Advances, Nuances, and Potential Pitfalls When Exploiting the Therapeutic Potential of RNA Interference

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The discovery of RNA interference (RNAi) holds the potential to alter the paradigm of medical therapeutics. With the ability to selectively silence the function of a gene, RNAi not only provides an indispensable research tool for determining the function of a gene, but also offers potential for the development of novel therapeutics that will inhibit specific genes involved in disease. New concepts in therapeutics have been uncovered through the study of RNAi. Nuances have emerged. For instance, global RNAi pathways can be affected by somatic mutations in cancer and cellular stress, such as hypoxia. Also, viral gene therapy can have unexpected effects on endogenous short noncoding RNA pathways. Therefore, it is important to understand where RNAi therapeutics enter the processing pathways. We highlight the evolving use of RNAi as a new class of therapeutics, such as for amyloidosis, and address some of the anticipated challenges associated with its clinical application.

OVERVIEW OF RNA INTERFERENCE (RNAi)

It was a huge surprise when it was discovered that RNA molecules could silence gene function in a specific fashion. Fire and Mello's discovery in 1998 that double-stranded RNA could regulate gene expression, a discovery that would later earn them a Nobel Prize in 2006, sparked a new field of research.[1] Although it had long been appreciated that long double-stranded RNA molecules could inhibit gene expression in a global and nonspecific fashion, what was paradigm shifting was the discovery that the process could be elicited by short sequences of RNA and that this inhibition was sequence-specific.

We now know that RNAi can be mediated by single- or double-stranded RNA. When it takes the form of short interfering RNAs (siRNAs), the sequence-specific degradation of messenger RNA (mRNA) is elicited by the base pairing of complementary RNA strands, each approximately 21 to 25 nucleotides in length (**Figure 1**).[2,3] These molecular complexes, which are termed small interfering RNA (siRNA) duplexes, can be delivered exogenously to cells as small duplexed intermolecular double-stranded RNA complexes. Importantly, this is a potent therapeutic tool. The challenge, however, is getting them into the cells. siRNA can also be generated through the cleavage of endogenous long intermolecular double-stranded RNA complexes or from a long single-stranded RNA that has taken the form of a

short hairpin RNA (shRNA), the stem and loop of intramolecular base-pairing. Importantly, the production of this latter synthetic shRNA from gene-therapy vectors (either viral or nonviral) is an efficient means of eliciting RNAi *in vivo*. This is an effective therapeutic approach, especially since viral vectors are a potent way of getting exogenous RNA molecules into cells.[4]

A related endogenous pathway also exists in mammalian cells. First discovered in 1993, microRNAs (miRNAs) are highly conserved across evolution and represent a potent regulatory pathway that involves 21 to 25-nt single-stranded RNA molecules.[5,6] Although not fully appreciated at the outset, we now know that hundreds of discrete regions within the human genome encode atypical genes that give rise to miRNAs.

Production of miRNAs

Individual miRNAs can increase or decrease in disease (**Figure 1**). Surprisingly, the processes that control the steady-state levels of mRNAs are related but not identical to the processes that control the steady-state levels of miRNAs. The production of miRNAs begins in the nucleus with the transcription of primary miRNA transcripts (pri-miRNAs). They are transcribed by RNA polymerase II, the enzyme that typically produces mRNA destined for translation into proteins. MicroRNAs are atypical genes, given that they do not encode a protein. Instead, they produce RNA

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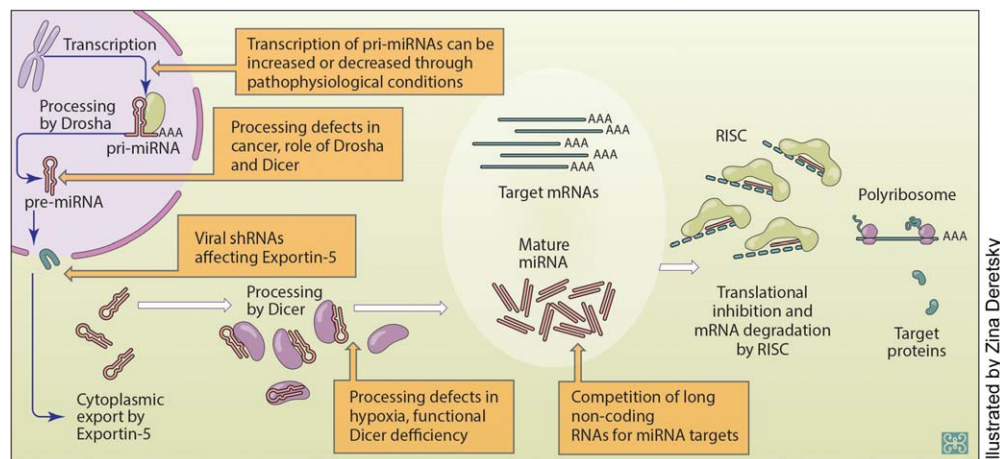
Figure 1 MicroRNA processing steps in health and disease. MicroRNAs are initially transcribed in the nucleus as primary microRNA transcripts (pri-miRNAs), where they are processed by Drosha into precursor microRNAs (pre-miRNAs). Pre-miRNAs are then exported into the cytoplasm by Exportin-5 in a Ran-GTP-dependent manner, where they are processed by Dicer into 22nt mature miRNAs. They are then incorporated into RNA-induced silencing complexes (RISCs), which are effector complexes that mediate miRNA-dependent translational inhibition and/or mRNA degradation. In disease these canonical pathways can be affected. Transcription of pri-miRNAs can be increased or decreased in pathophysiological conditions. This results in increases or decreases in mature miRNA levels, respectively. Cancer cells can be deficient in Drosha or Dicer. Viral gene therapy can produce so much synthetic shRNAs that they compete with pre-miRNAs for nucleo-cytoplasmic transport. Hypoxia can lead to functional deficiency of Dicer. Long noncoding RNAs can be differentially expressed in disease. These molecules, which do not encode a protein, can serve as molecular sponges for miRNAs, thus limiting their silencing effect on target protein-coding mRNAs.

species that regulate the expression of proteins. Transcription controls the level of expression of individual miRNAs, as with protein-coding mRNAs. However, maturation processes for miRNA precursors are also relevant. Important details regarding canonical miRNA biogenesis are now clear. These concepts are important because the pathways are regulated and can be deficient in disease. The pri-miRNAs are processed by the nuclear RNase III enzyme Drosha to generate 70 nt long precursor miRNAs (pre-miRNAs), which are then exported into the cytoplasm by Exportin-5/Ran-GTP or Exportin-1/CRM1. Here, pre-miRNAs are processed by the cytoplasmic RNase III enzyme Dicer to generate a mature 22 nt double-stranded miRNA duplex, with characteristic 2 nt 3′-overhangs. This complex is then loaded, with the aid of Dicer, onto effector RNA-induced silencing complexes (RISCs) that contain Argonaute proteins (AGOs), especially AGO1 and AGO2. As part of RISCs, the guide strand miRNA directs translational inhibition and/or mRNA degradation while the passenger strand is ejected and possibly cleaved. Importantly, depending on cellular context, either strand may be predominant in terms of steady-state levels and/or activity over the other. The majority of miRNAs are produced via the canonical biogenesis pathway. However, miRNAs can also be generated via alternative pathways that are independent of Drosha or Dicer. As an example, miR-451 is a Dicer-independent microRNA. Instead, AGO2 that is dependent on AGO2 directly cleaves pre-miR-451 to generate mature miR-451.[7]

The stability of miRNAs contributes to steady-state miRNA expression. Labile and stable miRNAs have been described. In this respect, miRNAs exhibit the same type of posttranscriptional regulatory control as protein-coding mRNAs; however, the molecular mechanisms that lead to the differential stability of miRNAs are not known. Curiously, the steady-state levels of a mature miRNA in a cell is also dependent on the abundance of the mRNA targets in that cell. Stated in other terms, the level of the mRNA target can effect of level of the miRNA.[2,3] A unique facet of miRNAs is that import and export from the cell is a regulated process that also controls steady-state levels of mature miRNAs.[8–10] This latter concept will be key, as blood levels of miRNAs may be very effective biomarkers in disease prevention, prognosis, and assessing response to pharmacologic therapeutic intervention.[11,12] As an example, it has been argued that the release of miRNA-143/145 from endothelial cells and the uptake of miRNA-143/145 by vascular smooth muscle cells is relevant to the biology of the atherosclerosis.[13] This is important because it provides a possible mechanistic link between how the hemodynamics of the circulation control gene expression and cellular phenotype in diseases of large blood vessels.[14,15]

MECHANISMS OF GENE SILENCING

How does RNAi change gene expression? In brief, siRNA and miRNA both result in decreased levels of functional protein within cells, albeit through different mechanisms. RNAi mediated via siRNA tends to affect steady-state mRNA levels, whereas RNAi mediated via miRNA mainly affects the efficiency with which mRNA is translated into protein.[4,16,17] Studies performed in mammalian systems initially suggested target mRNA degradation is the predominant activity of siRNAs.[16] As modifiers of gene expression, miRNAs exert a significant impact on global protein and mRNA levels.[18] However, the relative contributions and exact mechanism(s) of translational inhibition vs. mRNA degradation have not been clearly established for miRNAs. Most evidence points to translational inhibition as the predominant effect of miRNAs. However, it is not thoroughly understood whether miRNA-mediated translational inhibition occurs mainly at the initiation stage, postinitiation stages (including elongation,

termination, and cotranslational protein degradation), or both. Yet is also clear that miRNAs can lead to target mRNA degradation, at least in part by recruiting effectors of general mRNA decay pathways, such as decapping and deadenylation factors. Notably, it remains to be elucidated as to whether mRNA degradation typically precedes translational inhibition or vice versa. However, recent studies performed in flies, zebrafish, and human cell lines have indicated that translational repression precedes mRNA degradation.[16]

Where does the specificity come in? Target recognition is a key aspect of miRNA biology.[4,19] miRNAs recognize sequences via Watson–Crick base-pairing on the mRNA target. These *cis*-elements are only partially complementary. As noted, one of the RNA strands, termed the guide strand, becomes incorporated into RISC complex during miRNA processing. The guide strand provides exquisite specificity to RISC, which binds in a sequence-specific fashion and then degrades complementary target mRNA in the cytoplasm (and possibly in the nucleus). The specificity of this manipulation is relatively low, because target RNA sequences within the mRNA are short in length and are only roughly related to the complementary miRNA sequence. miRNAs exhibit partial complementarity to their target mRNAs, usually, but not exclusively, in the 3′-untranslated region (UTR) of the mRNA. Significantly, miRNAs exhibit complete complementarity at their 5′-end to their target mRNAs, centering on nucleotides 2 to 8 of the miRNA from the 5′-end. This is referred to as the seed region. Indeed, seed pairing of miRNAs to their target mRNAs is a critical parameter in determining miRNA/mRNA interactions, among others.[4,19] Interestingly, recent findings suggest that the rough endoplasmic reticulum, which is an important site for mRNA translation, is also a central nucleation site for miRNA- and siRNA-mediated gene silencing. Specifically, Dicer cofactors TRBP and PACT serve to anchor Dicer and miRNA- and siRNA-loaded AGO2 (i.e., RISCs) to the rough endoplasmic reticulum.[20] This physical juxtaposition of mRNA translation and miRNA-mediated translational inhibition contributes to the potency and pervasiveness of miRNA-mediated gene regulation.

Regulation of RNAi pathways in health and disease: challenges for RNAi therapeutics

Most endogenous miRNAs function as sophisticated conductors of genetic pathways by manipulating the translational regulation of many genes at the same time.[2,3,21] One miRNA can regulate many protein-coding mRNAs. Moreover, one protein-coding mRNA can be regulated by multiple miRNAs. miRNAs exert a profound impact on mRNA repression and contribute to the generation of thresholds in target gene expression both basally and in response to exogenous stimuli, many of which are relevant to disease.[3] Some miRNAs are specifically upregulated by a cellular stress. For instance, miR-210 is induced in most nucleated cells by hypoxia.[22] Many unique miRNAs have a distinct developmental and tissue-specific expression pattern, where they play a pivotal role in defining cell identity. Some miRNAs are very cell-enriched. As an example, miR-126 is highly enriched in vascular endothelial cells vs. other cell types.[22] Some miRNAs are only expressed during development. Finally, the efficacy of miRNA inhibition is dependent on the abundance of the mRNA target. Stated

in other terms, although not well studied *in vivo*, an increase in the level of mRNA targets in disease could blunt the effect of a constant level of an RNAi therapeutic.[2,3] Curiously, miRNAs expressed at levels differing by 2 to 3 orders of magnitude may elicit similar repressive effects on target mRNAs.[23]

Bioinformatics analyses suggest that up to 60% of human genes are regulated by miRNAs.[19] Indeed, it is surprising that miRNAs are not functionally relevant to all mRNAs, especially given the short target seed sequences (discussed above) and the long length of the 3′-UTR for most protein-coding regions. Importantly, the miRBase 21 database was released in June 2014 and lists 1,996 unique high-confidence mature miRNAs.[24] Therefore, if there are a lot of miRNAs that could potentially target a lot of protein-coding mRNAs, why are all mRNAs not regulated by miRNAs? A number of explanations have been offered[16] (**Figure 1**).

One important concept relates to the observation that mRNAs exist in the context of dynamic ribonucleoprotein (RNP) throughout their entire life cycle in the cell. RNA binding proteins (RBPs) are the major component of these RNPs.[16] It is now appreciated that miRNAs and RBPs compete for binding to mRNAs. "Naked" single-stranded RNA does not exist in cells. Rather, they interact dynamically with a myriad of cellular *trans*-factors and exist almost exclusively in the context of macromolecular complexes. The stability of an mRNA species is determined, to a large extent, by the identities of these dynamically interacting *trans*-factors, which interact with key *cis*-elements in the 3′-UTR of mRNAs as cotranscriptional and posttranscriptional events. These *trans*-factors mostly fall into one of three categories: RNA-binding proteins (RBPs), natural antisense transcripts,[25] and small RNAs such as miRNAs. An excellent example is the human endothelial nitric oxide synthase (eNOS) gene. This enzyme synthesizes NO in the vasculature and its expression is disrupted in diseases such as atherosclerosis and hypoxia-associated vasoconstriction.[26] The eNOS mRNA is a highly stable transcript, with a half-life of more than 24 hours in human endothelial cells. The eNOS mRNA is highly enriched in vascular endothelium.[27,28] Importantly, decreased eNOS mRNA stability is a major contributor to reduced eNOS expression and function under many critical pathophysiological conditions including hypoxia,[29] tumor necrosis factor-α, entry into cell cycle, and oxidized low-density lipoproteins.[30,31] However, until recently it has been unclear as to the biological rationale behind the remarkable stability of basal eNOS mRNAs. We recently found that the basal stability of eNOS mRNAs is mediated by the formation of a stabilizing RNP complex, which protects eNOS mRNAs against posttranscriptional inhibitory mechanisms, especially eNOS-targeting miRNAs.[32] Numerous other examples of RBPs that protect target mRNAs from miRNA-mediated repression are now known. Therefore, one reason that all protein-coding genes are not targets for endogenous miRNA-mediated repression is that they are protected by RBPs that prevent miRNAs from acting. This has important therapeutic implications because it predicts that some protein coding genes will be hard to repress with RNAi-based therapeutics. An additional nuance was uncovered in our work with hypoxia. Knockdown of hnRNP E1 in endothelial cells under basal conditions decreased eNOS mRNA half-life, mRNA

levels, and protein expression.[32] Importantly, hnRNP-E1 was a major RBP that formed part of the stabilizing RNP complex that protects eNOS mRNA from basal downregulation by microRNAs, specifically miR-765, which targets eNOS mRNA stability determinants.[32] We noted that the formation of a stabilizing RNP complex on eNOS was disrupted in hypoxia. Heterogeneous nuclear RNP-E1 (hnRNP-E1) could no longer engage the mRNA target due to changes in posttranslational modifications and alterations in subcellular localization, which resulted in eNOS now becoming sensitive to exogenous siRNAs and endogenous miRNAs. The broader implication of this specific work is that exogenous stimuli can change the sensitivity of mRNA targets to RNAi-mediated inhibition by modifying the formation of stabilizing or destabilizing RNP complexes.[16]

We now know that the global RNAi pathway is regulated in health and disease. Dicer is essential for development, as knockout of the single mammalian Dicer gene has been shown to be embryonically lethal by embryonic day 7.5 in the mouse, and mutants with hypomorphic alleles exhibit impaired angiogenesis. Disruption of Dicer using cell-specific knockout strategies reveals that Dicer function is critical in all nucleated cell types.[33] Moreover, Dicer status has prognostic implications in a number of human malignancies, especially breast and ovary cancer[34] (**Figure 1**). A general downregulation of miRNAs in tumors compared with normal tissues has been observed, and recent studies have focused on Dicer as a haploinsufficient tumor suppressor gene where deletion of a single copy of Dicer in murine tumors led to reduced survival compared with controls. It is important to stress that loss of two copies of Dicer in malignant cells is not compatible with tumor growth, perhaps mediated by profound falls in new blood vessel formation.[35] Therefore, using an RNAi-based therapeutic that requires processing by Drosha or Dicer might not work in a tumor where the RNAi machinery is functionally deficient.[36]

It has been found that injury to a cell can affect global miRNA processing. For instance, chronic hypoxia impairs Dicer expression and activity *in vitro* and *in vivo*, resulting in global consequences on miRNA biogenesis, especially in blood vessels[22] (**Figure 1**). Specifically, pri/pre-miRNAs accumulated in endothelial cells exposed to chronic hypoxia. Full expression of hypoxia-responsive/HIF target mRNAs in chronic hypoxia was dependent on hypoxia-mediated downregulation of Dicer function and changes in posttranscriptional gene regulation.[22] When Dicer was overexpressed in hypoxic cells, the induction of HIF-target genes (e.g., VEGF, GLUT1) was markedly blunted. Hypoxia significantly blunted the efficacy of Dicer-dependent vs. Dicer-independent RNAi-based therapeutics. This clearly has implications in human disease, where tissue hypoxia is a key feature of cellular injury in ischemic tissues and tumors.[22,37]

THERAPEUTIC APPLICATIONS FOR RNAI THERAPEUTICS
Understanding of miRNA biogenesis has enabled the development of several methods for harnessing RNAi pathways for therapy. Recombinant inhibitory RNAs are designed to mimic pri-miRNAs or pre-miRNAs, whereas chemically synthesized RNA oligonucleotides are designed to mimic Dicer products or substrates. Each type of RNA therapeutic mediates gene silencing but enters the pathway at a different step.

Viral diseases
The therapeutic potential of RNAi was first highlighted in studies against human immunodeficiency virus (HIV) infection *in vitro*[38–41] where receptors for the virus and/or the virus itself were targeted.[42,43] Strategies included silencing genes required for HIV entry or replication because this avoids the problem of genetic variability in HIV replication cycles. This approach led to clinical trials using lentiviral vectors expressing a shRNA targeting an exon shared by HIV *tat* and *rev* genes combined with two HIV specific RNA-based inhibitors.[44] In early-stage studies, these researchers were able to transduce hematopoietic progenitor cells *ex vivo* and then reinfuse them into patients, demonstrating successful engraftment of transduced cells without toxicity.[44] Vector expression was documented for up to 2 years in multiple cell lineages. Importantly, the expression of the introduced shRNA was very long-lived. Collectively, these results support the development of an RNA-based cell therapy platform for HIV.[44] The potent and specific viral inhibition by RNAi, the transduction potency of lentiviral vectors in combination with hematopoietic stem cells, and the improvement of lentiviral delivery systems represent significant advances towards clinical application of RNAi in the battle against HIV.

Both DNA and RNA viruses have evolved mechanisms to degrade, enhance, or block the effects of cellular miRNAs to benefit the viral life cycle. Importantly, it is not surprising that there have also been promising developments in RNAi-based therapies for hepatitis B and C virus which take account these processes.[45–49] Similar to HIV, the hepatitis viruses have a high mutation frequency during viral replication. Therefore, the current focus is to use miRNA or shRNA expression methods to target more than one viral transcript, as well as targeting host proteins that are key for viral propagation.[50,51]

As one powerful example of this approach, investigators have recently developed a novel hepatitis C therapeutic. The stability and replication of hepatitis C virus is dependent on an interaction between HCV and a liver-expressed miRNA.[52] MiR-122 is a liver-enriched miRNA that facilitates replication of hepatitis C viral RNA. Miravirsen is a 15-nucleotide locked nucleic acid–modified antisense phosphorothioate oligonucleotide complementary to miR-122.[53] It exhibits a high affinity and specificity for the 5′-region of mature miR-122, thereby inhibiting miR-122 biogenesis and function in hepatocytes.[54] In a placebo-controlled 18-week trial of 36 patients with hepatitis C, there was a significant drop in viral RNA within the treatment group with no detectable evidence of resistance. The safety of using miravirsen for long periods is being addressed.[55] Nonetheless, this study opens up a new paradigm for the treatment of hepatitis C and other viral infections by targeting a specific host miRNA necessary for viral propagation within a target host tissue. It is encouraging that miR-122 is only expressed in the liver, suggesting that other cell types should not be impacted by miravirsen treatment.

Amyloidosis
In 2013, Coelho *et al.* reported the use of siRNAs to treat patients with familial amyloidotic polyneuropathy in a Phase I clinical trial[56] (**Figure 2**). They were among the first to use a
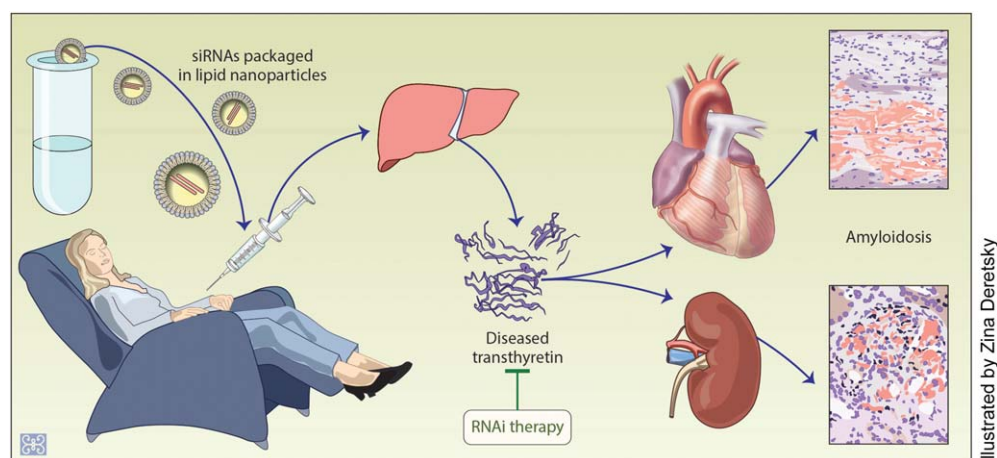
Figure 2 RNA interference proof of concept trial in human patients. Transthyretin amyloidosis is caused by the deposition of hepatocyte-derived transthyretin amyloid in the heart, kidney, and peripheral nerves. In many patients this is an autosomal dominant genetic disease. RNA interference (RNAi) was used to reduce the production of transthyretin from the liver in patients. Prompt, concentration-dependent, and durable lowering of transthyretin blood levels was observed using two transthyretin small interfering RNAs (siRNAs). RNA interference, using siRNAs, suppressed the production of both mutant and nonmutant forms of transthyretin, establishing the proof of concept that an RNAi therapy could target the mRNA transcribed from a disease-causing gene.

lipid nanoparticle envelope to facilitate the delivery of siRNAs to hepatocytes. They used siRNAs capable of inducing a robust and sustained knockdown of transthyretin production.[56] Transthyretin (TTR)-mediated amyloidosis is an inherited, progressively debilitating, and potentially fatal disease caused by mutations in the *TTR* gene. Transthyretin, derived from the liver, can form amyloid deposits in peripheral nerves, the gastrointestinal tract, heart, and kidneys. This results in intractable peripheral sensory neuropathy, autonomic neuropathy, and/or cardiomyopathy. More than 100 germline variants of the *TTR* gene are associated with autosomal dominant forms of the disease, known as familial amyloidotic polyneuropathy and familial amyloidotic cardiomyopathy. The most common mutation associated with familial amyloidotic polyneuropathy is V30M. TTR protein is produced primarily in the liver and is normally a carrier for retinol binding protein—known to transport vitamin A. Mutations in the TTR gene cause misfolding of the protein and the formation of amyloid fibrils that typically contain both mutant and wildtype TTR.

Coelho *et al.* demonstrated that a lipid nanoparticle could be used to deliver two independent siRNAs to the liver of patients with TTR amyloidosis.[56] Distribution of parentally administered lipid nanoparticles is predominantly to the liver, which is ideal for a liver-expressed mutant protein. When lipid nanoparticles were used to deliver siRNAs against the TTR mRNA to hepatocytes, they noted a robust and durable reduction in expression of both mutant and nonmutant forms of TTR. The encouraging results from this Phase I trial, performed in both control and symptomatic patients, are promising for the use of RNAi therapeutics. Importantly, the delivery method was specific to the liver, where TTR is produced. They noted only minimal toxicity.[56] Although the findings were successful in demonstrating that a single dose could rapidly and potently suppress hepatic production of TTR, the safety of long-term suppression of TTR with respect to its effect on levels of vitamin A and thyroxine remains

to be determined. This proof-of-concept trial demonstrates the potential for an RNAi-based therapy to target the mRNA transcribed from a disease-causing gene. Importantly, many hurdles remain for using these technologies for therapy, especially the need to provide long-term therapy and the need to address long-term toxicities and potential off-target effects (see below).

Metabolic diseases

One of the first organs tested for effectiveness of RNAi *in vivo* was the liver for hypercholesterolemia. In preclinical trials, siRNA complexed to liposomal particles that target expression of apolipoprotein B and proprotein convertase subtilisin/kexin type 9 (PCSK9- known as ALN-PCS) showed a significant low-density lipoprotein (LDL)-lowering effect.[57,58] Genetic studies have supported the hypothesis that lowering of PCSK9 by inhibiting its synthesis in hepatocytes should lower LDL cholesterol, thus potentially resulting in reduced risk for coronary heart disease.[59,60] Recently, a group of researchers performed a randomized, single-blind, placebo-controlled, Phase I dose-escalation study in healthy adult volunteers with serum LDL cholesterol of 3.00 mmol/L or higher.[61] Participants were randomly assigned to receive one dose of intravenous PCSK9 siRNA (with doses ranging from 0.015 to 0.400 mg/kg) or placebo. In the group given 0.400 mg/kg of PCSK9 siRNA, treatment resulted in a mean 70% reduction in circulating PCSK9 plasma protein and a mean 40% reduction in LDL cholesterol from baseline relative to placebo. Despite these encouraging safety findings, further large studies using multidose approaches are required in patients with high LDL levels, with or without conventional statin therapy, to confirm the safety and tolerability of siRNA-mediated inhibition of PCSK9.[61]

Neurological disorders

The blood–brain barrier limits access to the central nervous system (CNS) and thus it is difficult to target neural cells. While

direct injection of the RNAi formulation would be desirable for acute neurological disease like brain tumors, it may not be practical, as siRNAs have short half-lives and thus treatment would involve indwelling catheters. Viral platforms, however, provide lasting expression and may be ideal for chronic disorders of the CNS. In fact, there has been promising results of improvement in disease phenotype using vectors expressing therapeutic RNAi in preclinical studies in mice models of Huntington disease,[62] amyotrophic lateral sclerosis,[63–65] Parkinson's disease,[66] and Alzheimer's disease.[67] In nonhuman primate brains, viral vector-based systems seem to be safe and thus clinical trials using viral vectors expressing RNAi triggers are anticipated.[68]

Cancer

Since oncogenes are expressed at extremely high levels within many types of tumor cells, RNAi–based cancer therapies are attractive. One promising study in mice involved liposomal delivery of siRNA that targeted the tyrosine kinase receptor EphA2 gene, which is overexpressed in ovarian cells.[69] After giving this treatment twice weekly for a total of 4 weeks, a 50% reduction in tumor size was observed. Furthermore, when this liposomal RNAi-based therapy was combined with the chemotherapy agent paclitaxel, a 90% reduction in tumor size was seen.[69] A number of preclinical studies have now demonstrated favorable outcomes by silencing genes critical for tumor cell growth, metastasis, angiogenesis, and chemoresistance.[70] Therefore, the application of RNAi for cancer therapy is now being investigated in early phase clinical trials.

CHALLENGES IN THE UTILITY OF RNAI AS A THERAPEUTIC TOOL

As discussed, early proof-of-principle studies in animal models and early phase clinical trials have supported the use of RNAi as a therapeutic agent for gene expression inhibition without significant toxicity. There are, however, many practical and theoretical issues that must be addressed before RNAi can enter into routine clinical use. These include, among others, the need to understand how we can use chemical modifications of RNAi reagents to ensure they remain stable in biological fluids. Most short RNA species undergo rapid degradation and clearance from the body. Furthermore, the design of these RNAi formulations should enable delivery to a target tissue and but still allow loading of the RNAi into the RISC complex within the target cells of interest. Therefore, the screening and monitoring of these RNAi therapeutics for safety and efficacy, including assessment for potential off-target effects, must be carefully evaluated.

As noted above, it has been critically important that a detailed understanding of endogenous RNAi pathways has been emerging in parallel. If a tumor is functionally deficient in Dicer or Drosha, then the efficacy of RNA-based therapeutics will be affected. We now appreciate that a broad number of tumors have somatic gene deletions or epigenetic silencing of components of the RNAi machinery. Dicer-dependent therapies need Dicer activity, which can be genetically deficient or impaired because of cellular injury, such as in the setting of hypoxia (**Figure 1**). At baseline, we now appreciate that not all mRNAs are easily targeted by endogenous miRNAs or exogenous siRNAs/shRNAs because they are protected by stabilizing RNP complexes. Pathogens, especially viruses, have evolved devious mechanisms to evade endogenous and exogenous RNAi-mediated inhibition. Collectively, we now have a greater understanding of the nuances of the RNAi pathways.

Off-target effects and siRNA design

The development of RNAi as a therapy now focuses on avoiding off-target effects and efficient delivery to targeted tissues. Given the complex and poorly understood interaction networks between cellular genes, it is not surprising that silencing any given gene within a target cell may have unintended physiological consequences. The sequence of the RNAi therapeutic may be identical or almost identical to another miRNA sequence causing a phenotype.[71] Indeed, families of miRNAs can exhibit significant sequence identity. If these sequences are identical to seed regions of the endogenous miRNAs, this can cause miRNA-like unrelated gene silencing by pairing with complementary sequence in the 3′-UTR region of an unrelated protein-coding mRNA.[72–75] In this situation, siRNA may function similar to a miRNA, leading to off-target suppression of gene expression. Curiously, other noncoding RNAs, especially long noncoding RNAs, can serve as sponges to titrate off endogenous or exogenous RNAi species. Other off-targeting effects occur when dsRNA activates the antiviral type 1 interferon response.[76,77] siRNAs can activate the innate immune response by interacting with Toll-like receptors (TLRs) on the surface of cells[78,79] or endosomes.[80,81] TLR3 and TLR8 can recognize dsRNA and ssRNA, respectively. TLR activation by RNAi inhibits blood and lymphatic vessel growth, which can be advantageous in the setting of corneal vascularization where inhibition of angiogenesis is desired.[82,83] However, TLR activation can be harmful in other settings, such as in the process of inhibiting ischemia. Therefore, the design of the RNAi formulation must be carefully considered and tested to circumvent any of these off- target effects.

Grimm *et al.* evaluated the efficacy of long-term RNAi-based therapeutic agents in the livers of adult mice.[84] They used 49 distinct constructs consisting of viral DNA that encode synthetic shRNA species directed against six unique target mRNA species. Thirty-six constructs resulted in concentration-dependent liver toxicity, with premature death occurring in 23 of the mouse models. The toxic effects were not restricted to the specific shRNA expressed, the mRNA target against which it was designed, components of the vector, or a consequence of the activation of innate defense pathways against double-stranded RNA (which seem to have evolved as antiviral responses). Whether these findings are relevant to other types of tissue and organs will need to be addressed in future studies. As discussed above, precursor RNA species from which miRNA is derived, termed pre-miRNA, require processing to produce biologically active miRNA. Pre-miRNA is similar in structure to exogenous shRNA derived from viral gene-therapy vectors. Especially relevant, Grimm *et al.* argued that the export of shRNA and pre-miRNA from the nucleus used a common mediator, namely, exportin-5.[84] They provided compelling evidence that cells transfected with

vectors containing shRNA produced sufficient synthetic shRNA in the nucleus to block the normal export of endogenous pre-miRNA from the nucleus. Stated in other terms, the two structurally related molecules had to compete for a rate-limiting nuclear-export pathway. Consistent with this model, increases in the cellular levels of exportin-5 reduced the *in vivo* hepatotoxicity observed in mice treated with shRNA-containing vectors. Therefore, competition with the endogenous miRNA machinery represents yet an additional concern where shRNAs interfere with the normal processing and function of endogenous miRNAs.[84]

Delivery of RNAi therapeutics

The delivery of RNAi therapeutics to target cells at effective concentrations remains the most significant challenge. Ineffective delivery can potentially lead to only partial inhibition of gene expression. Others have argued that the critical problems of *in vivo* siRNA drug administration can be divided into two main issues: plasma stability and pharmacokinetics, as previously highlighted. Nucleic acid-based drugs are highly negatively charged, which requires approaches to shield the negative charge. Moreover, the molecular weight of siRNAs is typically greater than 13 kDa. In contrast, a typical antisense oligonucleotide weighs 6.5 kDa. Clearly, both are much larger than the conventional small molecule pharmaceutics.

The ability to turn siRNAs into therapeutic drugs is dependent on chemical modifications of the siRNAs that confer drug-like properties in order to facilitate safe and highly efficient delivery to diseased organs. Partial inhibition may be sufficient to provide therapeutic benefits in certain diseases such as Alzheimer's or Parkinson's disease. Partial inhibition alone may not provide therapeutic benefits in other diseases, such as cancer. Therefore, researchers have focused on modifying siRNA or attaching them to agents that will chemically stabilize them against RNA nucleases until they reach their target. The chemical nature of RNA has pushed the boundary of nucleic acid design. Backbone modification of bases (e.g., 2′-O-methyl) or chemical attachment of bulky conjugates (e.g., cholesterol or PEG) can improve the stability and bioavailability of siRNAs by protecting them against endo- and exo-nucleases. One of the most common methods is a lipid-based carrier or cholesterol conjugate to the siRNA. Researchers have demonstrated that cholesterol-conjugated siRNAs can inhibit the expression of the gene encoding proprotein convertase subtilisin/kexin type 9 (PCSK9- known as ALN-PCS).[61] Although this is promising technology, the primary concern with this method is lack of specificity for cell delivery, leading to potential adverse effects. Complexing siRNAs with carriers that recognize specific cell surface receptors, however, provides opportunities for targeting specific cells. Nanoparticle delivery of siRNA was first demonstrated in human Phase I clinical trials using transferrin–receptor targeting ligands showing a reduction in levels of the target mRNA.[85]

Other carriers for tissue-specific targeting include aptamers, antibodies, and proteins.[42,85–87] Song *et al.* developed a protamine-antibody fusion protein for systemic and targeted siRNA delivery.[88] They fused protamine, a protein that binds nucleic acids, to a Fab directed against the HIV-1 envelope protein and mixed the siRNA with the fusion protein. Treatment with the fusion protein mixed with siRNA targeted to the HIV-1 gag protein suppressed viral replication in infected primary T cells of mice.[88] Nucleic-acid aptamers, which are normally selected from a large random-sequence pool to bind to a specific target molecule, have also been explored for targeted siRNA delivery as an alternative to antibodies. Aptamers have advantages, such as high selective binding to proteins and receptors, ready-to-use chemical synthesis, process-compatible storability, and low immunogenicity.[89] McNamara *et al.* developed aptamer-siRNA chimeric RNAs for targeted delivery of siRNA.[90] The aptamer portions of the chimeras were introduced for specific binding to prostate-specific membrane antigen (PSMA), a cell-surface receptor overexpressed in prostate cancer cells and tumor endothelium, whereas the siRNA portion targeted the expression of survival genes. The chimeric RNA was demonstrated to bind only PSMA-expressing cells, resulting in depletion of siRNA target proteins and cell death. In addition, treatment with the chimeric RNA specifically inhibited tumor growth and mediated tumor regression in a xenograft model of prostate cancer.[90] The aptamer-siRNA chimera is a promising targeted approach for siRNA delivery because RNA is not recognized by antibodies. However, more varieties of RNA and DNA aptamers need to be developed in order to expand the use of the aptamer delivery approach.

Other delivery systems include nonviral vectors and viral vectors. Bacteria can be used as an innovative platform for RNAi delivery. A unique approach used minicells (i.e., bacteria-derived cells that have no chromosomes and are nonliving). Minicells were derived from *Salmonella* subspecies and targeted to tumor-cell surface receptors in mice for the delivery of siRNAs or shRNAs. This delivery method reduced tumor burden and improved mouse survival.[91]

The viral vectors used to deliver shRNAs or artificial miRNAs include oncoretroviruses, lentiviruses, adenoviruses, adeno-associated viruses, and herpes viruses. Lentiviruses and adeno-associated viruses (AAVs) are the most frequently used viral vectors for this purpose. Because lentiviruses integrate into the genome, they are advantageous when targeting dividing cells, so that therapeutic gene expression can be maintained through cell division. In contrast, AAVs may be advantageous for targeting nondividing or quiescent cells, such as neurons in the CNS. Viral vectors can permit some cell specificity based on the tropism of the virus and cell surface molecules that mediate viral entry. The choice of cellular promoters that express the shRNAs will also aid in directing cell-type specific expression. Despite the safety concerns associated with viral vectors, the long-term and possible regulatory expression of RNAi agents from tissue-specific promoters and relative ease of delivery makes them promising technology.[92] Because AAVs replicate episomally and do not integrate into the genome of the host, they are perceived as being safer because they do not cause insertional mutagenesis.

CONCLUSION

Approaches involving RNAi have revolutionized the control of gene expression and have been used as an experimental tool *in vitro*.

Importantly, promising new findings suggest that they now offer hope for *in vivo* use in humans, and that we may soon be able to harness this robust and specific gene-silencing mechanism as a therapeutic tool. We are confident that the inhibition or augmentation of specific miRNAs is now emerging as a viable option and there is the need to develop newer approaches to delivering these labile molecules. Moreover, there is still a lot to learn about endogenous RNAi processing pathways, especially how disruption of these pathways are contributing to disease phenotypes and affecting the efficacy of RNAi-based therapeutics.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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