Review

RNA interference: An emerging generation of biologicals

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RNA interference (RNAi) is a mechanism displayed by most eukaryotic cells to rid themselves of foreign double-stranded RNA molecules. RNAi has now been demonstrated to function in mammalian cells to alter gene expression, and has been used as a means for genetic discovery as well as a possible strategy for genetic correction. RNAi was first described in animal cells by Fire and colleagues in the nematode, *Caenorhabditis elegans*. Knowledge of RNAi mechanism in mammalian cell in 2001 brought a storm in the field of drug discovery. During the past few years scientists all over the world are focusing on exploiting the therapeutic potential of RNAi for identifying a new class of therapeutics. The applications of RNAi in medicine are unlimited because all cells possess RNAi machinery and hence all genes can be potential targets for therapy. RNAi can be developed as an endogenous host defense mechanism against many infections and diseases. Several studies have demonstrated therapeutic benefits of small interfering RNAs and micro RNAs in animal models. This has led to the rapid advancement of the technique from research discovery to clinical trials.

1 Introduction

RNA interference (RNAi) is a conserved mechanism in most eukaryotic organisms for RNA-guided regulation of gene expression, in which double-stranded ribonucleic acid (dsRNA) inhibits the expression of specific gene with complementary nucleotide sequences. It plays a major role in regulating development and genome maintenance. The RNAi pathway is a cellular mechanism to eliminate unwanted/invading genetic material and is thought to be evolved as a part of cell's innate immunity. Although the mechanism has only recently been uncovered, the concept of interference has been recurring in biology for over 20 years. It came into highlight during 1990s when scientists first discovered unexpected gene silencing phenomenon in plants and flatworms [1, 2]. Since then the RNAi pathway has been well-studied in certain model organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Arabidopsis thaliana*.

The knowledge of RNAi mechanism in mammalian cells in 2001 ignited an explosion of research to uncover new mechanisms of gene regulation for drug discovery. RNAi therapeutics provides a novel approach to conquer dreadful human diseases by addressing a target that is otherwise undruggable by existing methods of medicine. In this review we have focused on use of RNAi as a new class of biologicals.

2 History

The discovery of RNAi was preceded by unexpected outcomes in some experiments. In 1990, exogenous transgenes were introduced in petunias to alter the flower colors by up-regulating the activity of a gene for chalcone synthase, an enzyme involved in the production of specific pigmentation in petunia flowers. It was expected that the overexpres-

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Abbreviations: **miRNA**, microRNA; **nt**, nucleotide; **RNAi**, RNA interference; **siRNA**, small interfering RNA

sion of the gene would result in darker flowers, but in some cases complete loss of color was observed in flowers. This indicated that the introduced transgenes affected the expression of endogenous genes, thus decreasing the activity of chalcone synthase and resulting in partially white flowers. In the case of white flowers, both the endogenous genes and the transgenes were down-regulated [1]. This phenomenon was referred to as 'co-suppression' and later renamed post-transcriptional gene silencing (PTGS) [3]. After these initial observations in plants, many laboratories around the world searched for the occurrence of this phenomenon in other organisms.

During 1995, it was demonstrated that sense RNA was as effective as antisense RNA in suppressing gene expression in *C. elegans* [2]. Soon after in 1996, a similar phenomenon called 'quelling' was observed in the fungus *Neurospora crassa* [4]. Later, plant virologists working on improving plant resistance to viral diseases observed that the viral RNA produced by transgenes also inhibited viral replication. This phenomenon was labeled „virus-induced gene silencing" (VIGS). The set of such phenomena were collectively called post-transcriptional gene silencing [5, 6].

In 1998, Craig C. Mello and Andrew Fire reported a gene silencing effect after injecting dsRNA into *C. elegans* [7]. They observed that neither mRNA nor antisense RNA injections had an effect on protein production, but that dsRNA successfully silenced the targeted gene. As a result of this work, they coined the term 'RNAi'. This discovery resulted in the identification of the causative agent of a previously inexplicable phenomenon and for this Fire and Mello were awarded the Nobel Prize in Physiology or Medicine in 2006. Later, this phenomenon was studied in organisms ranging from worms [7] to trypanosomes [8] to flies [9]. Initial experiments in mammals proved disappointing, because introduction of dsRNA triggered the interferon response leading to alteration in cellular metabolism [10]. To avoid this, small RNAs were chemically synthesized and used. This resulted in the desired gene-specific silencing and established that RNAi also operates in mammalian cells [11, 12]. Subsequently, it was established that small interfering RNAs (siRNAs) are the mediators of mRNA degradation in the process of RNAi [13]. In 2003, therapeutic siRNAs were successfully used against hepatitis in a mouse model [14], followed by large-scale screening in mammalian and human cells [15, 16]. In 2004, FDA approved first clinical trials using RNAi. Since then a lot of research has been done in various model systems to develop RNAi for therapeutic purposes. The timeline high-

lighting landmark events in the discovery and development of RNAi is represented in Fig. 1 [17–35].

3 The micromanagers of RNAi pathway

Various types of small RNAs like siRNA, micro RNA (miRNA), repeat-associated siRNA (rasiRNA), short hairpin RNA (shRNA), small-modulatory RNA (smRNA), tiny non-coding RNA (tncRNA) and piwi-interacting RNA (piRNA) are involved in the RNAi machinery according to the needs of the cell.

3.1 siRNA

siRNA is a class of 20–25-nucleotide (nt)-long dsRNA molecules that play a variety of roles in cell biology. In addition to their role in the RNAi pathway, siRNAs also act in RNAi-related pathways, *e.g.*, as an antiviral mechanism or in shaping the chromatin structure of a genome. siRNAs were first discovered in 1999 as part of PTGS in plants [19]. Shortly thereafter, in 2001, synthetic siRNAs were shown to be able to induce RNAi in mammalian cells [11]. This discovery increased the interest in harnessing RNAi for biomedical research and drug development.

3.2 miRNA

miRNAs represent a class of noncoding RNAs encoded in the genomes of plants, invertebrates and vertebrates. They have been found in all multicellular organisms studied and their encoding genes make upto 0.5–1% of the predicted genes in these organisms (200–250 miRNAs in humans). miRNA are 21–23-nt-long single-stranded (ss) RNA molecules involved in regulation of gene expression. miRNAs are encoded by genes that are transcribed from DNA but not translated into protein (non-coding RNA); instead, they are processed from primary transcripts known as pri-miRNA to short-stem-loop structures called pre-miRNA and finally to functional miRNA. Mature miRNA molecules are partially complementary to one or more messenger RNA (mRNA) molecules, and their main function is to down-regulate gene expression. The presence of these short RNA transcripts was first observed in *C. elegans* in 1993 [17].

3.3 rasiRNA

Unlike siRNAs, which derive from both the sense and antisense strands of their dsRNA precursors, rasiRNAs arise mainly from the antisense strand
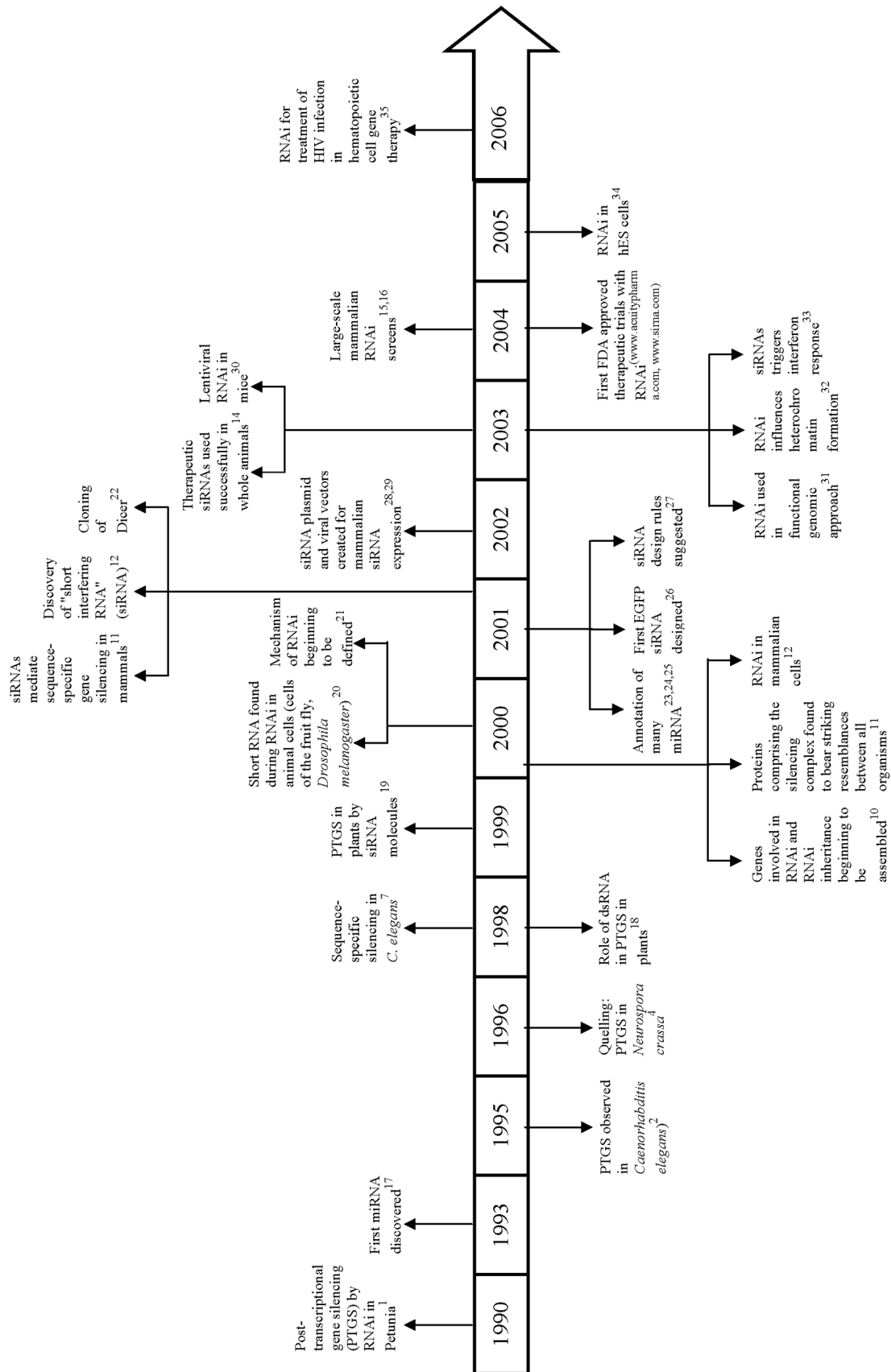
Figure 1. RNA interference - Landmark events in the discovery and development.

[36]. rasiRNAs are involved in the shutdown of expression of transposable elements, histone modification and DNA methylation modification. They function *via* partly overlapping components of the siRNA pathway.

3.4 shRNA

shRNA is a sequence of RNA that makes a tight hairpin turn that can be used to silence gene expression *via* RNAi. shRNA uses a vector for introduction into cells and utilizes the U6 promoter to ensure its expression. This vector is usually passed on to daughter cells, allowing the gene silencing to be inherited. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which then follows the RNAi pathway of the cell for gene silencing (http://en.wikipedia.org).

3.5 smRNA

smRNA are short, non-protein-coding, 20-nt dsRNAs, which are isolated from neural stem cells from the hippocampus of adult mice. This dsRNA has the same sequence as a 21–23-nt stretch of DNA called the neuron reactive silencer elements. This is evolutionarily conserved in vertebrates and bound by a regulatory protein that helps to prevent neuron-specific genes from being expressed in non-neuronal cells. In neuronal cells, where the neuronal genes are expressed, the smRNA interacts with the regulatory protein, and promotes gene expression and neuronal development [37].

3.6 tncRNA

This is a class of short 20–22-nt RNAs described in *C. elegans*. Unlike many miRNAs, the sequences of tncRNAs are not commonly conserved among related species. Although their functions are unknown, many are complementary to mRNA and might target them for degradation.

3.7 piRNA

piRNA is a class of small RNA molecules that is expressed uniquely in mammalian testes and forms RNA-protein complexes with Piwi proteins. These piRNA complexes have been linked to transcriptional gene silencing of retrotransposons and other genetic elements in germ-line cells, particularly those in spermatogenesis. Purification of these complexes has revealed that these oligonucleotides are approximately 29–30 nt long. They are distinct in size from miRNA and are associated with distinct protein complexes. It remains unclear

how piRNAs are generated, but their biogenesis pathway is distinct from miRNA and siRNA (http://en.wikipedia.org).

4 The RNAi pathway

The RNAi pathway is initiated by the enzyme Dicer, which cleaves dsRNA to short double-stranded fragments of 20–25 base pairs. One of the two strands of each fragment, known as the guide strand, is then incorporated into the RNA-induced silencing complex (RISC) and base pairs with an mRNA molecule and induces degradation of that by argonaute, the catalytic component of the RISC complex. The short RNA fragments are known as siRNA when they derive from exogenous sources and miRNA when they are produced from RNA-coding genes in the cell's own genome.

5 Biogenesis of si/miRNA

Two classes of short RNA molecules, siRNA and miRNA have been identified as sequence-specific post-transcriptional regulators of gene expression. Although both were initially discovered in unrelated studies, they are closely related in their molecular characteristics, biogenesis, effector functions, assembly into RNA-protein complexes and ability to regulate gene transcripts negatively in diverse eukaryotes [38–41]. Both of them share a common RNase III-processing enzyme, Dicer, and closely related effector complexes, RISCs, for post-transcriptional repression [42]. Dicer cuts long dsRNA into siRNAs and chops short precursor miRNAs with imperfect stem-loop structure to miRNAs [43]. Gene silencing can be induced by siRNA through sequence-specific cleavage of perfectly complementary mRNA, whereas miRNAs mediate translational repression and transcript degradation for imperfectly complementary targets.

The miRNA pathway begins with the transcription of a primary miRNA (pri-miRNA) from a miRNA gene. The 70–100-nt hairpin RNAs (pri-miRNA) are processed in the nucleus by the ribonuclease Drosha to become precursor miRNA (pre-miRNA). Once the pre-miRNAs are transported into the cytoplasm by exportin 5, a second ribonuclease, Dicer, digests the pre-miRNAs resulting in a 21–25-nt miRNA. At this stage, the miRNA binds the RISC, and aligns with the mRNA. Depending on the level of complementarity between the miRNA and the target sequence, the mRNA can either be translationally repressed (partial) or cleaved (identical). In plants, cleavage

appears to be the primary mode of action, while in mammals translational repressions seems to be the key method [38, 42, 44] (Fig. 2).

The siRNA pathway is an evolutionarily conserved response triggered by an externally introduced dsRNA. The dsRNA is cleaved by the ribonuclease, Dicer, into siRNA of approximately 21–23 nt. The siRNA is loaded into an RISC, which facilitates the separation of the two strands and alignment of the siRNA with its appropriate target mRNA. The siRNA has near perfect complementarity with its target mRNA, and the mRNA cleavage is directed at the site of complementarity [42]. Scientific researchers have utilized the siRNA pathway by artificially introducing either dsRNA or siRNA designed to degrade targeted mRNAs. These synthetic silencing reagents are utilized as molecular biology tools for novel gene identification, gene functional analysis, and biological pathways screens (Fig. 2).

6   Therapeutic application

RNAi can be developed as an endogenous host defense mechanism against many infections and diseases. This defense mechanism has been observed against virus infections in both plants [5, 6] and animals [45, 46]. Studies on the use of RNAi technology against multiple viruses, including human immunodeficiency virus (HIV), influenza virus, and human papilloma virus, have shown promising results by preventing the establishment of productive infection in susceptible cells. In addition to pathogenic viruses, RNAi technology has also been used to target specific cancer genes in melanoma [47], pancreatic adenocarcinoma [48] and leukemia.

The goal of RNAi-based therapy is to activate selective mRNA cleavage for efficient gene silencing. This can be achieved either using a viral vector to express shRNA that resembles micro-RNA precursors, or by introducing siRNAs that mimic the Dicer cleavage product into the cytoplasm.

Apart from siRNAs, miRNAs and anti-miRNA oligonucleotides have also been studied for their therapeutic importance. miRNAs act as natural antisense molecules by negatively regulating the expression of genes with sequences that are complementary to the miRNAs. miRNAs play an important role in the regulation of many genes in key pathways of various cellular processes like cell cycle control, apoptosis [49], hematopoiesis [50], adipocyte differentiation [51] and insulin secretion [52]. Many studies have established the link between miRNAs and human diseases such as cancer [53], neurological and developmental disorders [54],
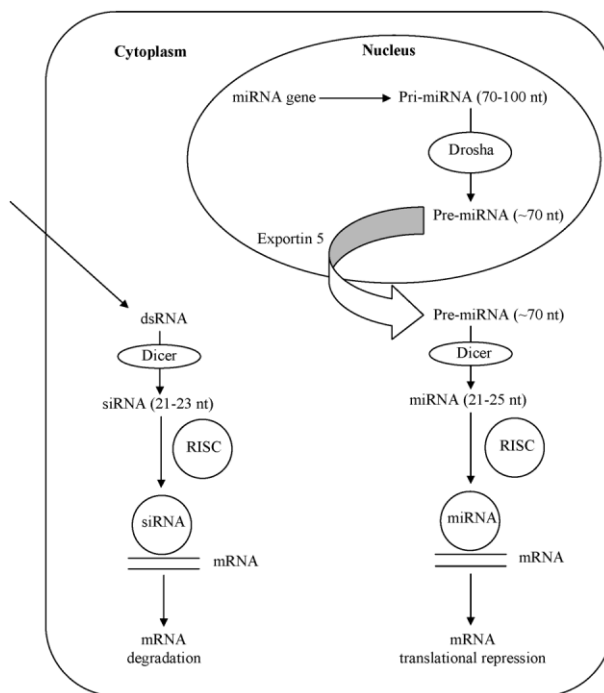

Figure 2. Biogenesis of siRNA and miRNA.

and viral [55] and metabolic diseases [52]. miRNAs play an important role in human cancer pathogenesis [56–58]. Dysfunctioning of an miRNA pathway has been observed to cause neurological disorders like Fragile X syndrome [59] and spinal muscular atrophy [54]. Numerous miRNAs are also involved in developmental regulation of gene expression [60]. miRNAs can also specifically inactivate host cell defense factors in small-sized viral genomes. Recent reports have described miRNAs cloned from a variety of viruses such as herpes viruses [61] and HIV-1 [62]. miRNAs are potential therapeutic targets for the treatment of diabetes and obesity [51, 52]. Modified synthetic anti-miRNA oligonucleotides (AMOs) are useful tools in specifically inhibiting individual miRNAs. This feature can be used to unravel the function of miRNAs and their targets [63].

Synthetic siRNAs harness the naturally occurring RNAi pathway in a manner that is consistent and predictable, thus making them particularly attractive as therapeutics. Moreover, as they enter the RNAi pathway later, siRNAs are less likely to interfere with gene regulation by endogenous miRNAs [64]. As a consequence, siRNAs are the class of RNAi therapeutics that are most advanced in preclinical and clinical studies.

6.1 Cancer

Cancer is a genetic disease in which mutational and/or epigenetic changes in a genome leads to stepwise deregulation of cell proliferation and cell death mechanisms. The ability of RNAi to silence disease-associated genes in cell culture and animal models has encouraged scientists to develop RNAi-based therapeutics to treat diseases including cancer. siRNAs are readily synthesized with low production costs compared to protein or antibody therapies. In addition, siRNAs have favorable pharmacokinetic properties and can be delivered to a wide range of organs [65]. However, their stability in blood and delivery methods are challenges that must be solved for developing effective RNAi reagents for cancer therapy.

Oncogenes expressed at abnormally high levels are attractive targets for RNAi-based therapies against cancers, and such approaches have effectively inhibited tumor growth *in vivo* in mouse models [66]. One successful study involved liposomal delivery of siRNAs targeting the tyrosine kinase receptor *EphA2* gene, which was overexpressed in ovarian cancer cells [67]. After biweekly delivery of siRNAs for 4 weeks, an up to 50% reduction of tumor size was observed. When RNAi therapy was combined with the chemotherapy agent paclitaxel, an up to 90% reduction in tumor size was observed [67]. An RNAi approach was also found to be helpful in overcoming two major problems: relapse of the disease, and P-glycoprotein-mediated drug resistance in cancer chemotherapy [67, 68]. siRNA treatment has also been shown to reduce the expression of the BCR-ABL oncoprotein in leukemia and lymphoma cell lines, leading to apoptosis in these cells [69]. With respect to future medical applications, siRNA-based therapy seems to have a great potential to combat carcinomas, myeloma, and cancer caused by overexpression of an oncoprotein or generation of an oncoprotein by chromosomal translocation and point mutations [70]. RNAi also provides a powerful new way to learn more about genes that trigger or inhibit cancer. Such efforts might pinpoint genes never before linked to cancer and generate novel ideas for treatments.

6.2 Neurodegenerative diseases

Neurodegenerative diseases represent one of the more attractive targets for the development of therapeutic RNAi. In this group of diseases, the progressive loss of neurons leads to the gradual appearance of disabling neurological symptoms and premature death. Currently available therapies aim to improve the symptoms but not to halt the process of neurodegeneration. Neurodegenerative diseases can be classified either as inherited, like Huntington's disease (HD), or sporadic, like Alzheimer's disease (AD) and Parkinson's disease (PD). The increasing prevalence and economic burden of AD and PD has boosted the efforts to develop interventions, such as RNAi, for therapeutic purposes.

HD is a rare inherited neurological disorder caused by a trinucleotide repeat expansion in the Huntingtin (Htt) gene, resulting in production of mutant Htt protein, which causes neuronal cell death in selected areas of the brain. Since HD is caused by expression of a single gene, the progression of the disease can be halted if that particular gene is silenced. A study showed that when the mouse model of HD was treated with siRNA, 60% knockdown in expression of the defective gene was observed, which in turn halted the progression of disease [71]. RNAi-based gene therapy may make the most sense for correcting dominant genetic defects, such as inherited neurodegenerative diseases like Huntington's disease or amyotrophic lateral sclerosis.

The discovery of genetic defects and the development of cellular and animal models for HD have revealed the patho-biological processes underlying AD and PD. However, there is still a lack of good animal models for these diseases and availability of screening tools to diagnose the disorders in their earlier stages. Initial studies of RNAi for AD in mouse model have shown some promising results but the detailed study has to carried out in nonhuman primates before moving to human trails [72].

6.3 Infectious diseases

RNAs have been shown to inhibit infection by HIV, poliovirus, and hepatitis C virus (HCV) in cultured cell lines [73]. Bitko and Barik [74] successfully used siRNAs to silence genes expressed from respiratory syncytial virus (RSV), an RNA virus that causes severe respiratory disease in neonates and infants.

Since then, many other viruses have been successfully targeted by RNAi like HIV-1, HCV, hepatitis B virus (HBV), severe acute respiratory syndrome coronavirus (SARS-CoV) and influenza A virus [75]. HIV *tat, rev, nef* and *gag* genes have been silenced, resulting in inhibition of viral replication in cultured cells [76–79]. In mice, reduction in HBV RNA and replicative intermediates has been demonstrated upon introduction of siRNAs or shRNA vectors [80, 81]. Likewise, the accumulation of influenza viral mRNAs was arrested following

addition of siRNAs specific for nucleocapsid or a component of the RNA transcriptase [82].

6.4 Macular degeneration

The first RNAi therapy to reach patients in clinical trials aims at a debilitating eye disease called macular degeneration. RNAi drugs can be delivered directly to the diseased tissue, *i.e.*, injected into the eye [83]. This direct delivery helps ensure that „naked" RNAi drugs, short strands of RNA that are not packaged and protected in membranes and which quickly break down in the bloodstream, can reach their target intact. Local delivery also makes it less likely that the drugs will have unanticipated, harmful effects elsewhere in the body.

The disease is triggered by a protein called vascular endothelial growth factor (VEGF) that promotes blood vessel growth. In patients with macular degeneration, too much of this protein leads to the sprouting of excess blood vessels behind the retina. The blood vessels leak, clouding and often entirely destroying vision. The new RNAi drugs shut down genes that produce VEGF, thus reducing the leakage from blood vessels and resulting in improved vision [83].

7 Challenges in RNAi therapy

Successful delivery at the site of action is the bottleneck for any of the discovered drugs in general and for targeted drugs in particular. As siRNAs do not cross the mammalian cell membrane unaided, there are two strategies used for delivering siRNAs *in vivo*. One is to stably express siRNA precursors, such as shRNAs, from viral vectors using gene therapy; the other is to deliver synthetic siRNAs by complexing or covalently linking the duplex RNA with lipids and/or delivery proteins. Direct, local delivery has the advantage that the dose of siRNA required for efficacy is lower when injected into or administered at, or near, the target tissue. It might also reduce any undesired systemic side effects. Some of the modifications made to allow the efficient delivery of siRNA are discussed here.

7.1 Naked siRNA

Naked siRNA refers to the delivery of siRNA in saline or other simple excipients, such as 5% dextrose. siRNA can be directly delivered to tissues such as eye, lung and central nervous system. Certain cells have ability to directly take up siRNAs into the cytoplasm where the RNAi machinery operates. The main advantage of this approach is ease

of formulation and direct administration to the target tissues [84]. This approach is being followed in the initial development of RNAi therapeutics and testing in clinical studies. In animal models of ocular neovascularization and scarring, intravitreal injection of saline-formulated siRNA was efficacious [83, 85]. Lipid-formulated siRNA reduced laser-induced choroidal neovascularization in mouse model of age-related macular degeneration (AMD) [84]. Intranasal instillation of siRNAs against viral genes reduced the viral load of RSV and parainfluenza virus [86]. Similarly, intranasal administration of siRNA in a nonhuman primate model of SARS-CoV infection inhibited viral replication in the lung [87]. Saline-formulated siRNA, when directly administered by intracerebroventricular, intrathecal or intraparenchymal infusion, resulted in silencing of specific neuronal mRNA targets in multiple regions of the peripheral and central nervous system [88].

7.2 Conjugation

Covalent conjugation of siRNAs to a targeting molecule has been used to deliver siRNA into cells and can be used for cell type-specific targeting. In conjugation, only one of the strands in the duplex is active and conjugates can be attached to the sense or passenger strand without disrupting the activity of the active antisense strand. Conjugates can be placed on either 5′- or 3′-end of the sense strand or on the 3′-end of the antisense strand. siRNA conjugates have been made using lipophilic molecules, proteins, peptides and aptamers. Conjugation to cholesterol has been adapted to achieve effective *in vivo* silencing by siRNAs [89]. Other natural ligands, like transferrin, folate, RGD (Arg-Gly-Asp) peptides, and membrane permeant peptides, such as penetratin and transportan, have been directly conjugated to siRNA duplexes to facilitate delivery, *in vitro*. siRNAs can also be conjugated to RNA aptamers for delivery to specific cell types [90].

7.3 Liposomes/lipoplexes

Liposomes are formulated to improve the pharmacokinetic property and decrease the toxicity of a drug. Polar drugs can be entrapped in the aqueous center of liposomes. Liposomes can fuse with cell membranes and enhance drug delivery into cells. When lipids complex with nucleic acids to form amorphous particles, they are known as lipoplexes. Lipoplexes are formed by mixing siRNAs with transfection agents like Lipofectamine 2000. Both liposomes and lipoplexes have been extensively used to deliver siRNA *in vitro* and *in vivo*. The local

and systemic administration of siRNA using liposomes and lipoplexes has shown promising results [91]. Local injection of liposomes and lipoplexes effectively delivered siRNA to target cells in the eye and nervous system, and to tumors [92–94]. An area of increasing interest is direct application of lipoplexed siRNA to mucosal surfaces such as the vagina and the intestine. Because of the relatively easy access to mucosal sites, the large number of viral infections that enter the body through mucosal portals, the general tolerability of siRNA-lipid formulations and the robust *in vivo* delivery, mucosal administration of lipid formulated siRNA will be a fertile area for developing future RNAi therapeutics.

7.4 Peptides and polymers

siRNA can also be complexed with cationic peptides and polymers by ionic interactions with their negatively charged phosphate backbones to form stable nanoparticles. To prevent aggregation and stabilize particle size, molecules such as polyethylene glycol (PEG) are incorporated. PEG groups also improve the pharmacokinetic profile of these particles by shielding positive charges on the surface that will stick to negatively charged cell membranes and result in rapid clearance from the circulation. One of the most widely studied polymers for delivery of nucleic acids is polyethylenimine (PEI). PEI polymers are synthetic, linear or branched structures with high cationic charge densities. The cationic polyplexes formed by PEI with siRNA interact with the cell surface electrostatically and are endocytosed into cells where they disrupt the low endosomal pH. PEI–siRNA complexes have been reported to show therapeutic benefit *in vivo* in a number of disease models [95, 96]. A main disadvantage of using PEI as a therapeutic delivery vehicle is the extreme toxicity seen at higher doses. Other synthetic polycations consisting of histidine and polylysine residues have also been evaluated for delivery of siRNA and seem to have improved *in vitro* efficacy compared with PEI.

Cationic peptides can also deliver oligonucleotides into cells [96]. Several peptide-based gene delivery systems, formed with peptides like penetratin, promote the uptake of noncovalently bound siRNAs *in vitro*. Peptide-based approaches can also be coupled with other delivery systems, such as liposomes, to enable a more targeted delivery of siRNAs [97, 98].

7.5 Antibodies

Antibodies can also be used for specific targeted delivery of siRNA. In *in vitro* studies, it was observed that the protamine-antibody fusion protein efficiently delivered non-covalently bound siRNA to HIV-envelop-expressing B16 melanoma cells or HIV-infected primary CD4 T cells. A protamine fragment binds to siRNA and Fab fragment of the HIV envelope antibody and mediates receptor-specific binding to cells expressing the HIV envelope protein. These siRNA antibody–protamine complexes, when administered intratumorally or intravenously, specifically delivered siRNA to subcutaneous tumor and retarded its growth [99]. Recently, fusion proteins that target all human white blood cells have revealed selectivity of this targeting strategy both *in vitro* and *in vivo* [100]. These studies demonstrate the potential for antibodies to direct siRNA selectively into cells *in vivo*.

8 Drug discovery and development in RNAi

The initial steps involved in identifying lead siRNA candidate are bioinformatics design, and *in vitro* studies to determine efficacy, to verify that unwanted effects are absent and to introduce chemical modifications to improve stability and specificity. Three important attributes to be considered during designing and selection of siRNA are potency, specificity and nuclease stability [84]. To establish the efficiency of RNAi as a therapeutic agent, another important parameter is efficient delivery of siRNA to the target tissue. The unwanted effects to be avoided or minimized are silencing of genes sharing partial homology to siRNA and stimulation of immune system due to certain siRNAs.

8.1 Potency

In the mammalian cells, RNAi can be activated by introducing siRNAs that mimic Dicer-cleaved, endogenous miRNAs. Effective siRNAs can be identified by empirical testing, which is based on their common features. This can be done using algorithms available on the web [101]. Algorithms can increase the chances of identifying active siRNA, but they are imperfect and can sometimes miss the most potent siRNAs. Another alternative to identify active siRNAs is to test all the sequences experimentally and select a group of candidates that induce effective silencing at the lowest concentrations. A study also suggests the use of slightly longer siRNAs as Dicer substrate for maintaining silencing activity, but longer siRNAs are more com-

plicated to synthesize and can activate undesired immune responses [102]. Designing the siRNAs can also be done by manipulation during strand selection [103]. Single nt substitution at the 5'-end of the duplex that favors its incorporation into RISC can increase the chances of identifying a potent duplex.

8.2 Specificity

RNAi-mediated silencing of gene expression can be highly specific. Nevertheless, siRNAs also recognizes and interferes with the expression of mRNAs that share partial homology with the target mRNA, although the alteration at the mRNA levels of off-target genes is usually less than threefold [104]. One approach for minimizing off-target effects is the careful design of siRNA to minimize complementary regions between off-target genes and either of the strands of siRNA duplex [105]. Chemical modifications of riboses like 2'-O-methyl modifications of guide strand can also be done to suppress off-target effects without significantly interfering with on-target silencing [106]. Thus, bioinformatics design and position-specific, sequence-independent chemical modifications can be used to reduce sequence-related off-target effects, while maintaining efficient target-mRNA silencing.

siRNA can also activate innate immune response and induce potential unwanted effect. dsRNA longer than 30 nt or smaller siRNAs at higher concentration are recognized by the serine/threonine protein kinase PKR, thus triggering the pathway and resulting in global translational blockade and cell death [107]. These dsRNA also have the potential to activate certain receptors on plasmacytoid dendritic cells, thus triggering the production of type I interferons and pro-inflammatory cytokines and induce nuclear factor-κB (NF-κB) activation [108]. To minimize this, the siRNAs can be transfected into plasmacytoid dendritic cells and the ones that induce interferon expression can be discarded. The presence of 2'-O-methyl modifications in the siRNA duplexes can also be used to minimize the binding of siRNAs to receptors and suppress the immunostimulatory activity [109].

8.3 Stability

In human plasma, naked siRNA has a half-life of minutes [110]. To convert siRNAs into drugs, it is necessary to prolong its half-life without affecting its biological activity. The siRNA duplex can be stabilized by simple chemical modifications. The min-

imum modification required to stabilize a particular duplex can be identified by studying its degradation fragments in the plasma, thus avoiding any associated toxicities and changes causing reduced activity. The introduction of phosphorothioate (P=S) backbone linkage at the 3'-end protects against exonuclease degradation, and 2'-sugar modification, like 2'-O-methyl or 2'-fluoro, provides endonuclease resistance [111]. These simple modifications can stabilize an siRNA duplex while maintaining silencing activity. Chemical modifications at the 2' position of the ribose, increases siRNA stability by providing resistance to RNase activity. 2'-O-Methylmodified siRNAs have been shown to provide greater protection against *in vivo* infection with HBV when compared with unmodified siRNAs [112].

When siRNAs are formulated into carrier systems for *in vivo* delivery, they need to be protected from nuclease digestion, especially if they are exposed to a nuclease-rich environment (such as blood) or if they are formulated with excipients that do not provide nuclease protection. The nuclease-stabilized siRNA show improved pharmacokinetic properties *in vivo* [112]. Several studies have showed that the chemical modifications in nucleobases, sugars, and the phosphate ester backbone of siRNA can reduce siRNA sensitivity to nucleases [113, 114]. A number of chemical modifications have been observed to increase nuclease resistance and were still able to induce siRNA-mediating gene silencing, provided that the modifications were absent in specific regions of the siRNA and included to a limited extent [115, 116]. Increased nuclease resistance of siRNA is expected to increase *in vivo* silencing effects, but a study showed that unmodified siRNA had a similar potency as the stabilized version after hydrodynamic injection in the liver [117]. In contrast, in another study chemical modifications were shown to enhance therapeutic effects in a mouse model of HBV infection. The indicators of viral infection were 30-fold higher in animals treated with unmodified siRNA as compared to chemically modified siRNA [112]. The effect of nuclease stabilization on the longevity of mRNA silencing still needs to be determined.

8.4 Delivery

The efficient delivery of siRNAs is a vital step in RNAi-based gene silencing. The effectiveness of the RNAi as a therapeutic agent greatly depends on the efficient delivery of siRNA to the correct intracellular location, to interact with the RNAi machinery within the target cell, within the target tissue responsible for the pathology. Each of these levels

of targeting poses a significant barrier. Several strategies have been developed to overcome these barriers, such as chemical modifications of siRNA, viral nucleic acid delivery systems, and nonviral nucleic acid delivery systems. The method widely used to facilitate the efficient delivery of siRNA is high-pressure intravenous (i.v.) injection [118, 119]. In this method, a large volume of siRNA solution is injected into the circulation in a relatively short time; this puts the recipient at risk for acute heart failure. Also, hydrodynamic injection delivers siRNA throughout the body, thus inducing undesirable suppression of gene expression in some organs [119]. Later on, mechanical methods like electroporation and sonoparation were developed to deliver siRNA to the target tissue [120, 121]. Electroporation is a convenient and efficient method in which electrical shocks are applied to the membrane, producing pores, and allowing the negatively charged external molecules to enter into the cytosol by electrophoresis [120]. Electroporation has been found useful for the intracellular delivery of siRNA to muscle [122], brain [120] and kidney [119]. This method requires the insertion of electrodes into the target area, thus involving invasive procedures and limiting its range of application. Sonoporation is a method in which ultrasound is used to increase the porosity of the cell membrane to allow large molecules in the surrounding medium to enter the cell [121]. This method has been used to deliver siRNA into whole organs like heart. The main problem encountered by this method is its low efficiency, which needs to be addressed [119].

9 Clinical trials

RNAi has rapidly advanced from research discovery to clinical trials (Table 1). Three RNAi therapeutics are currently under clinical investigation, with many others ready to enter trials soon. Initial trials have focused on well-validated therapeutic targets, such as VEGF pathway for wet AMD, and RSV genome for RSV infection. The only approved siRNA-based drugs are used in the eye, Vitravene for retinitis [123] and Macugen for AMD [124]. Direct injection into the vitreal cavity efficiently targets siRNA drugs to the retina. Also, since the ocular compartment is relatively free of nucleases, it permits the unmodified siRNA to be used in the vitreal cavity. The ease of drug delivery combined with the validation of VEGF as a therapeutic target has led to the rapid development of several RNAi therapeutics for eye diseases. Intravitreous injection of Cand5, an unmodified siRNA has completed a

Phase II trial in patients with serious progressive wet AMD and has been reported to provide dose-related benefits including near vision and lesion size. Cand5 is also being tested for efficacy against diabetic macular edema in a Phase II trial (www.acuitypharma.com). Sirna-027, a chemically modified siRNA has completed Phase I trials in patients with wet AMD and was reported to be well tolerated. It was also reported to stabilize or improve vision in some patients (www.sirna.com). The delivery of siRNA therapeutics directly to the lungs for targeting lung epithelial cells also has many advantages. Lung epithelial cells can uptake naked siRNA, mediate RNAi silencing and are nuclease free [86]. Delivery to the lung by inhalation directly targets the tissue epithelium, thus improving drug concentrations at the target tissue, reducing drug dose and decreasing the chances of systemic side effects. In many cases, naked siRNA in saline has been effective, but other approaches to optimize lung delivery have to be developed for different indications. The first pulmonary studies are directed at treating RSV, a serious neonatal respiratory infection. Two Phase I trials with ALN-RSV01, an siRNA targeting the viral nucleocapsid gene, have been completed and was found to be safe and well tolerated (Alnylam pharmaceuticals). Many RNAi-based drugs have shown promising results in preclinical and clinical trials and are expected to have a potential to be developed as a new class of therapeutics (Table 1).

10 Conclusion

The initial limitation in the use of RNAi technology as therapeutics is designing of an effective siRNA sequence. Even if the recommended rules for siRNA design are followed, it does not ensure effective silencing of the target gene. The efficacy of siRNA-mediated suppression of gene expression depends on a number of factors like the structure of siRNA, the receptiveness of the cell type to siRNA uptake and half-life of the target protein, to achieve optimal silencing [102, 125]. The other most important factor is the stability of siRNAs. Although siRNAs are relatively stable in cell culture conditions, they require enhanced nuclease and thermodynamic stability when in the blood circulation *in vivo*. Many types of chemical modifications of siRNAs are being explored to enhance the stability, but there are mixed opinions about which modification is most effective at enhancing stability without compromising target-silencing activity [126]. Advances are being made towards the goal of making siRNAs suitable for therapeutic purposes,

Table 1. RNAi drugs in the pipeline[a]

Disease/Condition	Company	Organ	Technology	Approach	Status
AMD	Sirna therapeutics	Eye	Chemically synthesized siRNAs	Blocking VEGF pathway	Phase II clinical trial
AMD	Acuity pharmaceuticals	Eye	Unmodified siRNAs	Blocking VEGF pathway	Phase II clinical trial
RSV infection	Alnylam pharmaceuticals	Lung	Direct RNAi (local delivery of siRNA)	Silencing nucleocapsid gene thus inhibiting virus multiplication	Phase II clinical trial
Hair removal	Sirna therapeutics	Skin	Chemically synthesized siRNAs	Blocking hair growth genes thus disrupting hair follicle integrity	Phase I clinical trial
Asthma	Sirna therapeutics	Lung	Chemically synthesized siRNAs	Targets Th2 cytokines, which play a critical role in inflammation and broncho-constriction in the airways	Phase I clinical trial
AIDS/AIDS related lymphoma	Benitec	Lymphoids	DNA-directed RNAi (ddRNAi)	Production of HIV-resistant white blood cells indefinitely	Phase I clinical trial
Hepatitis B	Nucleonics	Liver	Cationic-lipid delivery of siRNA	Destruction or elimination of all RNA species produced by HBV within an infected cell	Phase I clinical trial
Huntington's disease	Sirna therapeutics	Central Nervous System (CNS)	Adeno-associated viral vector delivered siRNA	Targets the expression of Huntingtin gene	Preclinical development
Hepatitis C	Sirna therapeutics	Liver	Multiple chemically modified siRNAs	Inhibiting viral replication and reducing the selection of drug resistant mutants	Preclinical development
Parkinson's disease	Alnylam pharmaceuticals	CNS	Chemically synthesized siRNAs	Effectively reduced α-synuclein levels	Preclinical development
Liver cancer	Alnylam pharmaceuticals	Liver	Liposomal delivery	Silencing genes for VEGF and kinesin spindle protein	Preclinical development
Influenza	Galenea	Lung	Chemically synthesized siRNAs	Preventing viral replication in airway epithelium	Preclinical development

a) AMD, Age-related macular degeneration; RSV, respiratory syncytial virus; VEGF, vascular endothelial growth factor

and RNAi as a emerging way to restore health, but to answer the question – Will RNAi be a new class of biologicals? – we still have to wait.

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