Vector Systems for the Delivery of Small Interfering RNAs: Managing the RISC**

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Introduction

The seminal observation by Tuschl and colleagues that small RNA duplexes can function in a potent and sequence-specific manner to mediate post-transcriptional gene silencing (PTGS) in mammalian cells has injected new vigor into the field of functional genomics.[1] Particularly exciting has been the generation of vector tools designed to permit stable knockdown of genes of interest in a variety of cell types. While it is clear from the recent literature that vector delivery of small interfering RNAs (siRNAs) is a tractable approach for silencing genes in cells, tissues, or entire organisms, the bearing that vector design may have on experimental outcomes is not fully understood. This minireview seeks to address a number of variables that may have repercussions on the success of vector strategies for gene knockdown.

What Biological Parameters Govern Post-Transcriptional Gene Silencing?

The fascinating complexities of pathways governing PTGS have been elegantly reviewed elsewhere.[2–4] One important principle, perhaps best illustrated by studies of endogenous noncoding microRNAs (miRNAs), concerns the intracellular compartmentalization of the various factors involved (Figure 1).[5] Precursor miRNAs (pre-miRNAs), which form imperfectly base-paired hairpins of ~70 nucleotides (nt) in size, are generated in the nucleus by excision from a longer RNA polymerase II (Pol II) transcript. Pre-miRNAs lack the ability to silence nuclear pre-mRNAs, and acquire effector activity only after being transported into the cytoplasm, where they undergo processing by Dicer. Dicer, an RNase III-related ribonuclease, is responsible for converting certain RNA duplexes, including those present in pre-miRNA stems, into ~21–23 nt fragments. The stable product of this reaction is a single-stranded miRNA that elicits gene silencing in association with an incompletely characterized high-molecular-weight protein complex by a mechanism of translational inhibition, which, in higher organisms, is thought to require base-pair mismatches between the miRNA and the target mRNA.

When seeking to experimentally manipulate the RNA interference (RNAi) pathway to suppress the expression of specific gene products, it is important to engineer reagents that are optimized for entry into the appropriate steps of the RNAi cascade. Chemically synthesized siRNA duplexes of ~21 nt, which can be introduced into a variety of cell types by transient transfection, are designed to enter the RNAi pathway downstream of Dicer. These reagents act exclusively in the cytoplasm,[6] where the effector siRNA strand is recruited into a large multisubunit protein complex termed the RNA-induced silencing complex (RISC). Like miRNAs, siRNAs function to guide a silencing complex to target mRNA sequences through base-pair interactions, resulting in the induction of PTGS. In the case of siRNAs, however, the mechanism of silencing is catalytic, involving numerous cycles of target mRNA cleavage by RISC.[7] This contrasts with translational suppression by miRNAs, which is stoichiometric and therefore less efficient. Crucial to the efficient induction of RNAi by siRNAs is the presence of perfectly complimentary base pairings between the siRNA and the target mRNA. Vector-delivery strategies commonly employ short hairpin RNA (shRNA) expression cassettes that resemble pre-miRNAs and undergo processing by Dicer, but like synthetic siRNAs, contain sequences designed to pair perfectly with the target mRNA to induce RNAi.

Taking into account what is understood about endogenous pathways of PTGS, it is apparent that for robust silencing to be achieved by using vector technologies, the shRNA transcript must be efficiently transported from the nucleus to the cytoplasm, be recognized as a Dicer substrate, and give rise to an siRNA product capable of associating with RISC and directing target mRNA cleavage. However, there remain many gaps in our understanding of crucial biological parameters that may impinge on vector RNAi strategies. For example, what governs the coupling between Dicer cleavage and siRNA loading into RISC, and why is it important that the effector siRNA strand bear a 5′-phosophorylated terminus?[8–11] Are there differences between cofactors involved in the miRNA versus siRNA pathways, or are these two pathways virtually identical, with the outcome specified solely by the degree of base-pair mismatch to target mRNA?[7, 12–14] What accounts for the observation that only one strand of a given miRNA duplex is maintained in the steady state,

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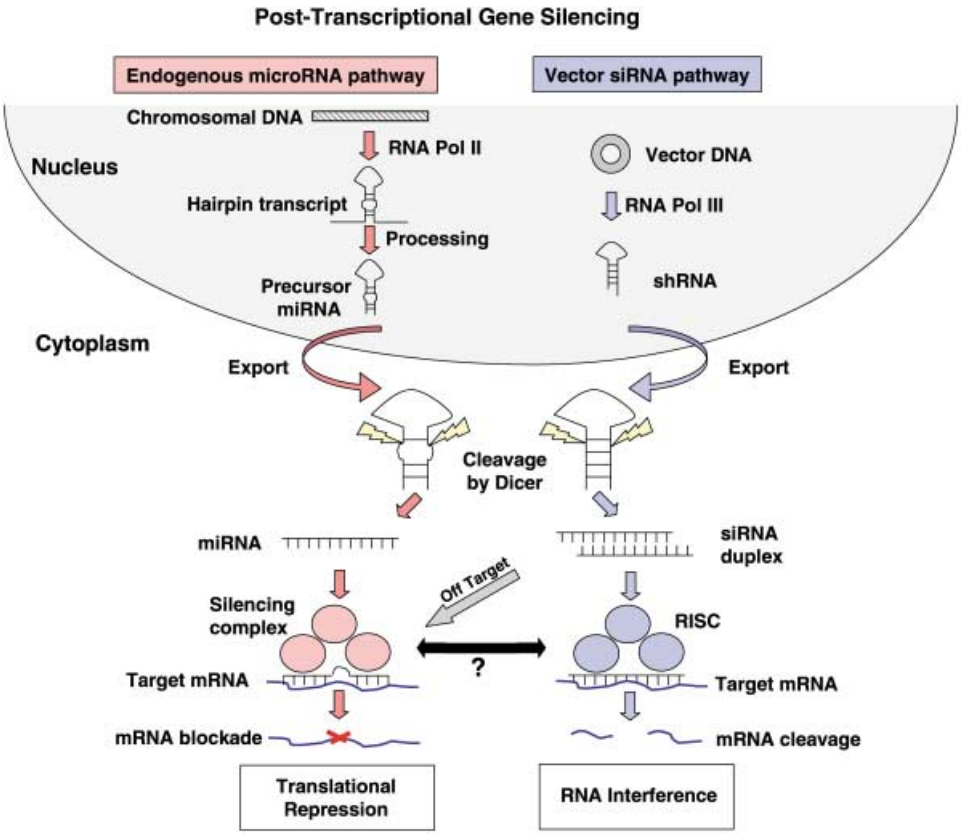

Figure 1. Interfering RNA expressed in the nucleus acquires effector activity in the cytoplasm. An endogenous precursor miRNA is excised from a Pol II transcript in the nucleus and transported to the cytoplasm. There it is cleaved by Dicer into a mature miRNA, designated as the RNA strand capable of associating with a silencing complex and inducing the translational repression of a target mRNA to which it binds with incomplete base-pair complementarity. Vector-delivered shRNA, typically transcribed from a Pol III promoter, also acquires effector activity in the cytoplasm upon being processed into an siRNA duplex by Dicer. This product is analogous to chemically synthesized RNA duplexes in which one siRNA strand is designed to preserve perfect base-pairing with the target mRNA, a feature that instructs the silencing complex to induce mRNA cleavage. The same silencing complex, RISC, may regulate both pathways, with the mode of suppression being dictated by the extent of homology between the siRNA and its target. Vector-expressed siRNAs have the potential to exhibit off-target activities in part by entering the miRNA pathway (gray arrow) and suppressing the translation of partially homologous mRNAs.

while the other is apparently degraded?[15, 16] And finally, given the fact that the miRNA pathway has evolved to tolerate and apparently require base-pair mismatching at the level of target mRNA, how much of a capacity exists for ectopically expressed siRNA to induce off-target effects? While the complete answers to these questions are not yet known, there are some intriguing hints in the recent literature that may help inform vector-based approaches aimed at suppressing specific gene products through RNAi.

What Is the Best Vector Type and How Should It Be Configured?

A number of considerations that come into play when selecting a vector system for RNAi experiments are universal to any gene-delivery approach, and will not be covered here in detail. Most importantly, the vector must be capable of entering target cells with reasonable efficiency, and appropriate reporters must be available to identify or select cells in which vector uptake has occurred. Viral vectors have the advantage of being highly versatile—they can be used in transfection experiments but can also be pseudotyped to permit entry into difficult-to-transfect target cells. A variety of successful retroviral[17–27] and adenoviral[28, 29] siRNA expression vectors have recently been described. One remarkable development has been the demonstration that lentiviruses expressing siRNAs can be used to infect blastocyst- and preblastocyst-stage cells and rapidly generate transgenic knockdown mice, a finding that has the potential to revolutionize in vivo reverse genetic analysis.[19, 23, 24] Nonviral shRNA expression vectors, transfected into embryonic stem cells[30] or injected into pronuclear zygotes,[31] have also been successfully employed to suppress gene expression in mice.

While there have been many impressive reports of robust gene knockdowns by different types of vector constructs in diverse mammalian cell types, few have reported on the comparative evaluation of different vector parameters.[18, 25, 32–37] However, there are some indications that the manner in which RNAi vectors are configured can quite substantially influence biological outcomes, particularly in the context of viral delivery strategies, in which the vector copy number per cell is often low and therefore differences in gene silencing efficiency are likely to be detected with greater sensitivity. Barton and Medzhitov, for example, have reported that p53 knockdown by retroviral RNAi vectors is considerably more pronounced in superinfected cells.[18] One strategy that they, and others working with similar viral vectors,[21, 23, 25, 27] have employed is to engineer an shRNA expression cassette in the 3′LTR (long terminal repeat) at a position in the U3 region that permits its duplication prior to proviral integration (Figure 2). This has the potential to double the gene dose of the shRNA transcript at a given multiplicity of infection, although further studies are necessary to quantitate the enhancement in siRNA levels and gene knockdown.

In another recent study, Hemann et al. obtained different potencies of p53 suppression when two different viral vectors were employed to express the same shRNA.[21] A cassette driving the expression of an shRNA against mouse Trp53 was placed

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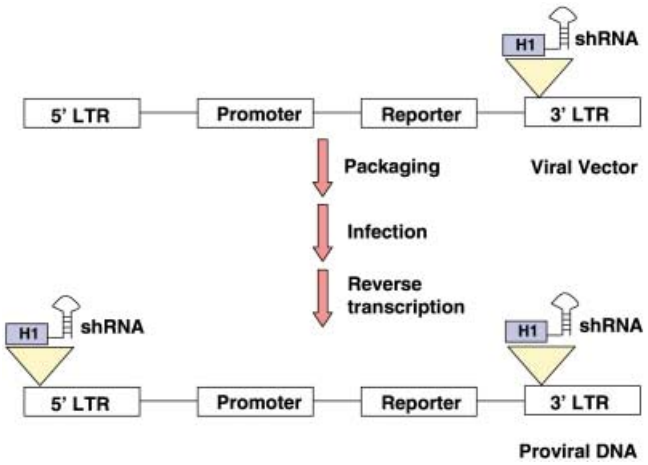

Figure 2. A self-inactivating viral system in which the shRNA cassette undergoes duplication. Retroviral vectors have been generated in which a promoter driving shRNA expression is engineered in the U3 region of the 3′LTR. During reverse transcription prior to proviral integration in target cells, the sequence information within this region, including the shRNA expression cassette, is copied to the 5′LTR. This results not only in the duplication of the shRNA cassette, but also in ablation of the promoter and enhancer activities of the 5′LTR. Both of these features may enhance the ability of these vectors to mediate RNAi.

either upstream of the puromycin selection cassette of pMSCV, or inserted into the 3′LTR of another retroviral vector, pBabePuro. Hematopoietic stem cells transduced with these vectors were evaluated in a murine lymphomagenesis model. While the two vectors induced similar rates of lymph-node enlargement, lethality was observed only in those mice receiving the pMSCV RNAi vector. This corresponded with apparently higher p53 knockdown by the pMSCV RNAi vector in transient transfection assays. Thus, even subtle differences in the abilities of different viral vectors to mediate RNAi may result in biological phenotypes that diverge in a pronounced manner.

There are several factors that may explain discrepancies in the levels of knockdown achieved with different vectors bearing identical siRNA cassettes. It is possible that proximity effects may influence the activity of the RNA polymerase III (Pol III) class promoters typically used to express shRNAs. One study indicated that Pol III promoters are attenuated when situated between retroviral LTRs, but exhibit robust activity when inserted into the U3 region of the 3′LTR (which results in their duplication; see Figure 2).[38] One interpretation of these results, which was not directly verified, is that the 5′LTR contributes a suppressive activity against the Pol III promoter, which is overcome when the promoter and enhancer activities of this LTR are ablated during vector self-inactivation. Interestingly, the 5′LTRs of retroviruses can downmodulate their downstream 3′LTRs in a process known as transcriptional interference,[39] and it is conceivable that similar proximity effects may occur in RNAi vectors, including nonviral vectors in which various promoter cassettes are arrayed. A related issue also worth considering, particularly if cell clones are to be derived, is that vectors may be susceptible to position effects that occur at sites of integration into chromosomal DNA.[19]

What Type of Promoter Should Be Used in the siRNA Expression Cassette?

Although the location of the siRNA expression cassette within a given vector clearly has the potential to influence experimental outcomes, the manner in which this cassette is designed deserves special attention. The first aspect to consider is the choice of promoter (Figure 3). Pol III promoters have been widely employed to express shRNAs in both viral and nonviral vectors.[17–27, 29–35] The familiar U6 and H1 members of the Pol III promoter family function to permit the ubiquitous high-level expression of small noncoding RNAs. In contrast to H1-based promoter cassettes, the efficiency of U6 transcription is more sensitive to the identity of the initiating (+1) nucleotide, which for optimal expression should preserve the endogenous guanosine.[37] Of particular importance from the perspective of RNAi, the products of Pol III are initiated at a precise nucleotide location and lack the trimethylguanosine 5′ cap and 3′-polyadenylated tail that are hallmarks of Pol II transcripts.

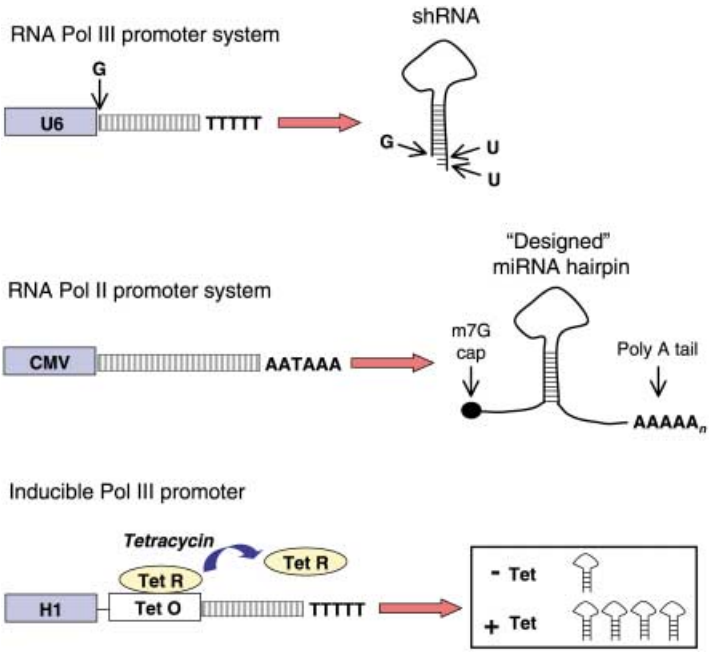

Figure 3. Different promoters can be used to express siRNAs. A common strategy is to express an shRNA from a Pol III promoter, which results in the production of a transcript with 3′-uridine overhangs that acts as a substrate for Dicer upon transport to the cytoplasm. Alternatively, Pol II promoters can be used to synthesize an artificially engineered miRNA-containing hairpin transcript that is 3′-polyadenylated and possesses a 5′-trimethylguanosine cap. Nuclear processing events release the stem-loop precursor miRNA from the transcript, allowing it to be exported to the cytoplasm where Dicer liberates the miRNA. This "artificial" miRNA is usually designed to possess perfect sequence complementarity to its target mRNA and thereby acts as an siRNA mimic, instructing RISC to induce mRNA cleavage. Finally, Pol III promoters have been successfully placed under the control of tetracycline-regulated transacting factors, allowing for the temporal regulation of siRNA expression.

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Instead, they terminate with an overhang of 1–4 uridine residues, signaled by a stretch of thymidine bases in the coding sequence. Thus, these promoters are ideal for the generation of compact and minimally modified shRNAs possessing 3′-nucleotide extensions at the base of the stem, a structure that is analogous to the overhangs found on bona fide Dicer cleavage products and to the free ends modeled in synthetic siRNA duplexes. Since the termini of shRNAs synthesized by Pol III resemble Dicer cleavage products, and since Dicer normally cleaves in a stepwise manner along double-stranded RNA with a periodicity of 21–23 nt, are Pol III-synthesized shRNAs particularly effective at attracting Dicer? Surprisingly, this may not be the case, at least when biochemically purified Dicer is assayed in vitro.[40] It is worth noting that the Dicer pathway does not appear to be a rate-limiting step in the ability of siRNA precursors (or miRNA precursors) to be processed, since these are often difficult to detect in the steady state.[33]

Another Pol III-class promoter that has been utilized in RNAi vectors is the $tRNA^{Val}$ promoter. When compared to a U6 promoter driving the expression of a similar shRNA, this element was found to significantly enhance the ability of the shRNA transcript to undergo nuclear export and enter the RNAi cascade.[41] Evidence for nuclear retention of shRNAs expressed from the U6 promoter (as chimeras with the first 27 nucleotides of endogenous U6 snRNA) has also been obtained by others.[32] While the nuclear export pathways governing endogenous noncoding RNAs have been well characterized,[42] the features of siRNA expression cassettes that regulate transcript trafficking are not understood, and further studies are required to help interpret these provocative observations.

While Pol III-based systems are well suited to the expression of shRNAs, Pol II-based siRNA cassettes have also been generated (Figure 3). This class of promoters is implicated in regulating the expression of endogenous miRNA-containing transcripts, in which silencing normally occurs at the level of translation due to base-pair mismatches with the target mRNA. "Designed" pre-miRNAs have been engineered by substituting regions of miRNA hairpin stems with siRNA cassettes possessing perfect complementarity to various target genes of interest.[7, 13] The prediction that these transcripts should transit through the Dicer pathway and trigger RNAi via RISC has been confirmed, suggesting that this is a viable alternative for RNAi vector design. One compelling advantage of Pol II promoters is the availability of tissue-specific versions for in vivo studies. Moreover, a single Pol II promoter can potentially be used to drive expression of both the miRNA and a reporter gene if, for instance, the pre-miRNA sequence is inserted into the 3′ untranslated region (UTR) of the reporter gene transcript. Further flexibility is conferred by the capacity to combine multiple miRNAs within the same Pol II transcript.[43] Of note, the ability of Pol II to efficiently synthesize transcripts containing active miRNAs does not necessarily extend to minimal shRNA-containing hairpins.[35, 37] In one report in which functional shRNAs were transcribed from a cytomegalovirus (CMV) promoter, the close juxtaposition of the shRNA between the promoter and a minimal polyadenylation termination signal appear to be critical for silencing activity.[28]

Although Pol II promoters have been comfortably manipulated within expression vectors for many years, a greater number of RNAi studies have employed vectors with Pol III-based expression cassettes. Pol III expression cassettes have also be successfully arrayed in tandem, an approach that has been used to individually express two siRNA strands of the same duplex,[44, 45] but which might be modified to permit two distinct shRNAs to be co-expressed (for example, to target two genes that act in a compensatory manner). Clearly, what is needed is a systematic analysis of the biosynthesis, nuclear export, and silencing activity of siRNAs produced by different promoters in a variety of vector systems. In a recent study, Boden et al. transfected cells with a vector panel in which different Pol III promoters, or the CMV promoter, directed the expression of an shRNA transcript targeting the *tat* gene of HIV-1.[37] Northern blot analysis revealed that the highest shRNA expression levels were obtained from either U6-based promoters or a modified $tRNA^{Met}$-derived promoter, while the H1 promoter yielded less steady-state shRNA product and the CMV promoter almost none at all.[37] Assuming that similar transfection efficiencies were obtained with these vectors, the data support the conclusion that a hierarchy exists among the abilities of various promoters to prime shRNA expression. Interestingly, the knockdown activity of the vector panel was found to correlate only partially with the steady-state levels of full-length shRNA;[37] this raises the possibility that differences exist in the ability of these transcripts to enter the RNAi cascade and give rise to active siRNAs.

Efforts to further optimize promoters for shRNA expression will likely prove fruitful in advancing the versatility of vector-delivery strategies. For example, fusing CMV enhancer sequences to a U6 promoter was found to augment the activity of an shRNA expression cassette.[46] In addition, several groups have developed tetracycline-regulated promoters of the Pol III class[47–50] (Figure 3). The ability to reversibly modulate the expression of siRNAs may be particularly powerful when targeting genes involved in growth regulation and when investigating the plasticity of cellular differentiation, providing that expression of the appropriate tetracycline-binding factor is also achieved.

How Should the Inhibitory RNA Transcript Be Designed?

Having considered the nature of the promoter used to express the interfering RNA, what about the sequence and structure of the siRNA transcript itself? Once again, different labs employing distinct design principles have achieved levels of gene suppression that appear equally impressive. Given this fact, how rigorous can these design principles be?

One of the most important aspects influencing the potency of a given "effector" or "antisense" siRNA strand is its ability to base pair with target mRNA (Figure 4). However, it is also clear that the efficiency of silencing can vary dramatically depending on which portion of the mRNA is targeted.[51, 52] Although some basic design principles have been suggested for chemically synthesized siRNAs,[1] the selection of target sequences remains largely empirical. It is not understood why a high proportion of chemically synthesized and vector-encoded siRNAs are ineffec-

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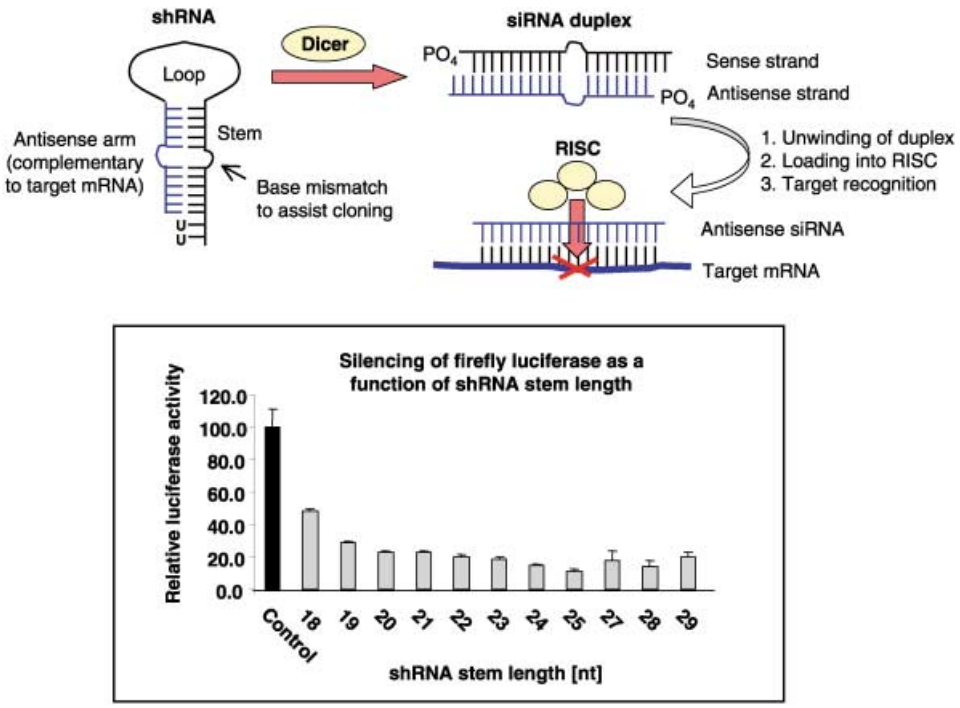

Figure 4. Features of the shRNA that may influence its function. Vector-expressed shRNA undergoes cleavage by Dicer, generating an siRNA duplex in which the antisense strand possesses perfect sequence complementarity to the target mRNA, a feature that confers the ability to trigger RNAi in association with RISC. In contrast, the sense siRNA, which by definition does not play a role in guiding RISC to the mRNA target, is sometimes designed to form an imperfectly base-paired shRNA stem, a modification that aids in DNA cloning and sequencing. Factors such as stem length, loop sequence, and base composition of the shRNA may modulate its ability to enter the RNAi pathway. Shown, as an example, is a dataset that we generated to address the relationship between shRNA stem length and RNAi potency. 293T cells (4×10^4) were co-transfected in triplicate with 30 ng of a firefly luciferase expression vector and 66 ng of different U6-driven shRNAs targeting the same region of firefly luciferase (5′-ATCACTTACGCTGAGTACTTCGAAATGTC-3′ = bases 151 – 180) but varying in stem length. Cells were also co-transfected with 3 ng *Renilla* luciferase to normalize for transfection efficiency, and the relative firefly luciferase signal for each condition was calculated in reference to a negative control empty vector after 48 h. Results, which are representative of 3 experiments, reveal a general trend in which longer shRNAs (up to 29 nt in stem length) are generally slightly more effective at suppressing target gene expression than shRNAs bearing shorter stems.

tive at mediating RNAi, although a number of explanations have been proposed. These include occlusion of target sites by mRNA secondary or tertiary structure, steric hindrance by mRNA-associated factors, and ineffective entry of the antisense siRNA into the RNAi cascade. Assuming that entry into the RNAi cascade is an important issue, is it possible that physical features of experimentally designed shRNAs such as stem length, loop sequence, and base composition might influence the efficiency of silencing?

On the issue of stem length, one point that is clear is that RNA duplexes longer than 30 nt trigger an interferon response, resulting in nonspecific translational suppression by the double-stranded RNA Responsive Protein Kinase (PKR) cascade. A variety of groups have experimented with shRNAs bearing stems of 19 – 23 nt in length, which is similar to the size of Dicer cleavage products.[17, 19, 20, 22–26, 30–34] Others have successfully employed stems as long as 29 nt, generating shRNAs resembling pre-miRNAs in size.[21, 35] We illustrate in Figure 4 an example of how we addressed the role of shRNA stem length using a reporter-based assay. We coexpressed, in 293T cells, the firefly luciferase reporter gene and a series of shRNAs directed against it that varied only in stem length (from 18 nt to 29 nt in 1 nt increments). Although all of the shRNAs targeted the same region of firefly luciferase, we reproducibly observed that shRNAs containing longer stems exhibited higher silencing activity than those possessing stems of less than 20 nt. While the differences were modest, the slight advantage conferred by longer stems, which has been mentioned by others,[35] may have a meaningful impact in experiments in which vector copy number is limiting. One point to consider, especially when working with larger stems, is that hairpin-containing DNA plasmids can be difficult to propagate and sequence. These difficulties can be mitigated by modifying several bases within the "sense" arm of the RNA hairpin to introduce G – U mismatches, which are stable in the context of an RNA alpha-helix.[35] Since Dicer has the capacity to cleave in the context of base mismatches,[12] these modifications should not impair entry into the RNAi pathway.

The next factors to consider are the size and nucleotide composition of the loop that is used to connect each strand of the shRNA stem. Brummelkamp et al. tested loops of 5, 7, and 9 nt length and concluded that 7 nt produced shRNAs with the greatest activity.[33] Others have successfully employed loops as small as 3 nt[25, 34] and as large as 23 nt,[35] although it should be noted that many "loops" that have been described in the literature contain some complementary base pairs. In terms of the nucleotide sequence of the loop, different restriction sites inserted into the loop region of shRNAs have been well tolerated in different assay systems, including palindromic sequences that may form additional hairpin structures. However, Kawasaki and Taira made the intriguing observation that the insertion of a loop motif from the miRNA *mir-23* greatly enhanced the cytoplasmic partitioning of an shRNA under the control of the U6 promoter.[41] We have also found that U6-driven shRNAs containing loop motifs from a variety of human miRNAs (including *mir-1*, *mir-19-b*, *mir-21*, *mir-23*, *mir-25*, *mir-26b*, and *mir-28*) exhibit enhanced silencing activity in transient transfection assays compared with shRNAs substituted with artificial loop sequences (e.g. containing palindromic or nonpalindromic restriction sites). Whether there are mechanisms through which miRNA features, such as loop structures, are recognized by nuclear export pathways remains a particularly intriguing area for further investigation.

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A final question concerns whether the base composition of the shRNA stem can influence its activity. Beyond the observation that the presence of base pair mismatches with target mRNA appear to regulate the mode of silencing,[7, 13, 14, 53] the available data do not clearly address whether the nucleotide sequence may influence processes other than target recognition. There is some evidence that local sequence determinants can influence the ability of double-stranded RNA to be recognized by RNase III class enzymes,[54] however extensive efforts to catalogue endogenous pre-miRNAs have identified few consensus-type sequences.[55] One perplexing observation is that often only one arm of a given pre-miRNA stem (i.e. either the 5′ or 3′ arm) can be detected in cell extracts;[15, 16] this implies that some aspect of miRNA precursor structure or sequence regulates miRNA half-life by dictating which strand stably associates with the silencing complex. By analogy, it is possible that in the case of vector shRNA there is also a bias at the level of RISC loading of one siRNA strand at the expense of the other; if so, this may be one explanation for why different shRNAs exhibit different levels of silencing activity. In a recently study, Lee et al. indeed found that only one arm of a vector-expressed shRNA could be detected in cell extracts; this suggests that the mechanisms regulating miRNA stability, though not yet well defined, may also apply to siRNAs.[27] It will be critical to address whether this is a general feature of most shRNAs and to determine whether features of shRNA nucleotide chemistry, such as the local annealing energies of base-paired regions, alter the relative abilities of the two siRNA products to stably associate with RISC. An exciting advance would be the ability to enhance RISC loading of the "antisense" effector siRNA by engineering base-pair mutations in the "sense" shRNA arm that alter the relative abilities of the two siRNA products to associate with RISC.

While flow cytometry, Western blotting, and real-time PCR are all viable approaches to compare the effectiveness of different RNAi vectors in suppressing endogenous gene expression, whether these assays are possible or practical may vary based on gene target, cell type, and efficiency of vector delivery. An alternative strategy is to subject vector reagents to preliminary functional screens in simple transfection-based reporter assays, which can be standardized for a wide variety of target genes. For example, genes can be epitope-tagged or fused to reporters such as the green fluorescent protein (Figure 5). Vector reagents, including viral constructs, can then be screened by transient co-transfection with the tagged target genes. An alternative approach that is even more versatile is to insert target sequences into the 3′-UTR of a reporter gene such as luciferase to generate a "tagged" mRNA chimera (Figure 5). Since this strategy does not involve the translation of target sequences, reading frame need not be preserved and any portion of the target mRNA can be screened. In either of these experimental approaches, the transfection mix should be spiked with another reporter vector to control for transfection efficiency and nonspecific (i.e. global) gene suppression. By using these approaches, which can be carried out in 96-well format with cell lines, candidate vectors that yield the best levels of knockdown compared with positive control reagents directed against reporter sequences can be

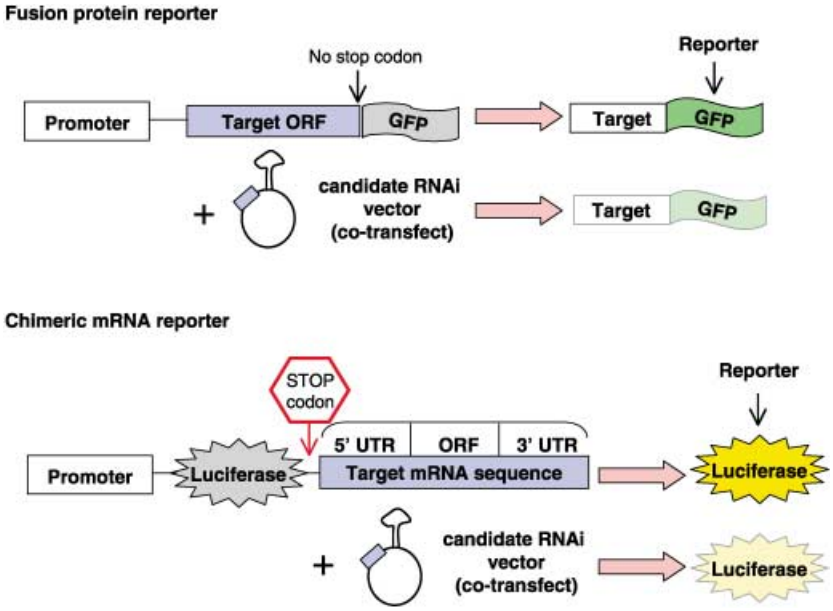

Figure 5. Reporter-based assays for target sequence selection. Two schemes are illustrated in which reporter genes can be utilized to screen candidate reagents for the ability to induce RNAi. In the first example, a fusion protein between the open reading frame (ORF) of the target gene and the green fluorescent protein (GFP) is generated that is assayed by co-transfection with siRNA vectors directed against the target ORF. Vectors that result in the greatest decrease in GFP signal can be selected for further analysis in a more relevant experimental model. Alternatively, a fusion transcript can be generated in which a target mRNA sequence is inserted within the 3′-untranslated region of a reporter such as firefly luciferase. Vectors encoding an active siRNA against the target sequence will induce a reduction in firefly luciferase activity that can be quantitated in reference to another reporter (e.g. *Renilla* luciferase) as an internal control. The advantage of this latter approach is that any portion of the target-gene mRNA can be utilized in the screen.

How Should Vectors Targeting Different Regions of the Same mRNA Be Evaluated?

A particularly frustrating bottleneck in the ability to deploy RNAi vectors is the difficulty in determining which portion of the mRNA to target, since there is a high level of variability in the degree of silencing achieved with reagents directed against different regions of the same transcript.[51, 52] Although various algorithms to aid in deciding which sequences to target are undergoing refinement, the selection process remains empirical, and at present multiple reagents must be generated for each target gene in order to achieve a statistical probability of success. Are there practical approaches that can help streamline the selection of candidate vector reagents?

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rapidly selected for more rigorous testing. By having in place an efficient screen that can be used to filter out ineffective vector reagents, greater efforts can be focused on the more daunting task of analyzing endogenous gene knockdown by using the best candidate vector reagents in relevant target cells. Efforts to assess the predictive ability of reporter assays such as these will be of value in helping streamline target sequence selection until the ideal of reliable in silico design can be achieved.

Unfortunately, being able to trigger an effective knockdown is not the only concern when screening for candidate target sequences. A more nebulous issue is how to assess and minimize potential off-target effects of a given vector siRNA. There are several possible mechanisms through which additional genes to the intended target may be suppressed. For example, it has been observed that some degree of base-pair mismatch may be tolerated in the induction of RNAi by RISC.[34, 43, 52] Imperfect base pairing between either member of an siRNA duplex and an off-target mRNA also has the potential to induce translational silencing (Figure 1), although it should be noted that siRNAs functioning as miRNAs tend to require tandemly arrayed target sites for efficient suppression of transiently expressed reporters.[13, 53] Obviously, sequence-homology searches should be performed on all candidate target sequences prior to vector generation, but at what level of stringency?

Recently, several groups have reported the results of microarray screens designed to detect off-target mRNA suppression in RNAi experiments. In two studies, gene silencing was extremely selective, at least at the level of mRNA.[56, 57] However, one report was more troubling, in that evidence was obtained that contiguous stretches of cross-hybridization as small as 11 nt between an siRNA and mRNA could trigger PTGS.[58] While these experiments were carried out with synthetic siRNAs, additional concerns have been raised by another recent study showing that a high proportion of plasmid and viral shRNA expression vectors induce an interferon response, particularly at higher doses.[36] It is therefore essential that appropriate measures be taken to control for nonspecific phenotypes in RNAi experiments. A number of practical measures were outlined in a recent RNA symposium,[59] the simplest of which is to titrate down the dose of knockdown reagent to the lowest possible level. Different doses of the same siRNA can produce dramatically different levels of off-target RNAi.[56, 58] Another experimental control is to demonstrate that the same phenotype is obtained when targeting distinct regions of the same mRNA or, more elegantly, to perform a rescue experiment by reconstituting expression of the targeted gene in the presence of the knockdown vector. This can be accomplished with relative ease if the vector regents are directed against UTR sequences, since the minimal open reading frame can then be ectopically expressed without serving as a target for RNAi. A final control that should be considered is examining whether vector siRNA induces the upregulation of genes associated with an interferon response, such as 2′5′-oligoadenylate synthetase.[36]

Summary

Although the RNAi field is in its infancy, there have been rapid advances in the vector delivery of small interfering RNAs to cells, tissues, and entire organisms. Success has been achieved through a variety of distinct approaches; however there are opportunities to further refine these technologies to more reliably elicit potent and specific RNAi. Pivotal to the continuing evolution of these revolutionary reverse genetic tools will be the attainment of a better understanding of the interplay between vector reagents and the elaborate cellular machinery of the RNAi pathway.

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