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Expression Strategies for Short Hairpin RNA Interference Triggers

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Abstract

Since the discovery that the triggers for RNA interference (RNAi), small interfering RNAs, could mediate silencing in mammalian cells without triggering a toxic response, RNAi has become the standard tool for sequence-specific knockdown of gene expression in molecular biology. This is due in part to the development of methods for promoter-based expression of RNAi triggers that can mediate stable silencing in mammalian cells. Numerous systems with slightly different characteristics exist, but despite incredible progress in a field that moves very rapidly, challenges still remain. The biggest challenge is to successfully and safely apply RNAi *in vivo*. Aside from potential issues of delivery, which is one of the most important considerations, successful application of short hairpin RNAs (shRNAs) *in vivo* requires expression systems that yield potent and specific knockdown of the target in the absence of toxicity. With a couple of exceptions, the current systems available for shRNA expression have not generally resulted in unexpected toxicities, while still providing strong knockdown of the intended targets; however, we do not know enough about how sequence-specific off-target effects will affect various cell and tissue types, or to what extent ectopic expression of RNAi triggers will perturb the endogenous RNAi mechanisms.

RNA INTERFERENCE TRIGGERING BY EXPRESSED HAIRPIN RNAs

TYPE I INTERFERONS that induce nonspecific silencing of mRNAs (Manche *et al.*, 1992) hampered the use of RNA interference (RNAi) in mammalian cells until small interfering RNAs (siRNAs), 19–25 base pairs in length, were shown to avoid this mechanism (Elbashir *et al.*, 2001a,b). At the same time, microRNAs (miRNAs), endogenous non-protein-coding genes that mature into duplexes that are similar to siRNAs, were discovered (Lee and Ambros, 2001; Lagos-Quintana *et al.*, 2003). Importantly, the miRNA precursors are hairpin structures, the strands of which are an imperfect stem joined by a loop, and these structures are subsequently processed into siRNA-like duplexes by Dicer (Hutvagner and Zamore, 2002; Bernstein *et al.*, 2003). Short hairpin RNAs (shRNAs) are characterized by base-paired stems and a loop region. These are processed by Dicer into siRNAs that are 21- to 23-base pair duplexes typically containing two-base 3' overhangs. Different types of promoters have been used for shRNA as well as siRNA expression in cells. The earliest reports took advantage of the simplicity and strength of RNA polymerase III (Pol III) promoters, particularly those derived from the U6 small nuclear RNA (snRNA) and H1 RNA genes (Lee *et al.*, 2002; Paul *et al.*, 2002; Yu *et al.*, 2002; Xia *et al.*, 2003). The sequence and structural characteristics of the stems and hairpins of the shRNAs expressed from these promoters may vary, and yet these systems are generally able to produce good target knockdown.

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AUTHOR DISCLOSURE STATEMENT

John Rossi is a cofounder of Dicerna Pharmaceuticals (an RNAi company) and cofounder of Calando Pharmaceuticals (an RNAi delivery company).

Many of the highly abundant cellular small RNAs such as tRNAs, 5S RNA, U6 snRNA, and U7 RNA are transcribed by RNA Pol III, which has been popularly used for shRNA expression. MicroRNAs, on the other hand, are largely transcribed by RNA Pol II (Lee *et al.*, 2004). Paddison and coworkers (2002) first showed that Pol II transcription of long double-stranded RNA (dsRNA) of about 500 nucleotides in cultured mammalian cells could result in sequence-specific posttranscriptional gene silencing. Expression of long hairpins transcribed by Pol III promoters has been demonstrated to trigger RNAi, but the siRNAs were produced in a gradient with the most abundant and active being at the end distal to the loop (Barichievsky *et al.*, 2007; Sano *et al.*, 2008). Most investigators use the U6 snRNA Pol III promoter or the H1 RNase P RNA promoter to express either a shorter hairpin or separate sense and antisense transcripts (Scherer and Rossi, 2003; Hannon and Rossi, 2004). A different approach is to base the hairpin on the structure of a microRNA intermediate (McManus *et al.*, 2002). This approach has gained favor because the miRNA type precursors go through the same processing and export pathway as the endogenous miRNAs, and can be expressed from either Pol II or Pol III promoters. Zeng and coworkers (2002) used a Pol II promoter to express natural as well as engineered miRNAs in human cells. Denti and coworkers (2004) used an shRNA expression system that is based on the snRNA U1 Pol II promoter, and showed that this can be effective for siRNA expression. More recently, Scherer and coworkers created tRNA-shRNA chimeras that use the endogenous tRNA 3'-end processing endonuclease to release shRNAs that subsequently are processed into siRNAs (Scherer *et al.*, 2007). This system was also shown to produce excellent strand selectivity, rendering the sense strand almost nonfunctional in producing siRNAs. At this time, it is not clear whether one type of shRNA expression system is best for general use. The best approach is to test two or more different expression systems to determine which is optimal for the intended purpose (Fig. 1).

Pol III-expressed short hairpin RNAs have been reported to induce an interferon response (Bridge *et al.*, 2003), but this may be a problem in the design of the vector, and elimination of an AA dinucleotide at the 5' end of the shRNAs abrogated the interferon response of the original offending shRNAs (Pebernard and Iggo, 2004). One possible explanation for these results is that the triphosphate on the 5' end of the shRNAs could trigger activation of retinoic acid-inducible protein (RIG)-I when expressed in certain contexts (Hornung *et al.*, 2006; Nallagatla *et al.*, 2007). On the other hand, endogenous expression of shRNAs in primary immune cells was shown not to activate the innate immune system while the same sequences applied by lipid transfection were potent activators (Robbins *et al.*, 2006). The expression of hairpins from vectors should nevertheless be carefully designed and tested to avoid activating the innate immune system.

In addition to delivery, which is always a challenge in therapeutics, the use of shRNAs *in vivo* still faces some important problems, especially concerning specificity. Perhaps most problematic is the fact that the mature products of shRNA expression are similar to miRNAs, and thereby have the potential to cause sequence-specific off-target effects on hundreds of genes simultaneously (Jackson *et al.*, 2003, 2006; Jackson and Linsley, 2004). The mechanism for this is largely due to the seed sequences of the processed shRNAs functioning as miRNAs (Jackson *et al.*, 2006). Aside from expressed shRNAs functioning as miRNAs, high-level expression of shRNAs leads to competition with endogenous miRNAs (An *et al.*, 2006; Grimm *et al.*, 2006; Castanotto *et al.*, 2007) and can cause lethality in animals (Grimm *et al.*, 2006). How then can these problems with shRNA expression be avoided or minimized? For off-targeting by seed sequence pairing to unintended targets, bioinformatic analyses of seed pairing to 3' untranslated regions (UTRs) in the transcriptome can be used as a first screen. The potential targets can then be monitored via microarray analyses or by Northern or Western blot assays to determine whether the predicted targets are real. Often, seed pairing of the ectopically expressed shRNAs will occur within a critical distance from an endogenous miRNA site, where it is more likely to trigger off-target RNAi (Saetrom *et al.*, 2007). Regarding activation of RIG-

For competition with endogenous miRNAs, the choice of promoter is critical. Pol II transcripts are capped, and therefore will not activate RIG-I via triphosphate interaction. Pol III transcripts with certain features may trigger RIG-I activation via triphosphate interactions if not designed properly. In this regard, the tRNA-shRNA fusions are perhaps the safest because the 5' triphosphate is processed from the tRNA by RNase P in the nucleus, and RIG-I is cytoplasmic.

COMPETITION WITH ENDOGENOUS miRNA PATHWAYS

High expression levels of any shRNA are likely to lead to competition with endogenous RNAi machinery (An *et al.*, 2006; Grimm *et al.*, 2006; Castanotto *et al.*, 2007). Because shRNAs and microRNAs share the use of exportin-5 (Lu and Cullen, 2004; Yi *et al.*, 2005) for nuclear export, this transporter can become saturated. In addition, shRNAs will compete with microRNAs for cytoplasmic Dicer and ultimately incorporation into the RNA-induced silencing complex (RISC) (Castanotto *et al.*, 2007). Thus, the most prudent approach for shRNA expression is to identify potent shRNAs that are capable of target downregulation when expressed at low levels (An *et al.*, 2006). A rapid, high-throughput shRNA synthesis system using the polymerase chain reaction (PCR) has been reported, and shown to allow facile screening of a large number of shRNA targets in a relatively short period of time (Castanotto *et al.*, 2002; Scherer *et al.*, 2004). When properly designed, expressed shRNAs represent a facile method for triggering RNAi in mammalian cells and may be a powerful approach for the treatment of a variety of chronic diseases both acquired and inherited.

shRNA EXPRESSION AND FUNCTION *IN VIVO*

The initial *in vivo* demonstration of shRNA functional triggering of RNAi was via hydrodynamic tail vein injections into mice (McCaffrey *et al.*, 2002) of anti-hepatitis C virus shRNAs; subsequently this group showed that human hepatitis B viral replication could be inhibited in mouse liver by delivery of anti-hepatitis B virus shRNAs (McCaffrey *et al.*, 2003). This approach is still used to rapidly assess expression of shRNAs in the liver, but is not well suited for studying shRNA expression in other organs. Viral vectors are particularly attractive for shRNA gene delivery. The two major classes of viral vectors used for shRNA gene delivery are the oncoretrovirus- and lentivirus-based vectors, which integrate into host cell chromatin, and the nonintegrating DNA viruses such as adeno-associated virus (AAV), adenovirus, and herpesvirus, which generally persist as episomes. The retroviral and lentiviral vectors are most useful for long-term applications whereas the nonintegrating vectors are most optimal when transient expression is required.

AAV vectors have also been used for liver-specific delivery of shRNA genes by simple low-pressure tail vein injection (Grimm *et al.*, 2005, 2006). Experiments using *ex vivo* manipulations of hematopoietic stem cells transduced with a lentiviral vector that carries shRNA or siRNA encoding Pol III expression units resulted in T cell maturation and the functional expression of RNAi triggers in SCID-hu mice (Banerjee *et al.*, 2003; Li *et al.*, 2005; Anderson *et al.*, 2007). Other applications of viral vector-mediated delivery of shRNA genes have been reviewed previously (Amarzguioui *et al.*, 2005; Kuhn *et al.*, 2007).

Inducible shRNA expression is obviously a safer route to shRNA expression *in vivo*. To date, several Pol III-inducible systems have been reported (Czauderna *et al.*, 2003; Gupta *et al.*, 2004; Amarzguioui *et al.*, 2005; Heinonen *et al.*, 2005) and inducible Pol II systems also exist (Unwalla *et al.*, 2004). A potential disadvantage of inducible promoters is that the inducer/repressor must be coexpressed in the cells or tissues targeted by the shRNA; “all in one” systems that harbor the inducible promoter and repressor genes in a single vector have been developed and should prove useful for *in vivo* studies (Aagaard *et al.*, 2007; Gray *et al.*, 2007).

CONCLUDING REMARKS

In conclusion, there are many choices for promoter-based shRNA or miRNA expression in mammalian cells. The major concerns of off-targeting and toxicity need to be considered in the context of the application. For simple target knockdown experiments in cell culture, appropriate controls are easy to carry out, and one should always use at least two shRNAs to the same target to verify the phenotype. Before proceeding to *in vivo* applications it is important to know that the shRNA or miRNA expression system delivers the required level of knockdown in cell culture without off-targeting or toxicity. Choice of the appropriate vector to deliver the shRNA or miRNA expression constructs is also critical, but will depend on the desired application. Finally, the use of constitutive or regulated systems for shRNA or miRNA expression should be taken into consideration. Once again this will depend on the goals of the experiment or therapeutic approach. As with any experiment or application of RNAi, carefully designed experiments and appropriate controls are the keys to success.

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FIG. 1. Expression strategies for shRNAs in mammalian cells. (A) The Pol III promoters based on U6 and H1 directly transcribe the shRNAs from the +1 position of the promoter transcription units.

Termination occurs within a stretch of several uracils in the transcript. The shRNAs contain 5'-triphosphates (Paul *et al.*, 2002). **(B)** U1 snRNA transcription unit for shRNA. The transcripts initiate at +1 and the first nucleotide is capped. U1 has a terminator element that is positioned immediately after the shRNA. The terminator is processed from the transcript (Denti *et al.*, 2004). **(C)** tRNA Pol III transcription unit for shRNA. The shRNA is appended immediately after the stem of the tRNA following the discriminator nucleotide (Scherer *et al.*, 2007). The shRNA is processed from the tRNA leaving a 5'-monophosphate. The tRNA transcription units have A- and B-box regulatory elements and terminate in a string of uracils. **(D)** Generalized Pol II transcription system for microRNA mimics. The promoter element can contain tissue-specific enhancers and a TATA element. The transcripts will initiate with a cap structure and a leader element. The microRNA mimic is processed from the primary transcript by Drosha/DGCR8, resulting in a pre-miRNA mimic (Zeng *et al.*, 2002).