Chapter 4
siRNA Versus shRNA for Personalized Cancer Therapy: Mechanisms and Applications

John S. Vorhies, Donald D. Rao, Neil Senzer, and John Nemunaitis

Abstract RNA interference (RNAi) is a natural process of gene regulation that can be harnessed to knock down gene and protein targets with high specificity and selectivity. Proteomic and genomic approaches to target identification will soon allow investigators to rapidly indentify biorelevant cancer signal transduction network hubs that are more likely to be susceptible to a therapeutically effective targeted attack by RNAi. At present, the principle methods of mediating the RNAi effect involve synthetic small interfering RNA (siRNA) oligomers and DNA vector driven expression of short hairpin RNA (shRNA). Both these methods can achieve robust and specific knockdown, but they have striking mechanistic differences with broad practical implications. shRNA can effectively target knockdown with low copy numbers and longer lasting effects than siRNA. Bifunctional design has similar benefits to standard shRNA but with greatly enhanced potency. Effective delivery and avoidance of unwanted off-target effects remain as challenges to the clinical development of siRNA and shRNA. This chapter compares and contrasts siRNA, shRNA, and bifunctional shRNA as candidates for personalized solid tumor therapeutics.

Keywords RNAi • Personalized • Cancer therapeutic

J. Nemunaitis (✉)
Gradalis, Inc., Dallas, TX, USA
and
Mary Crowley Cancer Research Centers, Dallas, TX, USA
and
Texas Oncology PA, Dallas, TX, USA
and
Baylor Sammons Cancer Center, Dallas, TX, USA
e-mail: jnemunaitis@marycrowley.org

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1 Introduction

Refinement of our basic understanding of cancer mechanisms is now shifting treatment development from traditional, broadly cytotoxic chemotherapy to more selective approaches. Tumors have long been grouped into clinical and histological subtypes, but significant variation in response to treatment still exists within these subtypes. The underlying variation in tumor gene expression patterns may explain the widely divergent responses to treatment regimens most often prescribed by histological type. Molecular subtyping has the potential to further refine patient treatment groups and to improve treatment outcomes, thereby, establishing a "so-called" personalized medicine approach.

As already efficient high-throughput methods are accelerated, the possibility of personalized medicine is becoming a reality. The ultimate goal of personalized therapy is to make the drug development process, from target identification to treatment, feasible in a timescale relevant to a single patient. This would allow physicians to consider each patient's tumor as a subtype of its own, characterizing it and delivering appropriate treatment.

RNA interference (RNAi) is an evolutionarily conserved gene-silencing mechanism that occurs endogenously when small sequences of double stranded RNA, termed microRNA, suppress the translation of partially complementary posttranscriptional mRNA. When exogenously induced, it can be a powerful mechanism for targeted knockdown of over- or constitutively expressed molecular targets. It also has significant practical advantages over small molecules and antibodies in terms of production, potency, and specificity. RNAi is young as a potential therapeutic modality and there are several candidate mechanisms for inducing it. The two primary modes for inducing RNAi are through the introduction of chemically synthesized double-stranded oligomers, called small interfering RNA (siRNA) or through the introduction of a DNA vector, which expresses a short hairpin RNA (shRNA) within the target cells. These two modes have important mechanistic advantages and disadvantages relevant in terms of clinical efficacy, durability, off-target effects, and delivery (Rao et al. 2009).

2 Personalized Cancer Therapy

Signal transduction networks in cancer are quite robust to random individual gene/protein target knockout owing to the presence of functional redundancy and a scale-free interaction topology. Random pathway component failure predominantly affects targets with low connectivity within the network, thereby having limited functional impact. Highly connected information-transfer nodes are particularly vulnerable to attack and constitute weak points in the network. This property is exacerbated in cancer because oncogenic change tends to make cells more highly dependent on a specific rewired pathway (Letai 2008). Exploiting the vulnerability that such pathway dependence creates is useful for its lethality to cancer cells and its decreased

likelihood of perturbing normal cell function. Personalized RNAi-based therapeutics are particularly well suited to take advantage of these mechanisms.

Our group is currently developing a model for personalized RNAi-based therapy: we harvested tumor and normal cells from cancer patients, comparing expression profiles for malignant versus normal tissue at the mRNA and protein level by microarray and proteomic analysis (Nemunaitis et al. 2007). The resulting expression data was further analyzed by a computational system developed by our team specifically for clinical application including, but not limited to, gene set enrichment analysis and network inference modeling platforms. This allowed us to prioritize overexpressed potential targets based on their probability of being highly connected, nonredundant points in the network. This individualized target fingerprint then served as the template for the design, synthesis, and validation of individualized therapeutic RNAi molecules with knockdown activity against these targets.

3 Mechanisms of RNAi

Whether induced by shRNA or siRNA, the RNAi silencing process, as it is currently understood, converges into a final common set of pathways mediated through short (19–23 bp) oligomers of duplex RNA with 2–3 nt 3′ overhangs on each strand. The two strands of the duplex are termed the guide (antisense) strand, which is complementary to the target mRNA sequence, and the passenger (sense) strand, which may be completely complementary to the guide strand or it may contain mismatches. Figure 1 summarizes the main entry points into this pathway by exogenous RNAi as well as the end-mechanisms of silencing.

3.1 siRNA

Effective exogenous induction of RNAi was initially demonstrated by the application of RNA oligomers (Fire et al. 1998). Once in the cytoplasm, siRNA associates with several proteins that make up the RNA-interfering silencing complex (RISC). Depending on various factors, including the duplex mismatching and the nature of the RISC, RNAi can proceed through "cleavage dependent" or "cleavage independent" pathways.

The major component of the RISC is the argonaute family of proteins (Ago1, Ago2, Ago3, and Ago4). Within this family, only Ago2 contains the endonuclease activity. The remaining three members of Argonaute family do not have identifiable endonuclease activity, and presumably function through a cleavage-independent manner (Farazi et al. 2008; Paroo et al. 2007). During RISC assembly in the cleavage dependent mechanism, the passenger strand is cleaved by the RNase H like activity of Ago2 and, provided thermodynamically favorable conditions,

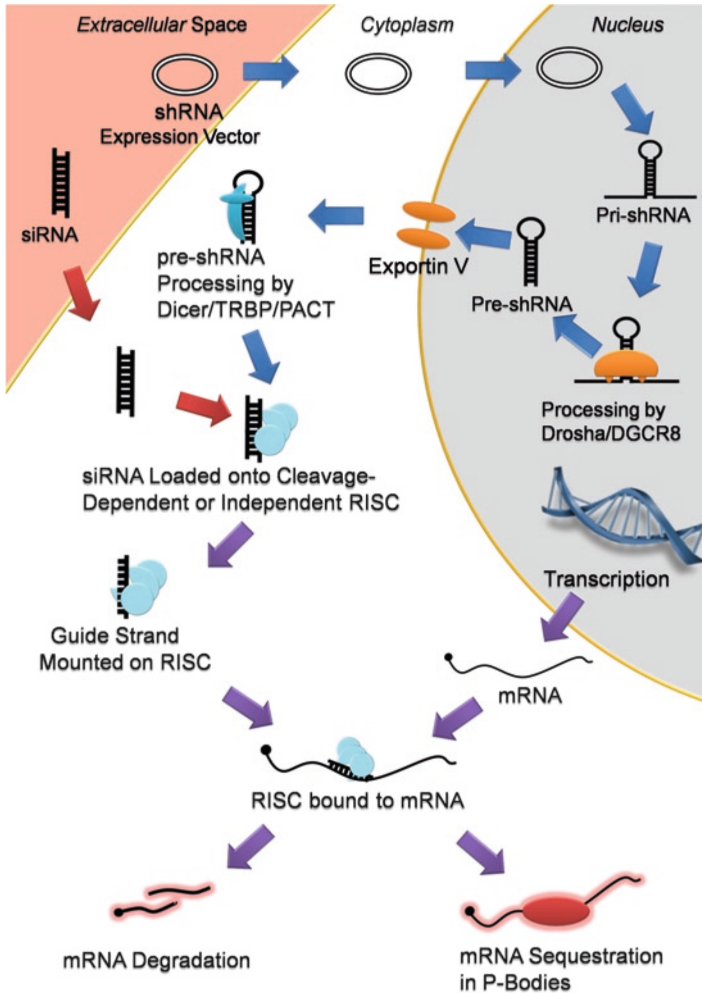

Fig. 1 Schematic of the cytoplasmic siRNA and shRNA mediated RNAi pathways. siRNA and shRNA are introduced in different ways, but they converge on a common set of pathways in the cytoplasm

the two strands of the duplex are separated. The RISC then scans mRNAs for target sites to which it binds and Ago2 cleaves the mRNA at a single site between nucleotides10 and 11 from the 5′ end of the guide strand, thereby initiating degradation. The RISC can then dissociate and execute multiple rounds of RNAi (Paroo et al. 2007).

If there are mismatches in the duplex RNA, a different, cleavage-independent RISC is assembled that lacks Ago2 endonuclease capacity. During the assembly of the cleavage-independent RISC, the passenger strand is induced to unwind and be

released by an ATP-dependent helicase. RISCs without endonucleolytic activity scan mRNAs and predominantly bind to partially complementary target sites located at the 3′ UTR, repressing translation through mRNA sequestration in processing bodies (p-bodies). Phosphorylation of Ago 2 on Serine-387 seems to affect its localization to p-bodies. The functional implications of this are still unknown, but it may represent an important regulatory step in RNAi via p-body sequestration. The exogenously applied RNAi constructs can be designed to participate in either or both pathways (Grimm 2009; Paroo et al. 2007).

Synthetic siRNA enters into the RNAi pathway at the stage of RISC assembly, but if the oligomer is longer than 19–23 bp, it requires processing by a multidomain RNase III-related endonuclease called Dicer before being loaded onto the RISC. Dicer preferentially binds to the 5′ phosphate of 2 nt 3′ over-hang and cleaves double-stranded RNA into 21 to 22 nucleotide siRNAs. It also forms an integral component of endogenous RNAi, processing pre-microRNA to mature miRNA and transferring the processed products to the RISC (Macrae et al. 2006; Carmell and Hannon 2004)

3.2 shRNA

Several years after exogenous RNAi was discovered, it was shown that RNAi could be induced by the in vitro transcription of shRNA using a T7 RNA polymerase or a U6 promoter on a plasmid construct (Yu et al. 2002; Miyagishi and Taira 2002). shRNAs, unlike siRNAs, are synthesized in the nucleus of cells similarly to miRNA. Thus, studies on the biogenesis of miRNAs have provided the groundwork for understanding the synthesis and maturation of shRNA.

shRNA is introduced as a DNA vector encoding an hairpin-like stem-loop structure. Once transcribed in the nucleus, if integrated into a miR30 scaffold, the hairpin containing the pre-shRNA-like construct is processed to a pre-shRNA by a complex containing the RNase III enzyme Drosha and the double-stranded RNA binding domain protein DGCR8 (Fig. 1). The complex measures the hairpin and allows precise processing of the long primary transcripts into individual shRNAs with a 2 nt 3′ overhang. This processed primary transcript or an exogenous pol III-based stem-loop structure is then transported to the cytoplasm by Exportin V via a Ran-GTP-dependent mechanism (Grimm 2009).

In the cytoplasm, the pre-shRNA undergoes a dicer-mediated endonucleolytic cleavage step, in which the loop of the hairpin is processed off to form a double-stranded siRNA with 2 nt 3′ overhangs. Dicer interacts with the double-stranded Tat-RNA-binding protein (TRBP) or PACT (PKR-activating protein) to mediate siRNA production from shRNA (Pillai et al. 2007; Paroo et al. 2007). The activity of exogenous siRNA, unlike shRNA, does not depend on the TRBP/PACT/Dicer complex. After this last processing step, the Dicer-containing complex coordinates loading onto the RISC. Once on the RISC, shRNA and siRNA should follow the same pathways.

3.2.1 Bifunctional shRNA

The concept of bifunctional shRNA rests on the hypothesis that an shRNA construct can be designed to utilize both cleavage-dependent and cleavage-independent RISCs. The design of the bifunctional shRNA expression unit consists of two stem-loop shRNA structures; one stem-loop structure composed of a fully matched passenger and guide strand duplex for cleavage-dependent RISC loading, the second stem-loop structure composed of a mismatched passenger strand (at the position 9–12) and guide strand for cleavage-independent RISC loading (Fig. 2).

Simultaneous expression of both cleavage-dependant and cleavage-independent shRNAs in cells should achieve a higher level of efficacy, greater durability, and more rapid onset than either siRNA or standard shRNA. Multitarget shRNA expression systems have been validated using in vitro (Cheng et al. 2009) cancer systems. There also exists evidence to suggest that this type of functional redundancy is active within the endogenous RNAi system as well. Most mRNAs have multiple miRNA target sites, which allow for cooperative downregulation. In vitro data also suggests that miRNA sequences with the same target and even the same sequence

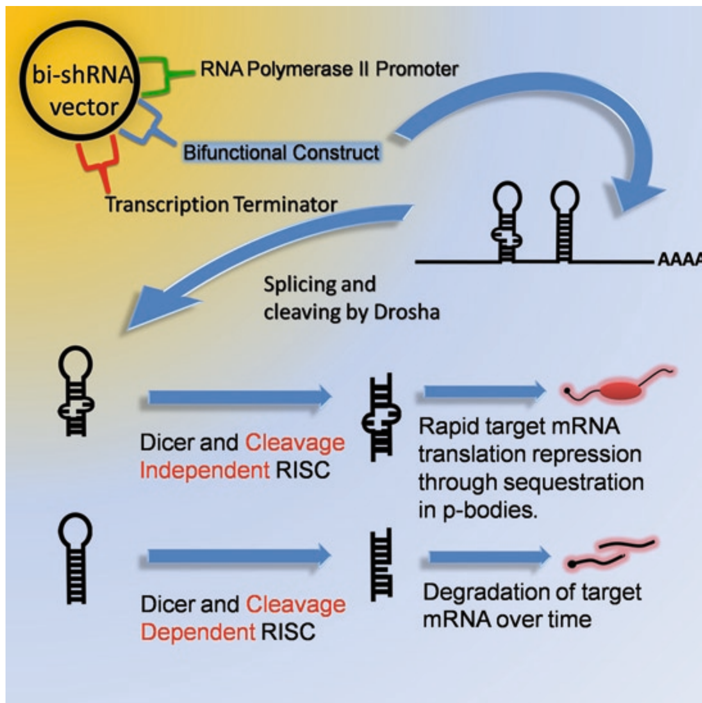

Fig. 2 Schematic of the bifunctional shRNA Vector design and mechanism. A construct that encodes two shRNAs for each targeted mRNA promotes translation repression through both cleavage-dependent and cleavage-independent RISCs

naturally associate with different RISCs in vivo (Azuma-Mukai et al. 2008; Landthaler et al. 2008).

4 SiRNA Versus shRNA

4.1 *Comparative Efficacy*

shRNA generally has higher efficacy than siRNA when directed to the same target and in vitro. McCleary and colleagues tested shRNA and siRNA directed against firefly luciferase in HeLa cells. More effective inhibition was seen with the shRNA. In an Hepatitis-C virus (HCV) model, 19 and 25 bp shRNAs were compared with 19 and 25 bp siRNA directed against the HCV internal ribosomal entry site using a luciferase reporter in the AVA5 cell line with stable expression (Vlassov et al. 2007). Both the shRNAs were more potent than either of the 19- or 25-bp siRNAs used. Takahashi et al. (2009) compared a luciferase-directed shRNA driven by different promoters to siRNA of the same sequence in melanoma cells and found that shRNA driven by a U6 promoter was at least 100-fold more potent and longer lasting than siRNA.

Comparison of siRNA and shRNA in vivo is difficult as equivalency of strand biasing may not be assured. Several studies have used luciferase reporter systems to quantify siRNA versus shRNA potency in vivo. McAnuff and colleagues (2007) found that siRNA and shRNA are equivalent in potency at 10 μg dose; however, on a molar basis, the shRNA was 250-fold more effective than the siRNA. In a murine HCV model siRNA and shRNA constructs were directed against the nonstructural protein 5B viral polymerase coding region fused with a luciferase gene. siRNA resulted in a 75% expression reduction while shRNA produced a 92.8% average reduction over three experiments (McCaffrey et al. 2002).

4.2 *Dicer/Drosha Expression in Cancer and RNAi Effector Suitability*

Low levels of Dicer and Drosha have been found in tumor samples from patients with ovarian cancer and breast cancer (Merritt et al. 2008; Grelier et al. 2009). In one of these studies (Merritt et al. 2008), shRNA was found to be less effective than siRNA in cells with low Dicer expression. These expression findings stand in contrast to other studies including those that have noted either no downregulation or upregulation of both Drosha and Dicer in ovarian tumors (Lin Zhang et al. 2008; Flavin et al. 2008). Further investigations of Dicer/Drosha expression in human cancers are needed, but these findings raise the possibility that, at least in some cases, the clinical efficacy of shRNA may be affected by the expression patterns of endogenous miRNA processing machinery (Rao et al. in press).

4.3 Off-Target Effects

There are multiple specific and nonspecific mechanisms through which siRNA and shRNA can cause effects other than the intended mRNA suppression. Specific off-target effects are mediated by partial sequence complementarity of the RNAi construct to mRNAs other than the intended target. Nonspecific off-target effects include a wide variety of immune- and toxicity-related effects that are intrinsic to the RNAi construct itself or its delivery vehicle.

4.3.1 Specific Off-Target Effects

In vitro, siRNA creates off-target expression patterns that are unique and consistent for a given sequence. They also appear to be unrelated to target knockdown (Jackson et al. 2003). Complementarity of the mRNA 3′UTR with nucleotides 2–7 at the 5′ end of either the siRNA passenger or guide strands has been shown to be a key determinant in directing off-target effects. This is reminiscent of the “seed” region within miRNA, which guides silencing through complementarity with the 3′UTR of an mRNA (Birmingham et al. 2006).

Although sequence optimization to reduce specific off-target effects will benefit both siRNA and shRNA discriminative functionality, unlike shRNA, siRNA oligomers can be chemically modified to reduce direct off-target effects. Various modifications can encourage preferential strand selection, limit the construct’s association with a certain class of RISC, or discourage seed region complementarity based off-target effects (Behlke 2008).

shRNA seems to cause fewer specific off-target effects than siRNA, potentially because of its use of endogenous processing and regulatory mechanisms (Rao et al. in press). The susceptibility of siRNA to cytoplasmic degradation may also lead to more off-target effects. In one study, shRNA and siRNA of the same core sequence directed toward *TP53* were applied to HCT-116 colon carcinoma cells in concentrations necessary to achieve comparable levels of target knockdown. Microarray profiling demonstrated a much higher degree of up- and downregulation of off-target transcripts in the siRNA transfected cells (M. Mehaffey, T. Ward, and M. Cleary, in prep.).

4.3.2 Nonspecific Off-Target Effects

Activation of the innate immune system in the case of exogenous RNAi is likely mediated through cytoplasmic and endosomal mechanisms attuned to recognize exogenous nucleic acids from infectious agents. Introduction of dsRNA longer than 29–30 bp into mammalian cells activates receptors sensitive to exogenous nucleic acids, such as Toll-Like Receptors (TLR), and induces the innate immune system, leading to global degradation of mRNA and upregulation of interferon (IFN)-stimulated gene expression. Though siRNA constructs are less immunogenic

than longer dsRNA, both siRNA and shRNA can induce a partial IFN response (Robbins et al. 2006).

Misinterpreting an immunologic effect of siRNA as a direct effect must be carefully avoided, as naked siRNA has been shown to activate the RNA-sensitive TLR-3 on the surface of vascular endothelial cells, triggering the release of IFN-γ and IL-12 that mediate nonspecific antiangiogenic effects in vivo (Kleinman et al. 2008). Activation of TLR 3 is not an issue for shRNA because the construct is presented on a DNA vector. However, TLR 9 is present in the endosome and is activated by unmethylated DNA CpG motifs, necessitating careful plasmid design to avoid immunoactivation (Robbins et al. 2009).

Sequence and chemical modification of siRNA (particularly the 2′ site) can attenuate the immune response (Robbins et al. 2009). shRNA is less likely to induce an inflammatory response through cytoplasmic dsRNA receptors because it is spliced by endogenous mechanisms. In an experiment that compared liposome-delivered siRNA and shRNA in primary $CD34^+$ progenitor-derived hematopoietic cells, it was shown that siRNA induced IFN-alpha and type I IFN genes, while the shRNA of the same sequence did not induce an immune response (Grimm and Kay 2007). Another study showed that modifying an shRNA by integration within an miR-30 scaffold could also decrease the IFN response (Bauer et al. 2009).

Over-saturation of nuclear membrane Exportin V and Ago2 by shRNA (particularly at high concentrations) can cause dose-dependent liver injury as a result of downregulation of critical endogenous miRNAs, which rely on the same proteins (Grimm 2009). siRNA avoids this problem and can achieve suppression of a target gene without disrupting endogenous miRNA levels (John et al. 2007). The over-saturation effect may be promoter related, as stable target gene suppression was subsequently demonstrated at high shRNA doses in a murine model for over one using a pol II promoter system (Grimm 2009). This indicates that selective promoter integration and careful dosing of shRNA is needed to avoid competitive inhibition of the endogenous miRNA biogenesis machinery.

5 Delivery Strategies for Clinical Translation

Clinical efficacy of an RNAi cancer therapeutic is limited by the properties of its delivery vehicle. In the case of siRNA, knockdown is directly related to the quantity of the oligomer that enters the tumor cells, whereas in the case of shRNA, the expression vector must reach the nucleus for gene silencing to be achieved. Issues of safety, selective tumor targeting, pharmacokinetics, and pharmacodynamics are also affected by the delivery vehicle. These include resisting host defenses, reaching the tumor while avoiding normal tissue, negotiating cell penetration, and, when apropos, endocytosis then endosomal/lysosomal escape and, in the case of shRNA, penetration of the nuclear membrane.

Viral vectors have received some clinical attention but concerns over efficient systemic delivery and immunogenicity may limit their clinical utility.

There are three major classes of nonviral delivery vehicle systems: synthetic polymers, natural/biodegradable polymers, and lipids (Vorhies and Nemunaitis 2009; Vorhies and Nemunaitis 2007; Whitehead et al. 2009). Hybrids of these can be effective. For instance, there is a cyclodextrin-based cationic polymer which has been used successfully to deliver siRNA targeted to RRM2 in various in vivo cancer models. The same formulation, now called CALAA-01 is currently in Phase I clinical trials (Heidel et al. 2007).

Lipid-based nanoparticles are also showing potential for clinical delivery of shRNA and siRNA. Silence Therapeutics has developed a lipid-based delivery vehicle specifically designed for the delivery of siRNA Targeting Protein Kinase N3 endothelial cells. This vehicle, called AtuPLEX, contains a mix of cationic and fusogenic lipids (Aleku et al. 2008). A Phase I trial is currently recruiting to investigate an siRNA therapeutic delivered with AtuPLEX.

6 Conclusions

Both siRNA and shRNA have excellent potential for clinical use within the emerging paradigm of scale-free biomolecular networks and their inherent integration of evolution and structure. However, several challenges remain that warrant further study at both the preclinical and clinical levels. Mechanisms influencing RNAi silencing efficacy, off-target effects, and delivery are crucial areas for further study, preclinical assessment and clinical translation. The transient effect of siRNA may be more suited to the treatment of infectious disease whereas the heightened potency and temporal and spatial control of shRNA may better suit it for the systemic treatment of malignancy. Finally, bi-shRNA represents an important therapeutic development which, by exploiting the latest advances in our understanding of RNAi mechanisms, may bring us closer to an optimized personalized cancer therapy.

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