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Gene Therapy Using RNAi

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1. Introduction

In the chapter, we hereby provide a brief review about RNA interference (RNAi) and elucidate its therapeutic applications. The enzymatic synthesis of double-stranded RNA in vitro will be discussed. We also describe how to design and select short interfering RNA (siRNA) sequences. In addition, we also introduce a DNA-directed RNAi (ddRNAi) system, which is designed for the generation of small hairpin RNAs in mammalian cells and is an efficient approach to allow fast and easy expression of hairpin oligonucleotides for therapeutic purpose. Moreover, various strategies for delivery of siRNA to target cells are discussed in this chapter. Under the consideration of distinct need of different diseases, use of transient or stable transfection onto living organism will be introduced. Finally, we will discuss the application of RNAi in clinical or preclinical trial. Some ongoing therapeutic models, efficacy or limitation for RNAi will be demonstrated and clarified.

1.1 Overview and mechanism about RNAi

Andrew Fire and Craig Mello published their break-through study on the mechanism of RNA interference in Nature in 1998 (Fire et al., 1998). RNA interference (RNAi) is a phenomenon in which double-stranded RNA (dsRNA) suppresses expression of a target protein by stimulating the specific degradation of the target mRNA (Fuchs et al. 2004). RNAi has been used to study loss of function for a variety of genes in several organisms including various plants, Caenorhabditis elegans and Drosophila, and permits loss-of-function genetic screens and rapid tests for genetic interactions in mammalian cells (Hannon, 2002; Williams et al., 2003). RNAi is generated by a multistep process (Figure 1). First of all, intracellular dsRNA is recognized by an RNase III (designated as "Dicer" in Drosophila) and cleaved into siRNAs of 21–23 nucleotides (Hammond et al. 2000). These siRNAs are then integrated in a complex (designated as "RISC", RNA-induced silencing complex). Each siRNA in RISC is specific and targeting to certain sequences of mRNAs, which is homologous to the integral siRNA followed by completely degradation of targeted mRNA. (Hammond et al., 2000; Bernstein et al., 2001). Actually, the target mRNA is cleaved in the center of the sequence complementary to the siRNA (Elbashir et al., 2001c). As a result, rapid degradation of the target mRNA and decreased protein expression was observed.

It is well-known that proteins are responsible for the physical and dynamic properties of a living cell, and defects in their function or regulation contribute to many diseases. In fact, the majority of drugs used at the present time are actually designed to inactive proteins.

Thus, gene therapy using RNAi should be effective in ablating protein function since mRNA is the template for the translation of multiple proteins. Much of the interest in RNAi is based on the fact that the RNAi mechanism operates upstream of protein production by eliminating the mRNAs coding for such proteins. Thus, scientists are eager to finds solutions for treating diseases of all kinds and sorts by using RNAi.

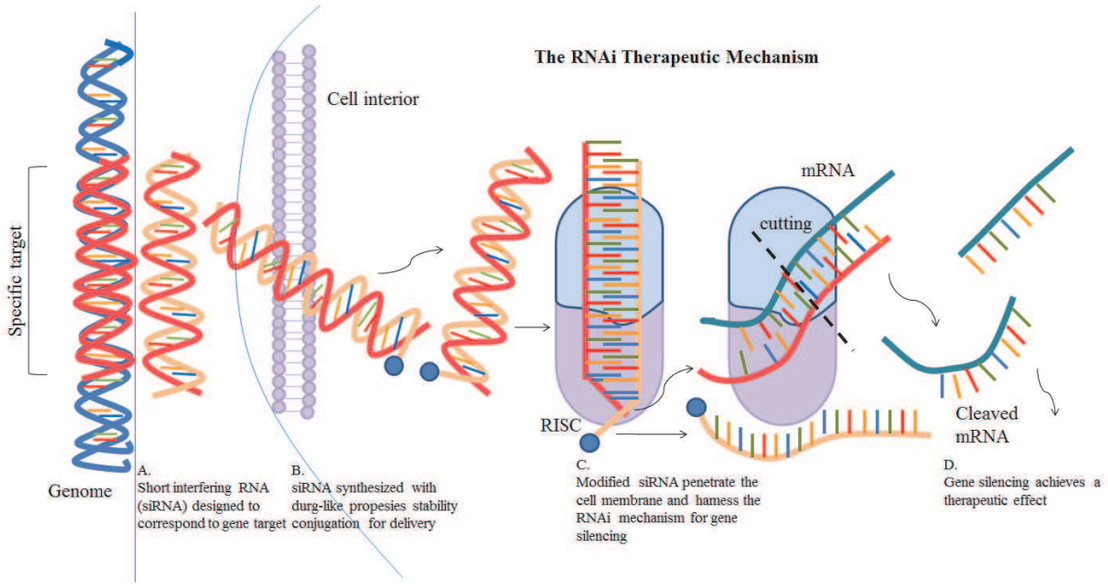

Fig. 1. Simplified schematic diagram of the proposed RNA interference mechanism. dsRNA processing proteins (RNase III-like enzymes) bind to and cleave dsRNA into siRNA. The siRNA forms a multicomponent nuclease complex, the RNA-induced silencing complex (RISC). The target mRNA recognized by RISC is cleaved in the center of the region complementary to the siRNA and quickly degraded.

1.2 siRNA design and optimization

Selecting an optimal target sequence is essential to the success of RNA interference. Since it is not possible to predict the optimal siRNA sequence for a given target, many siRNAs are usually required to be screened for optimal effects. Suggestions for the determination of sequences of siRNAs are usually being modified upon as knowledge of the RNAi continues to expand. The general recommendations are as follows: siRNA target sequences should be specific to the gene of interest and have GC content with about 20~50 percents (Henshel et al. 2004). According to Ui-Tei et al. (2004) and Elbashir et al., 2001a, siRNAs satisfying the following "RNAi target selection rules" are capable of effective gene silencing in mammalian cells: (1) Targeted regions on the cDNA sequence of a targeted gene should be located 50-100 nt downstream of the start codon (ATG). (2)Search for sequence motif AA(N19)TT or NA(N21), or NAR(N17)YNN, where N is any nucleotide, R is purine (A, G) and Y is pyrimidine (C, U). (3)Avoid targeting introns, since RNAi only works in the cytoplasm and not within the nucleus. (4)Avoid sequences with > 50% G+C content. (5)Avoid stretches of 4 or more nucleotide repeats. (6)Avoid 5URT and 3UTR, although siRNAs targeting UTRs have successfully induced gene inhibition.

Designing effective siRNAs is essential for effective RNAi knockdown. In addition to choosing the optimal sequence, other essential factors that affect the efficacy of siRNA

should be as well considered. Once a siRNA sequence is selected using the above algorithm of choice, it is important to perform homology examination against all the other genes from the organism of your study. In addition, the homology test should be able to identify short regions of sequence identity since any siRNA displaying homology with the potential to cause mis-targeted effects should be excluded. Despite so many advances in siRNA design platforms showing highly likely to be functional, the only way to make sure if designed siRNA have the silencing effects in target gene is to validate in an actual experiment.

1.3 Enzymatic synthesis of siRNA

Several methods to generate siRNA have been developed. These include the preparation of siRNA mixtures using RNase III or Dicer enzymes to digest longer double-strand RNAs (dsRNAs) (Andrew et al., 2005; Myers et al., 2003). The short RNAs generated as a result of these digestions have been demonstrated to be efficient in RNAi study. siRNA synthesis in vitro provides an alternative approach instead of using the potentially expensive chemical synthesis of RNA. Take our study for example, which is published in Journal of Cellular Biochemistry (Yang et al., 2007). To analysis the effects of CD36 in renal tubule cells, CD36 siRNA was designed and synthesized. CD36 is an 88-kD integral-membrane glycoprotein that is present on renal tubular; however little is known about its actual biological function. We use LLC-PK1 cells to mimic proximal tubule cells. Cells were cultured in 10% fetal calf serum supplemented medium for 1 day. The cells were then transfected by 100 nM of synthetic CD36 siRNA (sense sequence: CUAAGUUGCUGAGACAAGGdTdT, anti-sense: CCUUGUCUCAGCAACUUAGdTdT), which was purchased from commercial biotech company. To make sure the silencing effects come from the siRNA introduced, scientists are required to make proper control experiments. Usually, we can design reverse sequence for a negative control for siRNA study. For example, we designed the reverse control for CD36 siRNA with sequence as following. Sense sequence is 5′-dTdTGGAACAGAGUCGUUGAAUC-3′ and anti-sense sequence is 5′-dTdTGAUUCAACGACUCUGUUCC-3′. Transfecting procedure was performed according to manufacturer's instructions. Cells were mixed with siRNA complex for 24 h and cultured in serum-free medium for 36 h. These results were shown in Figure 2.

1.4 DNA-directed RNAi

DNA-directed RNA interference (so called "ddRNAi") involves the introduction of DNA templates in cytoplasma and generation of siRNA in vivo. ddRNAi is dependent on RNA polymerase III for the production of siRNA which can silence specific gene of interest with the sequence related to that of siRNA (Brummelkamp et al. 2002; Novarino et al. 2004).Target sequences of si/shRNA can be generated by using PCR followed by cloning into selected vectors (Yu et al., 2002). Take pSUPER RNAi System for example, the pSUPER RNAi System (OligoEngine inc) can generates long-term silencing phenotypes in mammalian cell lines by vector system, along with a pair of oligo sequences specific to the target sequences that are ligated into the vector, to produce dsRNA and to provoke persistent synthesis of siRNA in cytoplasma as shown in Figure 3.

Vector-based RNA silencing strategy provides a way to induce stable, long-term inhibition of gene of interest by generating siRNAs in vivo. After specific gene silencing, the biochemical change in transfected cells can facilitate scientists to study the role of specific

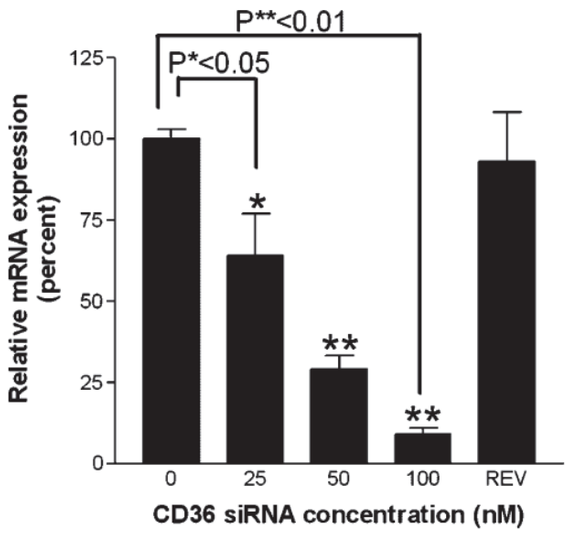

Fig. 2. CD36 mRNA is suppressed in LLC-PK1 using CD36 siRNA. CD36 mRNA was determined by quantitative real-time PCR in LLC-PK1 cell treated with CD36 siRNA (0, 25, 50, 100 nM) for 24 h following maintenance of cells in complete medium for 36 h. Bars represent mean +/- SEM of three experiments. It is evident that CD36 siRNA is a suitable approach applied in CD36 gene silencing. (Adapted from Yang et al., 2007)

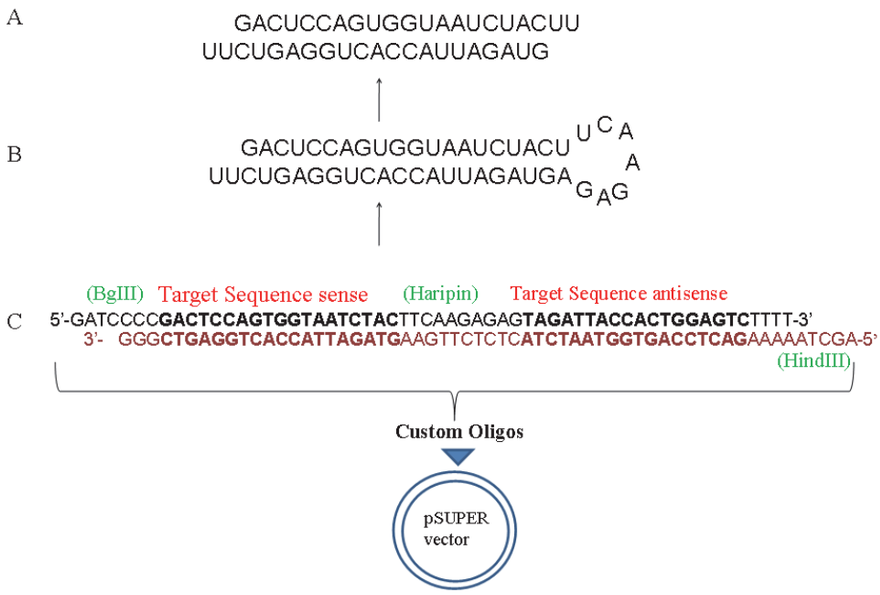

Fig. 3. (A) Demonstrating the sequence (sense strand) and its complement sequence (antisense strand) in the duplex form, with 3′ UU overhang. (B) showing the same sequences along with hairpin loop. The loop is ready to be degraded by Dicer enzyme following RNA transcription. (C) Demonstrating the forward and reverse oligos in relative orientation. (Adapted from http://www.oligoengine.com/)

gene or protein. On the vectors, markers, such as puromycin or neomycin can be performed for long-term suppression of genes of interest. Usually, the silencing time period for synthetic siRNAs is between 48–72 hours. Thus, synthetic siRNAs is suitable for transient silencing purpose. However, vector-based RNA silencing can induce stable and long-term silencing of target gene. Furthermore, to obtain a successful ddRNAi, several parameters should be noted. First, well prepare the vectors containing full-length shRNA or siRNA sequences. Avoid contaminating endotoxin carried from prokaryotes host. Second, make sure of a successful delivery of those vectors into cells and induction of the expression of shRNA constructs. Third, properly select and clone siRNA target sequences into expression vectors. This sequence consists of two inverted repeats separated by a short spacer sequence (loop sequence). After transfection and transcription by RNA polymerase, the inverted repeats anneal and form a hairpin structure, which is then cleaved by Dicer to form siRNA. Taken together, DNA-Directed RNAi strategies provide another method for quickly and easily screening various targets (Yeager et al., 2005).

2. Therapeutic delivery of RNAi

There have been several reports on gene expression can be silenced by the targeted destruction of specific mRNA molecules in a highly conserved process known as RNAi. It is a form of post-transcriptional gene silencing that has been described in a number of plant, nematode, protozoan, and invertebrate species. In the recent years, RNAi has emerged as a major regulatory mechanism in eukaryotic gene expression. Also, RNAi is a powerful tool to silence gene expression in a sequence-specific manner and has the potential to treat diseases (ex. cancer therapeutics or in tissue regeneration) which can be corrected by the decreased expression of specific proteins (Izquierdo, 2005). A single strand of exogenously introduced double-stranded small interfering RNA (siRNA; 20-30 nucleotides) guides an RNA-inducing silencing protein complex to degrade the mRNA with the matching sequence; thus, translation into the target proteins is silenced (Elbashir et al., 2001b; Hannon & Rossi , 2004; Novina & Sharp, 2004). Theoretically, siRNA can interfere with the translation of almost any mRNA, as long as the mRNA has a distinctive sequence, whereas the targets of traditional drugs are limited by types of cellular receptors and enzymes (de Fougerolles et al., 2007). In designing a particular RNAi, it is important to identify the sense/antisense combination that provides the most potent suppression of the target mRNA, and several rules have been established to give > 90% gene expression inhibition. RNAi technology can be directed against cancer and various diseases using a variety of genetic therapeutic strategies. The RNAi delivery strategies are often exploited in experimental biology to study the function of genes in target cells or organisms. Successful RNAi experiments are dependent on both siRNA design and effective delivery of siRNA duplexes into cells.

2.1 Current challenges in RNAi *in vivo*

There are several challenges that currently limit the use of RNAi in the clinic. Methods that overcome these are being developed and are discussed below.

2.1.1 Design and delivery of chemically modified siRNA

Unmodified, naked siRNA are relatively unstable in blood circulation owing to its native form, though they are more stable than single-stranded RNA. siRNA are rapidly degraded by nucleases, renal clearance, and non-specific uptake by the reticuloendothelial system

indicating that they have short half-lives in vivo. In order to enhance biological stability without affecting gene-silencing activity, chemical modifications have been introduced into the siRNA structure. Various chemical modifications to the backbone, base, and sugar of the siRNA for stability and activity against nuclease degradation have been reported. siRNA is an anionic macromolecule and does not readily enter cells by passive diffusion mechanisms. An appropriate siRNA delivery system enhances cellular uptake, protects its payload from enzymatic digestion and immune recognition, and improves the pharmacokinetics by avoiding excretion via the reticuloendothelial system and renal filtration (i.e. prolonged half-life in vivo) (Lorenz et al., 2004; Sioud & Sorensen, 2003; Sorensen et al., 2003). In addition, an appropriate delivery system localizes siRNA in the desired tissue, resulting in a reduction in the amount of siRNA required for efficient gene silencing in vivo, as well as minimized side effects. By these chemical modifications of siRNA, degradation of siRNA in blood and/or cells can be delayed from minutes to hours. Thus, gene-silencing activity in vivo can be sustained for several days in conjunction with an appropriate delivery system. Chemical modifications can also reduce off-target effects (Jackson et al., 2006) and alter thermal stability within the various critical sequence regions of the siRNA duplex for improved activity of siRNA (Khvorova et al., 2003; Reynolds et al., 2004; Schwarz et al., 2003). Also, this kind of backbone modification enhances the resistance of siRNA to enzymatic hydrolysis, immunogenicity and toxicology

2.1.2 Local versus systemic siRNA delivery

To achieve RNAi via siRNA delivery in vivo, it is critical for siRNA to be efficiently located in desired tissues/cells. The types of target tissues and cells dictate the optimum administration routes of local versus systemic delivery. For example, siRNA can be directly applied to the eye, skin or muscle via local delivery, whereas systemic siRNA delivery is the only way to reach metastatic and hematological cancer cells (Shim & Kwon, 2010). Recent study has indicated systemic siRNA delivery imposes several additional barriers compared with local delivery. Local delivery offers several advantages and makes achieving efficient RNAi over systemic delivery, such as low effective doses, simple formulation (e.g. no targeting moieties), low risk of inducing systemic side effects and facilitated site-specific delivery (Dykxhoorn et al., 2006). In other word, local administration is likely to be a more cost-efficient strategy for siRNA delivery in vivo than systemic administration. Generally, naked siRNA injected into the body has a very short half life, on the order of minutes, limiting its usefulness (Dykxhoorn & Lieberman, 2006). However, it has been found that some tissues are able to uptake naked siRNA to a much higher degree than other tissues (for instance the eye, central nervous system, and lung) making localized delivery by direct injection of siRNA to these sites a possibility. For example, initial clinical trials for RNAi-based treatment of age-related macular degeneration have exclusively used local injections of siRNA direct injection into the eye and central nervous system (Vives et al., 1997).

2.1.3 Nanotechnology platforms for gene therapeutics

The biomedical application of nanotechnology has been extensively used in drug or gene delivery system in the past few years. Nanotechnology is considered to be novel and potential technology that will have significant impact in all industrial sectors and across-the-board applications in gene therapeutics. Nanoshells are nanoparticle beads that consist of a silica core coated with a thin gold shell (1 nanoparticle). Nanoparticle carriers have the

potential to overcome the challenges of intravascular degradation, and can provide safe and efficient delivery of synthetic dsRNAs. On entering the bloodstream, nanoparticles encounter a complex environment of plasma proteins and immune cells. Nanoparticle uptake by immune cells in blood circulation, such as monocytes, leukocytes, platelets and dendritic cells, occurs through various pathways and is facilitated by the adsorption of opsonins to the surface of the particle (Kim, 2007). Additionally, the application of nanotechnology significantly benefits clinical practice in cancer diagnosis, treatment, and management. Especially, nanotechnology offers a promise for the targeted delivery of drugs, genes, and proteins to tumor tissues and protect them from environmental factors which may degrade it and it typically uptake by cells with a higher efficiency (Loo et al., 2005).

3. Methods of siRNA delivery

Despite quite efficient and reliable gene silencing in vitro, only limited RNAi has been achieved in vivo because of rapid enzymatic degradation in combination with poor cellular uptake of siRNA (Kirchhoff, 2008). Therefore, novel delivery systems which enable prolonged naked siRNA and improve accessibility to target cells via clinically feasible administration routes and optimized cytosolic release of siRNA after efficient cellular uptake, are indispensably required (Grimm & Kay, 2007). There are several methods and major hurdles in siRNA delivery which overcome them and are discussed below:

3.1 Naked siRNA

"Naked" siRNA delivery is direct injection of a saline or excipient solution containing non-complexed siRNA sequences to a target site. Although naked siRNA was influenced by enzymes and rapid renal clearance, recent studies have also demonstrated the ability to deliver naked siRNA to target cells or tissues (for instance the eye, central nervous system, and lung) via local injection system delivery.

3.2 Microinjection

Microinjection offers a potentially powerful method for the analysis of the dynamics of expression because it enables real-time regulation of transient expression of multiple genes. Recent papers have provided a brief review of RNAi and discussion of the benefits and drawbacks of using long double-stranded RNA (dsRNA) into mouse oocytes and early embryos by microinjection and preparation and testing of constructs for transgenic RNAi based on long hairpin RNA expression. Furthermore, microinjection can provide a useful method for quantitative analysis of transient gene expression in single cells using RNAi.

3.3 Biolistics (gene gun)

The gene gun (also known as biolistic or bioballistic particle delivery) is the most novel physical transfection method. This technique is based on DNA (or RNA) become "sticky," adhering to biologically inert particles such as metal atoms (usually tungsten or gold), and then are accelerated to high velocity to get into target tissues or cells (Klein et al., 1992; Uchida et al., 2009). This approach allows DNA (or RNA) to penetrate directly through cell membranes into the cytoplasm or even the nucleus, and to bypass the endosomes, thus avoiding enzymatic degradation. Gene guns are so far mostly applied for plant cells.

However, there is much potential use in animals and humans as well. This technology was useful to transfection of cells and assessed by analyzing gene expression. Optimal transfection conditions were determined to be between 75 and 100 psi of helium pressure, 1.0 to 1.6 μm gold particle size and 0.5 mg of gold particle amount with a loading ratio of 4 μg DNA/mg gold particles (Uchida et al., 2009). These findings will be useful and suitable in the design of gene gun device, and bring further improvements to the in vivo transfection studies including gene therapy and vaccination.

3.4 Liposome

To increase the half-life of siRNA in vivo it can be encapsulated within liposomes or complex with cationic lipid to form siRNA-laden nanoparticles. Liposome are vesicles composed of phospholipids bilayer membranes that can enclose various substances, including DNA, siRNA, peptides, proteins, aptmers, chemicals, and drugs. Research on using liposomes to encapsulate and deliver chemotherapeutics has been performed since the late 1970s, and in the early 1980s they were extensively studied as potential vectors for gene therapy (Felgner et al., 1987). Recently, liposomes containing siRNA were modified with a peptide that targeted MCF-7 breast cancer cells, and were shown to effectively silence the expression of the PDMR14 gene that plays a role in breast cancer carcinogenesis, with minimal uptake and silencing effect in other non-cancerous cells (Bedi et al., 2011). Cationic liposomes can condense nucleic acids into a cationic particle when mixed together. This cationic lipid/nucleic acids complex (also called lipoplex) can protect nucleic acids from enzymatic degradation and deliver nucleic acids into cells by interacting with negatively charged cell membrane. Cationic liposomes have also been applied with substantial success for the in vitro as well as in vivo delivery of siRNA. Studies have been reported utilizing the advantage RNAi technology in vivo is successful knockdown of ApoB in nonhuman primates by systemically delivered siRNA in stable nucleic acid-lipid particles. The siRNA-lipid complexes showed significantly enhanced cellular internalization and endosomal escape of siRNA. Thus, the resulting ApoB siRNA-carrying liposome were stable during circulation in vivo after i.v. injection and reduced ApoB expression and serum cholesterol levels (Zimmermann et al., 2006).

3.5 Protein transduction domain-mediated siRNA

CPPs, also referred protein transduction domains (PTD) are short cationic peptide chains with a maximum of 30 amino acids. CPPs are able to penetrate biological membranes, to trigger the movement of various biomolecules across cell membranes into the cytoplasm and to improve their intracellular routing thereby facilitating interactions with the target. CPPs have been reported to deliver a wide variety of cargo (e.g. plasmid DNA, oligonucleotide, siRNA, protein, peptide, liposome, nanoparticle…), they are rapidly taken up into primary cells and most tissues by macropinocytosis in clinical trials. Most cellular uptake studies of PTD in the literature based on fluorescence microscopy of fixed cells and flow cytometry analysis report that internalization of PTD (Suzuki et al., 2002; Torchilin et al., 2001). As RNAi is one of the most promising strategies for gene therapy, further advances in CPP-based RNA delivery are expected in the near future.

3.6 Cationic polymers and polypeptide

Nucleic acids such as siRNA are easily complexed with synthetic cationic polymers e.g., polyethylenimine (PEI), biodegradable cationic polysaccharide (e.g. chitosan) and cationic

polypeptides (e.g. atelocollagen, poly-L-lysine and protamine), via attractive electrostatic interactions. Polymer reduction can also allow for the release of nucleic acids into the cytoplasm. The use of reducible polymers and peptides is therefore a rapidly emerging strategy for enhanced transfection efficiency and cytoplasm-sensitive gene delivery. Various reduction-sensitive cationic polymers have been developed for gene delivery because they are highly stable in physiological conditions and rapidly reduced in the cytosol. For example, i.v. injection of siRNA-atelocollagen complexes silenced luciferase expression in germ cell tumor xenografted in mice and inhibited tumor growth (Minakuchi et al., 2004). In another study, the vascular endothelial growth factor (VEGF) siRNA-atelocollagen complexes successfully suppressed the secretion and expression of VEGF in human prostate PC-3 carcinoma cells, leading to the potent suppression of tumor growth in its xenograft model. This result clearly demonstrated that a novel VEGF blockade system by RNAi is valid as a therapeutic (Takei et al., 2004). Previous papers have demonstrated overexpression of RhoA in cancer indicates a poor prognosis, because of increased tumor cell proliferation and tumor angiogenesis. Furthermore, Pille´ et al showed that anti-RhoA siRNA inhibited aggressive breast cancer more effectively than conventional blockers of Rho-mediated signaling pathways. Likewise, the study has also demonstrated that intravenous administration of chitosan-RhoA siRNA complexes resulted in effective gene silencing in subcutaneously implanted breast cancer cells in nude mice (Pille et al., 2006). Tumor necrosis factor expression in systemic macrophages was silenced in mice after i.p. administration of chitosan⁄siRNA complexes, thus downregulating systemic and local inflammation (Howard et al., 2009).

3.6.1 Polyethylenimine

Polyethylenimine (PEI) is a synthetic polymer that can be either linear or branched, PEI is a well-known cationic polymer utilized as a non-viral vector because of its strong binding affinities to nucleic acids and proton buffering effect. In particular, 25 kDa PEI has been widely used in gene delivery. PEI with a high percentage of free amine groups which are positively charged and it is one of the most popularly investigated synthetic cationic polymers for nucleic acid delivery in vitro and in vivo. Paper has demonstrated that the noncovalent complexation of synthetic c-erbB2 / neu (HER-2) siRNA with low molecular weight polyethylenimine efficiently stabilizes siRNAs and delivers siRNAs into tumor cells via i.p. administration and results in a remarkable reduction of tumor growth, where where they display full bioactivity at completely nontoxic concentrations (Urban-Klein et al., 2005). Angiogenesis is the formation of new blood vessels from preexisting microvessels. In particular, tumor growth and metastasis were found to be dependent on angiogenesis. Thus, anti-angiogenic therapy has become an important route for cancer treatment (Cvetkovic et al., 2001; Lu et al., 2005). Likewise, a recent clinical trial examined the delivery of siRNA-laden polyethylenimine- based polymer modified with a targeting VEGF has been injected directly into subcutaneous tumors, and the silencing of VEGF and subsequent diminishment tumor growth was confirmed (Kim et al., 2008). For inhibition of VEGF expression, several gene therapeutic approaches using antisense oligonucleotide, ribozyme, and siRNA have been suggested as potential strategies for anti-cancer therapy (Lu et al., 2005; Rhee & Hoff, 2005; Takei et al., 2001). In another study, polyethylenimine-conjugated siRNA against secreted growth factor pleiotrophin (PTN) reduced tumor growth and cell proliferation without a measurable induction of siRNA-mediated immunostimulation after intracerebral

or subcutaneous administration in an orthotopic glioblastoma nude mice model (Grzelinski et al., 2006). Overall, polyethylenimine seems to be a promising nonviral platform for siRNA delivery in vitro.

3.6.2 Chitosan

Chitosan, a linear and cationic polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), is one of such biodegradable polymers, which has been extensively exploited for the preparation of nanoparticles for oral controlled delivery of several therapeutic agents. In recent years, the area of focus has shifted from chitosan to chitosan derivatized polymers, such as trimethylchitosan for the application of non-viral and non-toxic gene delivery. Paper has been shown quaternised chitosan transfect breast cancer cells; with the most efficient at gene delivery. In addition, chitosan's properties also allow it to be used in trans-dermal drug delivery.

3.6.3 Polypeptides

Polypeptides, such as poly-L-lysine and protamine, have also commonly been used to deliver siRNA. Poly-L-lysine (PLL) is a cationic polypeptide with the amino acid lysine as the repeating unit (Watanabe et al., 2009). PLL can be synthesized using various strategies leading to PLLs with different molecular weights (typically 1 kDa up to > 300 kDa), in linear conformation or as highly branched polypeptide (Garnett, 1999; Yoshida & Nagasawa, 2003). Also, protamine, a natural arginine-rich cationic polypeptide, condenses negatively charged nucleic acids and has been used as an efficient gene-delivery carrier (Vangasseri et al., 2005). Recent studies have investigated the low molecular mass protamine (LMWP)/VEGFsiRNA complex treatment, which possessed membrane translocating potency, the peptide could carry and localize siRNA inside tumors via i.p. administration and successfully inhibited the expression of VEGF, thereby suppressed the growth of hepatocarcinoma tumors in tumor-bearing mice (Choi et al., 2010). The data in this study clearly demonstrate that cell penetrating peptide-based systems not only enhance cellular uptake of siRNA, but also facilitate the suppression of VEGF expression without causing detectable systemic side effects. Furthermore, The LMWP is a potent transducible cell penetrating carrier for gene products including siRNA, and that potent growth inhibitory effects of the LMWP / VEGFsiRNA complex in vitro and in vivo. In addition, previous paper has also developed an efficient and low immunostimulatory nanoparticle formulation [liposomes-protamine-hyaluronic acid nanoparticle (LPH-NP)] for systemically delivering siRNA into the tumor (Chono et al., 2008). Overall, we expected that LMWP peptide and protamine could be used as a tool for siRNA delivery and may be applicable in an anti-angiogenic regulator to treat cancer in the future.

3.7 Electroporation

Electroporation, or electropermeabilization, is a popular in vitro technique for introducing siRNA or plasmid DNA into living cells. The application of electric pulses opens pores in the cell plasma membrane through which siRNA or DNA can pass and directly enter into the cytoplasm. Then, the pores close again and the DNA is trapped within the cell (Neumann et al., 1982). So, it is a significant increase in the electrical conductivity and permeability of the cell plasma membrane caused by an externally applied electrical field,

and is usually used in molecular biology as a way of introducing some substance into a cell, such as loading it with a molecular probe, a drug that can change the cell's function, a piece of coding DNA or siRNA. Many research techniques in molecular biology require a foreign gene or protein material to be inserted into a host cell. Since the phospholipid bilayer of the plasma membrane has a hydrophilic exterior and a hydrophobic interior, any polar molecules, including DNA and protein, are unable to freely pass through the membrane (Hou, 2001). Electroporation is a dynamic phenomenon that depends on the local transmembrane voltage at each point on the cell membrane. It is generally accepted that for a given pulse duration and shape, a specific transmembrane voltage threshold exists for the manifestation of the electroporation phenomenon (from 0.5 V to 1 V). This leads to the definition of an electric field magnitude threshold for electroporation (Eth). That is, only the cells within areas where E≧Eth are electroporated. If a second threshold (Eir) is reached or surpassed, electroporation will compromise the viability of the cells, i.e., irreversible electroporation. This procedure is also highly efficient for the introduction of foreign genes in tissue culture cells, especially mammalian cells. For example, it is used in the process of producing knockout mice, as well as in tumor treatment, gene therapy, and cell-based therapy.

3.8 Calcium phosphate precipitation

A number of non-viral vectors have been explored till now. Some of these include cationic lipids, cationic polymers, peptides, other synthetic vectors, and precipitated calcium phosphate. Among these, the technique of calcium phosphate co-precipitation for in vitro transfection is used as a routine laboratory procedure. Calcium phosphate (CaP) is a well used non-viral vector for in vitro transfection of a wide variety of mammalian cells with little toxicity (Sokolova & Epple, 2008). Especially, DNA or siRNA-calcium phosphate coprecipitates have been used for many years as an efficient method to introduce genetic material in to cells, and the calcium phosphate particles have attracted much attention in gene therapy. Nucleic acids alone cannot penetrate the cell membrane, therefore special carriers like cationic polymers or inorganic nanoparticles are required. Single-shell and multi-shell calcium phosphate nanoparticles were prepared and functionalized with DNA and siRNA. The delivery activity is probably related to the fact that CaP rapidly dissolves in the acidic pH (Bisht et al., 2005). Endocytosed CaP is expected to deassemble in the endosomes and release its cargo into the cytoplasm. Many investigators have attempted to improve the manufacture of the CaP precipitate with limited success (Olton et al., 2007). In past study has reported that calcium ions play an important role in endosomal escape, cytosolic stability and enhanced nuclear uptake of siRNA through nuclear pore complexes. The siRNA encapsulated inside the nanoparticle is protected from the external RNase environment and could be used safely to transfer the encapsulated siRNA under in vivo conditions (Maitra, 2005). Thus, CaP precipitation is a dynamic and reversible process, and it can have multiple applications in gene function, regulation, and therapy and achieve its full potential in transfectional mammalian cells.

4. Application of RNAi

4.1 Clinical trials

There are many RNAi therapeutics approved by the U. S. Food and Drug Administration in various diseases in clinical trials (Table 1). Two *ex vivo* studies provided by City of Hope

Medical Center have designed for treating HIV infection through targeting on HIV tat and rev. IV transfer of in vivo lentiviral infected cells was used to treat patients with HIV infection. The expression of shRNA of tat and rev can knockdown the viral mRNA in T lymphocytes.

Four of these therapeutics for treating solid tumors are CALAA-01, siG12D LODER, ALN-VSP02, and Atu027. CALAA-01 provided by Calando Pharmaceuticals is a targeted therapeutic designed to inhibit tumor growth and/or reduce tumor size. It consists of a duplex siRNA to repress the expression of M2 subunit of ribonucleotide reductase. Another component AD-PEG-Tf combines the siRNA molecule with the transferrin receptor of the tumor cell surface to target the solid tumors. The majority of pancreatic ductal adenocarcinomas involve mutations in the KRAS oncogene, especially most common in G12D mutation. siG12D LODER provided by Silenseed Ltd is designed to inhibit oncogene KRas G12D mutation specifically. ALN-VSP02 provided by Alnylam Pharmaceuticals and Atu027 provided by Silence Therapeutics AG are both designed to inhibit growth and survival of cancer cells by tumor angiogenesis. ALN-VSP02 is a systemically delivered therapeutic packaged in Tekmira's SNALP delivery formulation and containing two siRNAs designed to target VEGF and kinesin spindle protein (KSP), also known as eglin 5 (Eg5), which disrupts cell division and induces apoptosis. In addition, in vitro studies revealed that Atu027-mediated inhibition of protein kinase N3 function in primary endothelial cells impaired tube formation on extracellular matrix and cell migration (Aleku et al., 2008).

There are five RNAi therapeutics approved by the U.S. FDA to treat ocular diseases, four designed in targeting VEGF and its receptor and one in caspase 2. Bevasiranib provided by Opko Health, Inc. involves in three clinical trials in treating macular edema and degeneration by 2-dT-modified siRNA on 3' end. Similarly, Allergan designed a siRNA targeting on VEGF receptor 1 to treat the same disease. In addition, a synthetic siRNA provided by Quark Pharmaceuticals, QPI-1007, inhibits expression of caspase 2 in retinal ganglion cells to prevent glaucoma, non-arteritic anterior ischemic optic neuropathy (NAION), and other ocular diseases.

There are two trials approved by FDA in treating kidney diseases using the same drug, I5NP, containing siRNA for p53, which arrests cell cycle progression and promotes cellular apoptosis. I5NP is provided by Quark Pharmaceuticals and designed to knockdown p53 to prevent cellular apoptosis of kidney in treating acute renal failure as well as in delayed graft function during kidney transplantation. Pachyonychia congenita is a rare keratin mutant disease, which affects the nails, skin, oral mucosae, larynx, hair and teeth. The majority of pachyonychia congenita involves mutations in the keratin gene, such as K6a, K6b, K16 or K17. A clinical trial using siRNA was applied by Pachyonychia Congenita Project. And, the siRNA was developed and provided by a small company, TransDerm, Inc., to inhibit the expression of the keratin K6a N171K mutation specifically.

4.2 Other approaches

4.2.1 Host protein screening and controlling of viral infection

Large siRNA libraries are constructed to knockdown all proteins known to be encoded by the human and mammalian genomes. Therefore, using siRNA library screening to identify the interacting proteins related to viral infection is a new approach. Several studies were carried out to screen the host factors of viruses, such as HIV, West Nile virus, Dengue virus, hepatitis C virus and influenza (Hirsch, 2010).

Disease	Sponsor	Product	Target gene	Target cell/tissue	Type of RNAi	Status
HIV	City of Hope Medical Center	rHIV7-shI-TAR-CCR5RZ-transduced autologous HPCs	HIV tat and rev	$CD34^+$ Cells	Lentivirus (*in vitro*)	Active, not recruiting
HIV	City of Hope Medical Center	pHIV7-shI-TAR-CCR5RZ treated CD4 cells	HIV tat and rev	T lymphocytes	Lentivirus (*in vitro*)	Phase 0 , terminated
Solid tumors	Calando Pharmaceuti-cals	CALAA-01	M2 subunit of ribonucleotide reductase	Solid tumors	Nanocom-plex, synthetic siRNA	Phase I , recruiting
Pancreatic cancer	Silenseed Ltd	siG12D LODER	KRAS oncogene G12D mutation	Pancreatic ductal adenocarci-noma	Polymeric matrix, synthetic siRNA	Phase I , recruiting
Solid tumors	Alnylam Pharmaceuti-cals	ALN-VSP02	Vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP)	Solid tumors, liver	SNALP particle, synthetic siRNA	Phase I , recruiting
Advanced solid tumors	Silence Therapeutics AG	Atu027	Protein kinase N3	Advanced solid tumors	Liposome, 2'-O-methyl modified synthetic siRNA	Phase I , recruiting
Age-related macular degeneration	Allergan	AGN211745	VEGF receptor-1	Macula cells (retina)	Chemically modified siRNA	Phase II , completed
Diabetic macular edema	Opko Health, Inc.	Bevasiranib	VEGF	Retinal vasculature	3'-2 dT-capped synthetic siRNA	Phase II, completed
Age-related macular degeneration	Opko Health, Inc.	Bevasiranib	VEGF	Retinal vasculature	3' 2 dT-capped synthetic siRNA	Phase III, withdrawn
Macular degeneration	Opko Health, Inc.	Bevasiranib	VEGF	Retinal vasculature	3' 2 dT-capped synthetic siRNA	Phase II, completed
Optic neuropathy	Quark Pharmaceuti-cals	QPI-1007	Caspase 2	Retinal ganglion cells	Synthetic siRNA	Phase I , recruiting

Disease	Sponsor	Product	Target gene	Target cell/tissue	Type of RNAi	Status
Pachyonychia congenita	Pachyonychia Congenita Project (TransDerm, Inc.)	TD101	Keratin K6a N171K mutation	Skin		Phase I , completed
Hypercholesterolemia	Tekmira Pharmaceuticals Corporation	PRO-040201	ApoB		SNALP particle, synthetic siRNA	Phase I , terminated
Acute renal failure	Quark Pharmaceuticals	I5NP	P53	Kidney	Synthetic siRNA	Phase I , completed
Delayed graft function	Quark Pharmaceuticals	I5NP	P53	Kidney	Synthetic siRNA	Phase I/II , recruiting

Table 1. RNAi theraputics in clinical trials. (From the U.S. National Library of Medicine, A service of the U.S. National Institute of Health)

Human immunodeficiency virus type 1 (HIV-1) belongs to the family of retroviruses, infects human CD4+ lymphocytes and causes an indolent-type of infection and terminally ended to acquired immunodeficiency syndrome (Alimonti et al., 2003; Guadalupe et al., 2003). Because RNAi provides a strong justification to become a novel but remarkable therapeutic strategy in inhibiting gene expression, including viral mRNAs, many scientists participate in developing new methods using RNAi to treat HIV replication (Berkhout, 2009; Soejitno et al., 2003). In addition to HIV, RNAi is also used in controlling infection of several viruses, such as hepatitis B, hepatitis C, influenza, parainfluenza, CVB3, JEV, HSV2, RSV, PIV, SARS, foot-and-mouth disease and West Nile viruses (Chen et al., 2008; Khaliq et al., 2010; López-Fraga etal., 2008).

4.2.2 Neurodegenerative diseases

Neurodegenerative diseases are characterized by incorrect expression or selective dysfunction of proteins and eventual death of distinct subpopulations of neurons occurred in the central nervous system, such as Alzheimer's, Huntington's and Parkinson's diseases. They are solely caused by the inheritance of genetic mutations and several strategies using RNAi to target the specific mutant genes to treat these diseases in cellular and animal models were developed (Boudreau & Davidson, 2010; Maxwell, 2009). In contrast to these diseases, prion disease is caused by the conversion of a highly conserved prion protein across species, PrPc, into a partially protease-resistant isoform, PrPsc, which aggregates in the brain and is associated with pathogenesis of a neurodegenerative disease (Prusiner, 1991; Prusiner & DeArmond, 1991). However, the function of PrPc is not clear and its ablation represents no deleterious effects. Therefore, RNAi therapeutics should be useful strategies to target and remove normal PrPc to decrease the conversion and aggregation in the adult brain (Verity & Mallucci, 2010).

4.2.3 Immune diseases

As described above, siRNA-based therapeutics have entered clinical trials in treating many diseases. However, it has not been reported that siRNA can be used as an effective

alternative to the present medications, such as corticosteroids, for managing allergy in vivo (Suzuki et al., 2009). It has been known that CD40/CD40 ligand (CD40L) interaction enhances Th2 cytokine production. Suzuki and colleagues have developed siRNA-based therapeutics to inhibit CD40 expression and found that decreased CD40 levels attenuated allergy through inhibition of functions of dendritic and B cells and generation of regulatory T cells (Suzuki et al., 2009). Bronchial asthma is a common inflammatory disease associated with allergy and activation of T helper type 2 cells, which secret cytokines to mediate local or systemic inflammation. One of inflammatory factors, interleukin 13 has been identified as a major driver of asthma pathology. Walker et al. used STAT6 siRNA to inhibit its expression and therefore found decreased downstream IL-13 as well as IL-4 and CCL26 protein expression in lung epithelial cells (Walker et al., 2009). The main inflammatory cytokines IL-1 and TNF-α are considered as potential targets for rheumatic diseases therapy. NF-κB is one of the key transcription factors associated with inflammatory pathway. siRNA specific for NF-κB p65 subunit is used in animal models to treat rheumatic diseases. It has been found that NF-κB downstream signaling molecules, such as cyclooxygenase-2, nitric oxide synthase-2, and matrix metalloproteinase-9 (de França et al., 2010; Lianxu et al., 2006).

4.2.4 Cancer gene therapies

Numerous scientists have designed shRNA or siRNAs in various cancer cell and animal models to develop the applications of RNAi in cancer therapeutics. He and colleagues have discussed genes associated with tumor growth and metastasis as well as adjuvant therapies for tumor radiation and chemotherapy (He et al., 2009). In addition to genes discussed by He et al., cyclooxygenase-2 (COX-2) is also a candidate for RNAi suppression, because it involves in tumor angiogenesis. Several in vitro studies have revealed that overexpression of COX-2 inhibits cellular apoptosis and promotes tumor angiogenesis. Therefore, COX-2 may play an important role in developing new vasculature, which involves in tumor growth and metastasis (DuBios et al., 1998; Liu et al., 2001; Tsujii et al., 1998; Wang & DuBois, 2010). Similar to identifying the interacting proteins related to viral infection (Hirsch, 2010), RNAi also paves the way for large scale loss-of-function genetic screens in mammalian cells to understand the cancer biology (Ashworth & Bernards, 2010). It also provides a novel and viable tool help in the identification of targets for treating cancer diseases and of those patients that are respond to a given therapy (Ashworth & Bernards, 2010; Micklem & Lorens, 2007; Nijwening & Beijersbergen, 2010).

5. Conclusion

RNA interference offers great promise for treating various disease states or helping to repair damaged tissue. Especially, initially in vivo studies demonstrated effective tumor suppression in nude mice by chemically synthesized siRNAs. More recently, many researchers have used non-viral or viral vectors for transcription of siRNAs / shRNAs in vitro and in vivo. RNAi technology has quickly been advanced from research discovery to clinical trials as effective gene silencing and therapeutic strategy since its discovery in 1998. Nevertheless, viral siRNA delivery raises several safety and preparation concerns such as immune responses and limited large-scale production. Non-viral siRNA have been developed to enhance transfection efficiency, minimize cytotoxicity and improve the bioactivities of nucleic acids, and then had competent in tackling the barriers in siRNA circulation, permeation into target tissues (ex. eye, central nervous system, muscle, and

lung) specific binding to target cells and optimized intracellular trafficking. Recent advances clearly indicate that interdisciplinary approaches using biology, chemistry and engineering play crucial roles in achieving efficient and targeted siRNA delivery in vivo. In certain tissues, it has been demonstrated that a simple injection of naked siRNA will silence gene expression specifically in that tissue. In addition, to achieve local gene silencing in other tissues, a variety of approaches have been pursued to help stabilize the siRNA and to encourage cellular uptake by administration to the site of interest; they include chemical modification of the siRNA or complexation within liposomes or polymers to form nanoparticles. Overall, the field of siRNA delivery has been very focused on the use of nanoparticles.

The application of nanoparticles in medicine is an emerging field of nanobiotechnology (Kim, 2007). As a result of their small size, nanoparticles can penetrate the cell wall and deliver drugs or biomolecules into living systems, usually for a therapeutic purpose. Many different kinds of nanoparticles are known, many have been tested on biosystems, and some approaches have made it into clinical trials (Chen et al., 2010; Strong & West, 2011). Thus, this multifunctional, unique nanoparticulate carrier has the potential to detect diseases, deliver medications, and monitor the ability to change the current scenario of various diseases research and diagnosis in real time. Despite all this, the discovery and characterization of RNAi is not only as a powerful molecular biological tool to suppress the expression of a target gene, but also as an emerging therapeutic strategy to silence disease genes (Harrington, 2001). This work is not intended to give a comprehensive review of the antisense world, but rather to focus on what we know about the mechanism and function of RNAi, and how this molecule can best be applied as a therapy against various diseases.

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7. References

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The aim of this book is to cover key aspects of existing problems in the field of development and future perspectives in gene therapy. Contributions consist of basic and translational research, as well as clinical experiences, and they outline functional mechanisms, predictive approaches, patient-related studies and upcoming challenges in this stimulating but also controversial field of gene therapy research. This source will make our doctors become comfortable with the common problems of gene therapy and inspire others to delve a bit more deeply into a topic of interest.

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