RNAi mechanisms and applications

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Within the past two decades we have become increasingly aware of the roles that RNAs play in regulation of gene expression. The RNA world was given a booster shot with the discovery of RNA interference (RNAi), a compendium of mechanisms involving small RNAs (less than 30 bases long) that regulate the expression of genes in a variety of eukaryotic organisms. Rapid progress in our understanding of RNAi-based mechanisms has led to applications of this powerful process in studies of gene function as well as in therapeutic applications for the treatment of disease. RNAi-based therapies involve two-dimensional drug designs using only identification of good Watson-Crick base pairing between the RNAi guide strand and the target, thereby resulting in rapid design and testing of RNAi triggers. To date there are several clinical trials using RNAi, and we should expect the list of new applications to grow at a phenomenal rate. This article summarizes our current knowledge about the mechanisms and applications of RNAi.

INTRODUCTION

RNA interference (RNAi) is a regulatory mechanism of most eukaryotic cells that uses small double-stranded RNA (dsRNA) molecules as triggers to direct homology-dependent control of gene activity (Figure 1) (1). Known as small interfering RNAs (siRNA), these ~21–22 bp long dsRNA molecules have characteristic 2 nt 3′ overhangs that allow them to be recognized by the enzymatic machinery of RNAi, which eventually leads to homology-dependent degradation of the target mRNA. In mammalian cells, siRNAs are produced from cleavage of longer dsRNA precursors by the RNase III endonuclease Dicer (2), or they can be synthesized by chemical or biochemical methods. Dicer is complexed with RNA-binding proteins, the TAR-RNA-binding protein (TRBP), PACT, and Ago-2, which are involved in the hand-off of siRNAs to the RNA-induced silencing complex (RISC) (3). The core components of RISC are the Argonaute (Ago) family members. In humans there are eight members of this family but only Ago-2 possesses an active catalytic domain for cleavage activity (4,5). While siRNAs loaded into RISC are double-stranded, Ago-2 cleaves and releases the "passenger" strand, leading to an activated form of RISC with a single-stranded "guide" RNA molecule that directs the specificity of the target recognition by intermolecular base pairing (6). Rules that govern selectivity of strand loading into RISC are based on differential thermodynamic stabilities of the ends of the siRNAs (7,8). The less thermodynamically stable end is favored for binding to the PIWI domain of Ago-2.

MicroRNAs

An important arm of RNAi involves the microRNAs (miRNAs). These are endogenous duplexes that posttranscriptionally regulate gene expression by complexing with RISC and binding to the 3′ untranslated regions (UTRs) of target sequences via short stretches of homology, termed "seed sequences" (9,10). The primary mechanism of action of miRNAs is translational repression, although this can be accompanied by message degradation (11). The miRNA duplexes possess incomplete Watson-Crick base pairing, and the antisense strand cannot be chosen by cleavage of the passenger strand as it is for siRNAs; therefore the antisense strand must be chosen by an alternative mechanism (12–14). miRNAs are endogenous substrates for the RNAi machinery. They are initially expressed as long primary transcripts (pri-miRNAs), which are processed within the nucleus into 60–70 bp hairpins by the Microprocessor complex, consisting of Drosha and DGCR8 (15,16) into pre-miRNAs. The pre-miRNAs are further processed in the cytoplasm by Dicer and one of the two strands is loaded into RISC, presumably via interaction with one of the Dicer accessory proteins (3). Importantly, it is possible to exploit this native gene silencing pathway for regulation of gene(s) of choice. If the siRNA effector is delivered to the cell it will "activate" RISC, resulting in potent and specific silencing of the targeted mRNA. Because of the potency and selectivity of RNAi, it has become the methodology of choice for silencing specific gene expression in mammalian cells.

RNAi as a Therapeutic Approach for Treatment of Disease

Control of disease-associated genes makes RNAi an attractive choice for future therapeutics. Basically every human disease caused by activity from one or a few genes should be amenable to RNAi-based intervention. This list includes cancer, autoimmune diseases, dominant genetic disorders, and viral infections. RNAi can be triggered by two different pathways: (*i*) an RNA-based approach where synthetic effector siRNAs are delivered by various carriers to target cells as preformed 21 base duplexes; or (*ii*) via DNA-based strategies in which the siRNA effectors are produced by intracellular processing of longer RNA hairpin

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transcripts (reviewed in References 17 and 18). The latter approach is primarily based on nuclear synthesis of short-hairpin RNAs (shRNAs), which are transported to the cytoplasm via the miRNA export pathway and are processed into siRNAs by Dicer. While direct use of synthetic siRNA effectors is simple and usually results in potent gene silencing, the effect is transient. DNA-based RNAi drugs, on the other hand, have the potential of being stably introduced when used in a gene therapy setting, allowing, in principle, a single treatment of viral vector-delivered shRNA genes.

The first clinical applications of RNAi have been directed at the treatment of age-related macular degeneration (AMD), which causes blindness or limited vision in millions of adults annually (19,20). Therapies based on RNAi are also currently being developed for viral infection, including human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV and HCV), and respiratory syncytial virus (RSV) (21). Strategies for the treatment of neurodegenerative diseases and cancers are also well under way.

Although successful in vivo studies have shown the potential effectiveness of RNAi-based therapies, other studies have illustrated specific approaches to avoid when adopting an endogenous cellular mechanism for therapeutic benefit. Unwanted side effects have included activation of Toll-like receptors (TLRs) and type 1 interferon responses, and competition with the endogenous RNAi pathway components (22). These findings indicate that although RNAi is potentially a revolutionary mechanism for treatment of disease, due caution is necessary when interpreting results from RNAi-mediated target knockdowns.

The challenge of cell- or tissue-specific delivery of siRNAs is also crucial when investigating the utility of RNAi-based therapies for a given disease; various strategies for nonviral and viral delivery of RNAi triggers have shown to be effective in their respective disease models. The relative advantages and disadvantages of using synthetic siRNAs versus expressed shRNAs must also be taken into consideration when designing RNAi-based therapies for a particular disease.

Chemically synthesized siRNAs are commonly screened for effective knockdown of a specific target gene. To increase siRNA stability, chemical modifications are introduced, such as 2′-O-methylpurines or 2′-fluoropyrimidines (23). When initially designing a siRNA molecule, computational algorithms are routinely used that incorporate various parameters, including siRNA duplex end stabilities for proper strand selection and mRNA secondary structures for target site accessibility. To improve the potency of an RNAi response, siRNA duplexes can also be designed to mimic substrates for Dicer processing (24). Longer siRNAs (e.g., 27-mers) are incorporated into the Dicer loading step of the RNAi pathway and may facilitate the activation of RISC. 27-mers are designed asymmetrically to exhibit a 2 nt 3′ overhang on one end and a blunt region on the other (25), which guides Dicer processing and biogenesis of the proper guide strand, since the PAZ domain of Dicer recognizes the overhang end. Furthermore, because of the efficiency of 27-mers in mediating gene silencing, a lower concentration of siRNAs can mediate a potent RNAi response.

From the perspective of therapeutic applications of RNAi, the most important concern is delivery of the siRNAs to the appropriate tissue. Numerous recent publications have shown that siRNAs can be systemically delivered to various tissues with resultant knockdown of target RNAs. Intravenous injection of siRNAs for systemic delivery is accomplished through conjugation of siRNA molecules to a cholesterol group or the packaging of siRNAs into liposomal particles. Systemic delivery using these approaches is effective

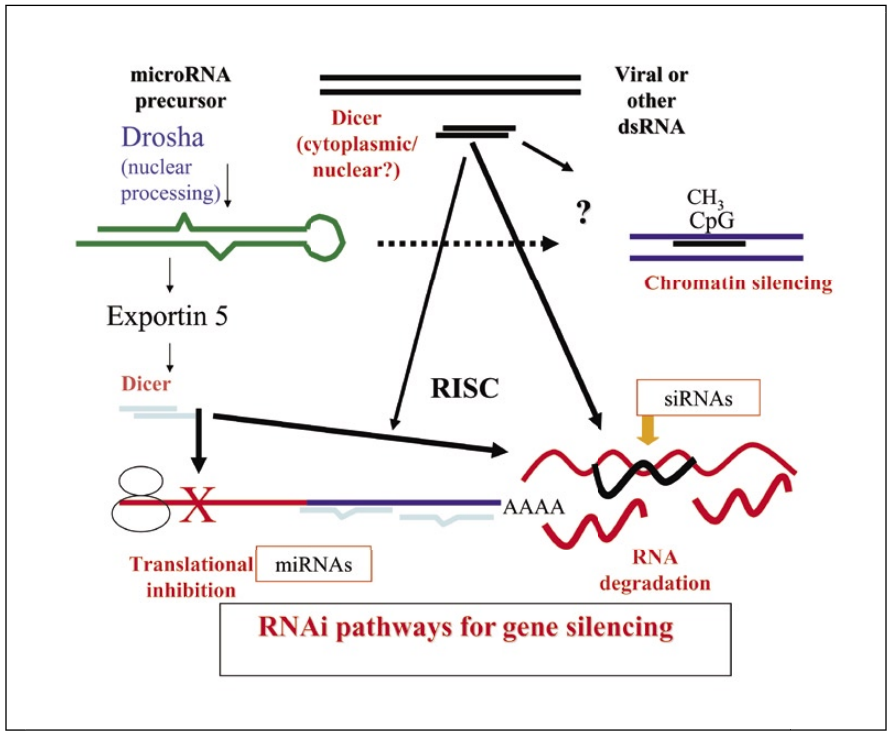

Figure 1. Cellular pathways of gene silencing by RNA interference. RNAi is multifaceted, and there are various pathways in which small double-stranded RNA (dsRNAs) regulate gene expression. The endogenous micro-RNA (miRNA) pathway begins with Pol II-transcribed primary miRNAs that are processed in the nucleus to pre-miRNAs, exported to the cytoplasm, and processed again into functional miRNAs. The primary function of miRNAs is to inhibit translation via incomplete Watson-Crick base pairing to the 3′ untranslated regions of targeted mRNAs. Alternatively, perfectly duplexed small interfering RNAs (siRNAs) can be produced intracellularly or supplied exogenously to cells. The guide strand is incorporated into the RNA-induced silencing complex (RISC), where it guides sequence-specific degradation of the target transcript, irrespective of where the base pairing occurs. The miRNA and siRNA pathways are interchangeable, and the important determinants are the positions within the message and the extent of base pairing with the targeted transcripts. siRNAs can also trigger transcriptional gene silencing via interactions with chromatin, wherein they guide histone and DNA methylation leading to inactive chromatin.

for delivery to the liver and jejunum, but may not be appropriate for delivery to other organs. In a proof-of-concept study, siRNAs targeting apolipoprotein B (APOB) were used to modify cholesterol metabolism. The 3′ hydroxyl group on the siRNA passenger strand was chemically linked to a cholesterol group, and these conjugated siRNAs effectively knocked down gene expression by >50% in the liver and 70% in the jejunum (26).

Another approach for systemic delivery involves the use of specialized lipid bilayers called stable nucleic acid-lipid particles (SNALPs), which incorporate chemically modified siRNAs (27). Cationic and neutral lipids comprise the bilayer, along with an outer hydrophilic coating of polyethylene glycol (PEG). In one study, monkeys were administered a single dose of siRNA-containing SNALPs, which lowered cholesterol levels for 11 days or longer, with <10% of APOB expression remaining in the liver of this nonhuman primate model (28). No noticeable toxicities were observed, suggesting the potential utility of this method in systemic delivery.

For the in vivo efficacy of siRNA molecules, the dosage of delivered siRNAs is a practical consideration, and selective delivery of siRNAs to specific tissues would potentially lower the effective dosage required. Targeting cell surface receptors is an advantageous approach, as it would lower the siRNA dosage and potentially avoid off-target effects from siRNA delivery to irrelevant tissues. The coupling of siRNAs to aptamers or antibody fragments, or the use of nanoparticles coated with receptor-specific ligands, allows for the specific delivery of siRNA payloads to targeted cells and tissues. For targeting of HIV-infected cells, siRNAs were coupled to heavy chain antibody fragments (Fabs) that recognize the HIV envelope glycoprotein gp120. Positively charged protamine was conjugated to Fab molecules, and the negatively charged siRNAs interacted electrostatically with the protamine to form a Fab-siRNA complex. This antibody-based approach demonstrated >70% knockdown of p24 group-specific antigen protein (Gag) when targeting cultured T lymphocytes infected with HIV-1 (29). A different targeting approach took advantage of a peptide from rabies virus, which specifically binds to the acetylcholine receptor. When this peptide was conjugated to a polyarginine peptide that binds siRNAs, delivery of siRNAs to the central nervous system was accomplished, resulting in inhibition of a fatal encephalitis viral infection (30).

Aptamers, which are structured RNA ligands, can be designed to bind specifically to cell surface receptors and be covalently linked to siRNAs for specific in vivo delivery. One method used aptamers that bind to the prostate-specific membrane antigen (PSMA) expressed on the surface of prostate cancer cells. When conjugated to siRNAs, these aptamer-siRNA hybrids effectively reduced tumor growth in mice (31). A similar approach using both bioti-

nylated siRNAs and aptamers bound to the biotin-binding protein streptavidin made use of 27-mer siRNAs to potently induce gene silencing (32).

Coating nanoparticles with cell type–specific ligands is another powerful approach to systemically deliver RNAi-inducing molecules. In an important proof-of-concept study, Ewing sarcoma tumors were targeted in vivo with transferrin ligand-coated nanoparticles (33). These nanoparticles were constructed using cyclodextrin-containing polycations (CDPs) specifically designed to incorporate negatively charged siRNA molecules. For added stability and to prevent aggregation, PEG polymers were attached to the outer surface using terminal adamantane groups. Transferrin ligands were then covalently linked to the adamantane-PEG chains, and the nanoparticle design allowed for self-assembly into uniform, ~50 nanometer-sized nanoparticles. The nanoparticle-incorporated siRNAs targeted the *Ews-Fli1* (*Ewing sarcoma breakpoint region 1-flightless 1 homolog*) gene fusion product and were shown to inhibit tumor l formation in mice (33).

Concluding Remarks

In summary, the progression from the initial discovery of RNAi to its clinical applications has been astounding. Understanding the fundamental biology of RNAi has led to its widespread applications in basic research and subsequently in applications for the treatment of disease. Within the next few years we should expect to unravel more RNAi-mediated regulation of gene expression, and will also see RNAi-based drugs approved for use in the treatment of disease. In addition, RNAi has proven to be a powerful tool for the study of gene function and has opened new areas of basic investigation. In the near future we should see continued development in our understanding and application of this remarkable cellular mechanism for posttranscriptional regulation of gene expression.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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