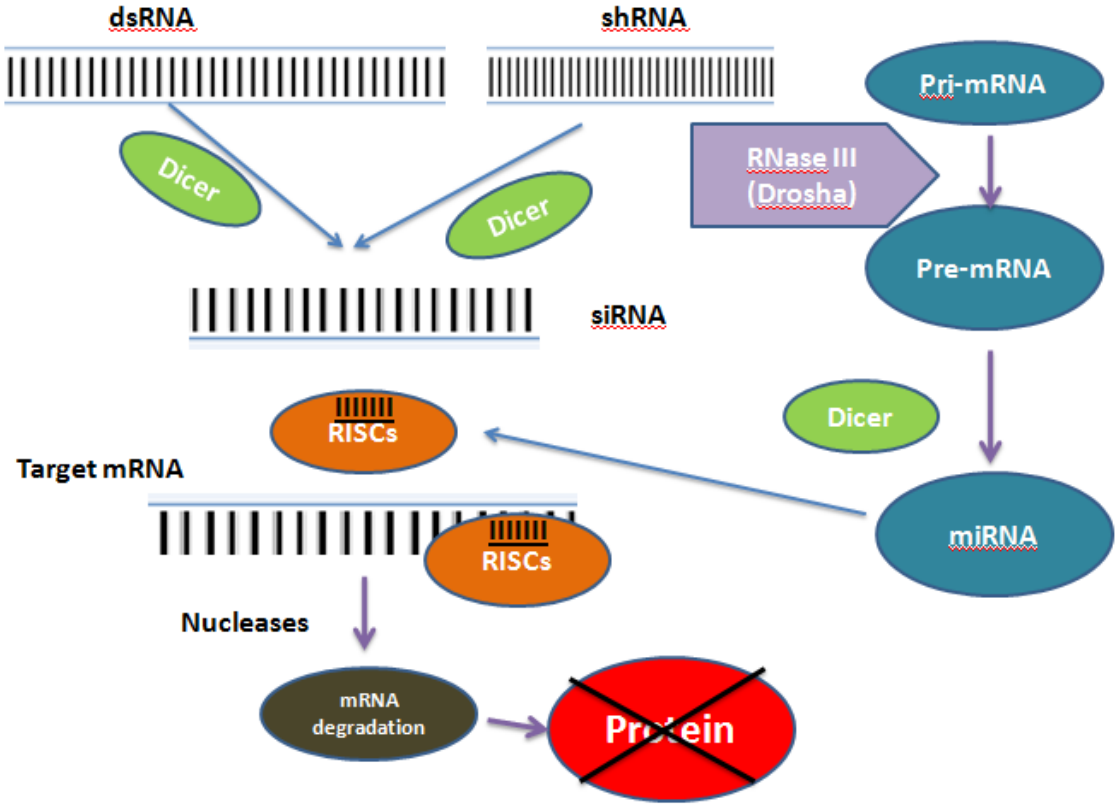

Figure 2: Mechanism of Gene Silencing by RNAi

Application of RNAi in Mammals and In Treatment of Disease

In 2004, the first human clinical trial of RNAi therapy was initiated for the treatment of age-related macular degeneration (AMD) with siRNA targeting VEGF-receptor 1 delivered intravitreally[19]. It can be also used as tool for target validation. One of the most critical applications for RNAi in mammalian cells is used as a tool for validating the targets of small drug molecules and identification of new drug targets. Small molecule drugs or the trigger of RNAi against that target can then be developed or a molecule that triggers RNAi could also be exploited as a therapeutic agent[20]. Although RNAi has the potential to be an important and widely used therapeutic tool in the future, it is currently a widely used as experimental tool for targeted knockdown of gene expression[21].

RNAi therapeutics:

RNAi technology is being evaluated as a potentially useful method to cure diseases including cancer, infection, respiratory disease, neuronal disease, and autoimmune disease[22] . RNAi can be potentially used to treat or prevent diseases affecting the airways, such as lung cancer, various types of respiratory infectious diseases, airway inflammatory diseases and cystic fibrosis. Silencing CD40 by RNAi prior to or after immunization with collagen II was shown to inhibit collagen-induced arthritis (CIA). Similarly, siRNAs to a different target were administered within lipoplexes to target delivery to phagocytic cells following intravenous injection and diminished the severity of CIA[23]. In this study, twice-weekly administration of siRNAs against Cathepsin L prevented cyclophosphamide induced autoimmune diabetes. Another group achieved disease amelioration in the experimental autoimmune encephalomyelitis model of multiple sclerosis by intravenous administration of unconjugated siRNAs[24]. Gene suppression by siRNA transfection in HeLa cells has been shown to reach its strongest effect after approximately 42 h before slowly tapering off after 66 h[25]. Huang et al's results suggest that lipidoid-formulated CLDN3 siRNA has the potential as a therapeutic for ovarian cancer, in which they have proved the efficacy of lipidoid-formulated CLDN3 siRNA in three different ovarian cancer models, by intratumoral injection to result in dramatic silencing of CLDN3, significant reduction in cell proliferation, reduction in tumor growth, and a significant increase in the number of apoptotic cells, and by intraperitoneal injection to result in a substantial reduction in tumor burden in MISIIR/Tag transgenic mice and mice bearing tumors derived from mouse ovarian surface epithelial cells[26]. Love et al., which enables siRNA-directed liver gene silencing in mice at doses below 0.01 mg/kg and inhibits the expression of five hepatic genes simultaneously, after a single injection. The potential of this formulation was further validated in nonhuman primates, where high levels of knockdown of the clinically relevant gene transthyretin were observed at doses as low as 0.03 mg/kg[27].

RNAi therapy for Cancer:

siRNA-mediate knockdown of PRAME (preferentially expressed antigen of melanoma) in a human Chronic Myeloid leukemia (CML) cell line K562 results in cell cycle arrest and subsequent leukemic cell apoptosis[28].

RNAi-based strategies for metabolic syndrome treatment:

The technical application of RNA interference offers great potential for the specific treatment of a huge variety of diseases including the metabolic syndrome, one of the most challenging threats to human health associated with our civilization. In order to develop novel and powerful

strategies for the treatment of the metabolic syndrome, it is essential to define a set of specific gene products that may be targeted by RNA interference. Based on currently available in vitro and in vivo data, it is easy discuss the feasibility of candidate genes involved in the pathophysiology of the metabolic syndrome as potential targets for a rational RNA interference[29].

RNAi based strategies for anti viral therapy:

Recently gene transfer approaches have been designed to directly target viral replication. One such approach is based on the use of interference RNA (RNAi), in which genes are silenced in a sequence dependent manner. RNAi has already been investigated in models of hepatitis viral infections with great success. It was demonstrated that siRNAs can alter the course of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infection by mediating viral RNA degradation, resulting in inhibition of viral RNA translation and replication. Treatment with plasmid or virus-encoded shRNAs for specific targeting of HBV sequences reduced the levels of serum HBV surface antigen (HBsAg) and HBV core antigen (HBcAg), and decreased viral genomic DNA and viral RNA transcription in the liver of mice. The 5' untranslated region of the HCV viral genome, as well as the NS3 and NS5B genes, have been demonstrated to be effective targets for synthetic siRNAs or vectors expressing shRNAs. However, a common problem in these studies is viral escape that derives from the high mutation rate of the viral genome[30]. Considering the potential of RNAi as antiviral agent, few groups have evaluated RNAi based antiviral agents against rabies[31,32]. The sequence-specific knockdown of viral genes in infected cells generated great interest in development of siRNAs-based antiviral agents. siRNA-based antiviral activity has been reported for many RNA and DNA viruses *in-vitro* and *in-vivo* including, human immunodeficiency virus, poliovirus, hepatitis B virus, hepatitis C virus, human papilloma virus, dengue virus, hepatitis delta virus, murine gamma herpes viruses, severe acute corona virus and respiratory syncytial virus infections[33]. In plants, it has been recognized that post-transcriptional gene silencing and RNAi play critical roles in genome surveillance, protecting the cell from inappropriate expression of repetitive sequences, transposable elements and viruses[34]. In fact, certain plant viruses, including potyviruses, potato virus X and cucumber mosaic virus, have evolved proteins that antagonize the RNAi pathway, providing some of the strongest evidence that RNAi can serve as an innate cellular antiviral mechanism. To date, RNAi has been used effectively to inhibit the replication of several different pathogenic viruses in culture, including: RSV (respiratory syncytial virus) [35], poliovirus, Hepatitis B virus[36], and HIV-1[37].

RNAi based therapy for neural and neuromuscular diseases

1. Muscular dystrophy: RNAi knockdown of genes in Drosophila and C. elegans to mimic loss of function mutations is helping to elucidate the mechanisms of a number of muscle wasting diseases. These are degenerative disorders marked by progressive paralysis of body muscle ending in premature death. [38].
2. Spinal muscular atrophy RNAi suppression of SMN in Drosophila S2 cells resulted in a significant increase in apoptosis [39].
3. Alzheimer's disease Although in vivo delivery of RNA interference remains a significant obstacle, new data show that RNAi blocks gene function in vivo, suggesting a potential therapeutic approach for humans. Some groups have demonstrated the efficacy of RNAi therapy in Alzheimer's disease[40].

Poly (Q) diseases:

There are at least nine human neurodegenerative disorders that are caused by expansion of the CAG trinucleotide repeat RNAi has recently been performed on two prevalent poly(Q) diseases, Huntington's disease (HD) and spinobulbar muscular atrophy (SBMA). Nollen et al prepared transgenic strains that expressed poly(Q35) expansion fused with yellow fluorescent protein and used it in a global RNAi screen. This screen identified 186 genes that resulted in premature appearance of protein aggregates. The genes identified are mainly involved in RNA metabolism, protein synthesis, protein folding, protein degradation and protein trafficking[41].

RNAi based therapy for Parkinson's disease:

Viral-mediated RNAi has been used to block dopamine synthesis in mid-brain neurons of adult mice. This study used an adeno-associated virus vector in which a U6 promoter drove the expression of shRNA directed against tyrosine hydroxylase, an enzyme required for the production of dopamine. This was injected stereotactically into the substantia nigra of one side of the brain and a similar vector promoting the expression of a randomized shRNA injected into the other side. GFP (green fluorescent protein) expression was observed in both halves of the brain, but dopamine staining was reduced only in the side of the brain into which the anti-tyrosine hydroxylase shRNA had been injected. The resultant behavioural deficits included loss of motor performance bilateral shRNA knockdown of dopamine synthesis resulted in reduced motor activity in response to amphetamine (a well-established dopamine dependent behaviour) and poorer performance in the rotarod test. Although only 30–40% protein knockdown was reported, these results are similar to previously established toxin induced models of dopaminergic neuron

loss, suggesting that RNAi-induced knockdown of dopamine synthesis furnishes a reasonably faithful model of Parkinson's disease[42].

RNAi therapy in Asthma:

Delivering siRNA to the respiratory system has also improved the therapeutic feasibility of RNAi for asthma[43]. The siRNA is targeting key cytokines that play a critical role in inflammation and bronchconstriction in the airways. Knocking out these cytokines by systemic or local (aerosol) delivery to the respiratory tract would be of obvious benefit in treating the disease. The sirna is currently developing siRNAs formulated to provide a long duration of activity after local administration to the lungs. The siRNA has collaborated with the National Jewish Medical and Research Center to test chemically modified, systemically delivered siRNAs in preclinical animal studies. The results showed a statistically significant reduction (66%) in airway hyperesponsiveness for the RNAi treatment group compared to the control group. Following additional preclinical studies, the company anticipates initiating human trials in 2006.

RNAi therapy in Diabetes:

Diabetes is a disease that prevents proper utilization of sugars due to the inability of the body to produce or properly use insulin. It is the sixth leading cause of death in the United States, with over 200,000 deaths due to complications from the disease. There are over 18.2 million affected individuals in the U.S. (6.3% of the population). The siRNA therapeutics to address other liver-associated disease indications such as diabetes. This group also claimed that they had promising results for the application of RNAi technology in the treatment of diabetes using systemically delivered siRNA to reduce phosphatase 1B (PTP-1B) in liver, a validated target in diabetes associated with insulin resistance[44]. The siRNA has seen promising results for the application of RNA interference (RNAi) technology in the treatment of diabetes. Preclinical studies of siRNA's systemically delivered short interfering RNAs (siRNAs) showed a 72% reduction of PTP-1B (phosphatase 1B), a validated target in diabetes that is associated with insulin resistance.

miRNAs:

The miRNA precursors are transcribed by RNA polymerase II as long primary transcripts named pri-miRNAs. Pri-miRNAs can contain one or more immature miRNAs. The pri-miRNA is processed by a nuclear RNase III called Drosha generating imperfectly pairing stem–loop molecules of approximately 70 nucleotides known as pre-miRNAs. Pre-miRNAs are transferred to the cytosol by exportin 5 (Exp5) where another RNase III, Dicer, generates a mature double-stranded miRNA of approximately 22 nucleotides. Following Dicer cleavage, the resulting RNA duplex is loaded into RISC. Argonaute (Ago), one of the proteins of the complex, separates the

two strands of the double-stranded RNA. The guide strand remains in RISC as a mature miRNA, whereas the other strand (the passenger strand) is degraded[9, 45-47]. miRNAs are naturally occurring small non-coding RNAs of 20 to 25 nucleotides in length, cleaved from 70 to 100 nucleotide long hairpin pre-miRNA precursors and distinct from other small RNAs such as interfering RNAs[9, 48, 49]. MicroRNAs are relative newcomers to the rapidly evolving fields of pathobiology of cancer and tailored therapy for specific cancers[49-51]. Various miRNAs have been demonstrated to play specific roles in cancer cell differentiation, survival, tumor progression and metastasis[52-54]. Altered miRNA expressions have been observed in Hepatocellular carcinoma (HCC). The different expression profiles of miRNAs in HCC suggest that miRNAs may serve as either novel potential targets acting directly as oncogenes or therapeutic molecules working as tumor suppressor genes. There is a reduced abundance of miR-26 in human HCC compared with paired noncancerous tissues. It has been shown that an AAV vector encoding this miRNA caused selective apoptosis of cancer cells and inhibited the progression of tumors in a c-myc transgenic model without toxicity[55]. Recent findings indicate that restoration of other miRNAs such as miR-122, miR-195 and miR-101 (which are downregulated in a significant proportion of human HCCs) could have therapeutic effect against HCC as they control the expression of genes implicated in oncogenic pathways[56]. miRNAs are noncoding endogenously transcribed RNAs that undergo a well-characterized series of processing steps that generate short single stranded (20–22) RNA fragments that bind to complementary regions within a range of targets and in turn lead to mRNA degradation or attenuated translation as a result of trafficking to processing bodies. miRNAs has the role in liver disease including nonalcoholic fatty liver disease, viral hepatitis, and hepatocellular carcinoma and also used as biomarkers of liver injury and neoplasia[57, 58]. Within the liver, the physiological importance of miRNAs has been demonstrated in metabolism[59], immunity[60], viral hepatitis, and oncogenesis. In addition, findings illustrate the importance of RNAi as an experimental tool for gene silencing[57]. Cell differentiation is regulated, in part by a miRNAs. These regulatory molecules can directly affect differentiation in both normal stem cells and cancer stem cells[61-63]. The miRNAs act as regulatory molecules in eukaryotic cells by binding to a noncoding region within target messenger RNAs (mRNAs),namely the 30-untranslated region (30 -UTR). Through this mechanism miRNAs regulate self-renewal, differentiation, and division of cells via post-transcriptional gene silencing[64]. The miRNAs play important roles in many cellular processes such as development, stem cell division, apoptosis, disease, and cancer.

siRNA: Commonly, RNAi studies carried out in mammalian cells achieve gene depletion using chemically synthesized small-interfering RNAs (siRNAs). These siRNAs, which are double-stranded RNA molecules that are commonly about 21 base pairs in length, are introduced into cells by transfection[5].

The Challenge of Delivery

Among these, oligonucleotide therapies using siRNAs, shRNAs, RNA aptamers, and ribozymes have attracted particular attention because of their potential to allow specific targeted delivery of antitumor drugs without significant toxicity or other systemic side effects[65-67]. Usually, RNAi delivery systems are composed of non-viral as well as viral systems[68, 69]. Viral systems are showed higher gene transfer efficiency, however, it has defective efficiency to target specific cells. Also, after transfer to the target cells, their remained viral elements can be regulate immunogenic, cytopathic and recombinogenic effects[69-71]. Non-viral systems for successful delivery of siRNA both in vitro and in vivo including in nonhuman primates and humans. The delivery of siRNA by using lipid-based systems, chitosans, polymeric micelles, siRNA conjugates and peptide delivery systems. Non-viral systems are constructed with polymers, liposomes, peptides, and polysaccharides for increased transfection efficiency. However, applications of non-viral systems have a still unsolved problems like as; 1) low packaging and gene transfer efficiency, 2) low colloidal stability, 3) target cell internalization, 4) endosomal escape(71). Another approach under study is the generation of nanoparticles composed of cationic lipids or biocompatible polymers[68, 72] siRNAs were also delivered hydrodynamically, that is by intravenous injection of a large volume to enforce delivery to various organs[73]. siRNAs were complexed with atelocollagen to permit robust delivery in Non-obese diabetic (NOD) mice[74]. Some non-viral vectors have great potential for combating the delivery obstacle. As a novel class of lipid like materials lipidoids have the advantages of easy synthesis and large library of compounds. Cell penetrating peptides and chitosans have been used for the delivery of bioactive molecules for many years, but they are showing great promise for the delivery of siRNA. The hybrids of inorganic particles and the conjugates of siRNA have indicated the complex utilization different materials may provide another solution to the delivery problem. The most exciting thing is some clinical trials are undergoing, which provokes the hope of real curing method by using RNAi mediated by some non-viral vectors. However, the delivery of siRNA remains to be the biggest challenge, because it is imperative for siRNA to reach the cytoplasm of the targeted cells to become effective and induce silencing. As naked RNAs cannot penetrate cellular lipid membranes by themselves[75]. Therefore, siRNA must be enclosed in

carriers such as viral vectors and non-viral vectors to be transported to the targeted cells in vitro or in vivo. The successful application of siRNA, is largely dependent on the development of a delivery vehicle which should be administered efficiently, safely, and repeatedly. Viral systems usually give high transfection efficiencies, safety concerns from potential mutation, recombination, oncogenic effect, and high cost, however, greatly limit their therapeutic applications. In contrast, non-viral vectors are believed to cause less safety problems due to their relative simplicity, though nonspecific cytotoxicity associated with cationic liposomes has been observed[76-78].

Viral vectors:

Viral vectors utilize the virus infection mechanism for efficient gene transfer into host cells.

1. Adenovirus: Bain et al. reported the feasibility of using Adv vector for gene silencing in islets and β cell lines[79] Adenovirus delivery systems have several features that make them attractive for use in gene therapy, including the ability to rapidly infect a broad range of human cells, produce high levels of gene transfer and relative ease of manipulating them using recombinant DNA techniques[80]. However, reports of some side effects of using adenovirus in humans, new approaches are being developed to switch to non-viral delivery systems[68].
2. Lentivirus: It is a type of retroviral vector, which reverse transcribes into DNA, be actively transported into the nucleus, and integrated into the genome of target cells. Therefore, stable gene expression or silencing could be achieved by using lentiviral vector[81]. shRNA constructs can be delivered by lentiviral vectors that mediate integration of the construct into the cellular genome to ensure long-term gene suppression[82].

Nonviral vectors used for the RNAi

1. Naked siRNA: This includes the delivery of both un modified siRNA and modified siRNA, formulated in saline or other simple excipients such as 5% dextrose. Since unmodified siRNA is susceptible to nuclease degradation, chemically modified siRNA was introduced initially to address this issue by increasing the nuclease resistance. The siRNA can also be chemically modified to improve potency, increase specificity, reduce immune response and reduce off-target effects.
2. Lipid-based delivery vectors:
   a) Cationic lipoplexes and liposomes: Lipoplexes are easy to prepare and generally have good transfection deficiency due to their deficient interaction with the negatively charged

cell membranes. However they also present some disadvantages such as poor stability and poor reproducibility[83].

b) PEGylated lipids: Griesenbach et al. assessed the in vivo efficiency of GL-67 in delivering siRNA into the lung of mice via the intranasal route [84].

c) Neutral lipids: Moschos et al. applied this strategy to pulmonary delivery of siRNA. The cholesterol-siRNA conjugates were delivered to the mice by intratracheal administration [85].

d) Lipids particles: An alternative strategy to improve the safety profile of lipid-based system is to encapsulate the siRNA inside the neutral lipid particles

e) Lipid-like molecules: Lipids have long been used for the delivery of genes and siRNAs, since the first cationic lipid (N-[1-(2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride) (DOTMA) was introduced[86]. Recently, a new approach to the lipid-like materials for siRNA delivery vectors using combinatorial methods was reported[87]

3. Cell-penetrating peptides (CPP): CPPs have been shown to be very promising to deliver siRNA, as they could significantly improve cellular uptake of various therapeutic molecules both in cultured cells and in vivo[88, 89]. The peptides are either covalently attached to siRNA through disulphide bond formation, or bind to siRNA through electrostatic interaction to form complexes in a non-covalent manner. It has been suggested that covalent attachment of CPP to siRNA may have a negative effect on cellular delivery as the biological activity of the peptides may have been altered during chemical modification[90].
4. Polymer-based delivery vectors:
   a) Chitosans: Numerous studies on DNA and siRNA delivery with chitosan as a carrier biomaterial have shown effective expression and silence reporter genes in vitro and in vivo [91, 92].
   b) Polyethylenimine(PEI): Merkel et al. investigated the use of PEI and PEI–PEG polyplexes for siRNA delivery to the lung[93].
   c) PLGA poly(D,L-lactide-co-glycolide): The production siRNA containing PLGA nanoparticles is done by spray-drying with controlled size distribution that are intended for inhalation[94].
5. The hybrids based on inorganic particles: Numerous nanosized inorganic particles have been development of siRNA conjugates with both of therapeutic and used as diagnostic tool.
   a) Gold nano-particles: Giljohann et al. have developed the first polyvalent RNA–gold nanoparticle conjugates through the connection of ethylene glycol spacer and an

alkylthiol group, which can be used to effectively regulate genes in the context of RNA interference [95]. Lee et al. developed a novel nanoparticulate delivery system based on gold nanoparticles modified with PEG, small interfering RNA conjugated to the nanoparticles via biodegradable disulfide linkages and poly(b-amino ester)s coating on the surface of the nanoparticles. The delivery system facilitated high levels of in vitro siRNA delivery [96].

b) Magnetic nanoparticles: The mRNA level of target genes (green fluorescence protein and human survivin) was suppressed to below 15% by systemic injection of the magnetic nanoparticle–siRNA conjugate, compared to saline treated controls.

c) Quantum dots (QDs) and UCN: Positively charged polyethylenimine (PEI) was covalently conjugated on the surface of QDs to complex with cyanine dye labeled vascular endothelial growth factor siRNA (cy5-VEGF siRNA), and Fluorescence resonance energy transfer(FRET) was achieved between cy5-VEGF siRNA and PEI conjugated QDs (QD625) to demonstrate that PEI conjugated QDs can be utilized as a useful siRNA carrier to analyze intracellular trafficking and unpacking pathway as well as to effectively silence a target gene [97].

6. Chemical conjugates of siRNA: Chemical modification of siRNA by using small drug molecules, aptamers, lipids, peptides, proteins, or polymers could increase its intracellular delivery and avoid its degradation by RNA nucleases [98].

a) Disulfide bond linked: In a study the double-stranded GFP-siRNA with a phosphothioethanol (PE) portion was reversibly modified via the reducible disulfide bond and incorporated the resulting siR-NA-S-S-PE conjugate in nanosized PEG-PE micelles which showed good stability against nucleases and release easily from these nanoparticles [99].

b) Lipophilic modification: The lipophilic conjugates of siRNA have been believed to induce intracellular RNAi without the significant loss of gene silencing activity.

Notable problems with the use of RNAi in vivo:-

1. Gene silencing by RNAi can be highly specific, but requires adequate controls. The use of at least two independent shRNA sequences is required to confirm that the observed phenotype is attributable to silencing of the target gene and not caused by a so-called off-target effect[100].
2. Depending on the integration site of the transgene, expression can display considerable variation within a single cell lineage or between cell types. This problem is circumvented by targeted transgenesis of ES cells[101].

3. It may sometime incomplete gene silencing [3].
4. It is not always straightforward to select a suitable targeting sequence [102].

Off-target effects:

The innate immune systems could be activated by several elements including dsRNA, plasmid or viral vectors, cationic delivery carriers. The activation of immune system causes the inhibition of global gene expression and false positive gene silencing effect. For an example, introduction of long dsRNA into cells leads to the activation of PKR, and results in the activation of innate immune system and type-I IFN production[103]. High levels of siRNA and shRNA could also compete for intracellular miRNA/shRNA process machinery. The saturation of epxortin-5 and RISC component, Argonaute-2 will cause cell toxicity[104]. Although the use of shorter siRNAs could minimize the activation of immune response, sequence dependent activation of TLR7 and 8 could not be eliminated[105].

CONCLUSION:

RNA interference is nowadays most efficient method for gene knockdown used in animal systems. RNA interference can be used in treatment of various diseases like cancer, metabolic syndrome, viral, neural and neuromuscular disease, poly (Q), parkinson, asthma, and diabetes etc. by suppression of gene expression. The delivery of the various RNA like miRNA, siRNA, shRNA by viral as well as by using nonviral vectors. It provides targeted gene silencing so chances of errors are less.

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