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Minireview

Approaches for chemically synthesized siRNA and vector-mediated RNAi

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Abstract Successful applications of RNAi in mammalian cells depend upon effective knockdown of targeted transcripts and efficient intracellular delivery of either preformed si/shRNAs or vector expressed si/shRNAs. We have previously demonstrated that 27 base pair double stranded RNAs which are substrates for Dicer can be up to 100 times more potent than 21mer siRNAs. In this mini-review we elaborate upon the rationale and design strategies for creating Dicer substrate RNAs that provide enhanced knockdown of targeted RNAs and minimize the utilization of the sense strand as RNAi effectors. Expression of shRNAs or siRNAs in mammalian cells can be achieved via transcription from either Pol II or Pol III promoters. There are certain constrictions in designing such vectors, and these are described here. Additionally, we review strategies for inducible shRNA expression and the various viral vectors that can be used to transduce shRNA genes into a variety of cells and tissues. The overall goal of this mini-review is to provide an overview of available approaches for optimizing RNAi mediated down regulation of gene expression in mammalian cells via RNA interference. Although the primary focus is the use of RNAi mediated cleavage of targeted transcripts, it is highly probable that some of the approaches described herein will be applicable to RNAi mediated inhibition of translation and transcriptional gene silencing.

Keywords: Dicer; siRNA; Pol III; Pol II; Inducible shRNA expression; Viral vector; PTGS; TGS

1. Introduction

RNA interference (RNAi) is a process, first described in the worm *Caenorhabditis elegans*, whereby the presence or introduction of long double-stranded RNA (dsRNA) in cells results in the degradation of homologous mRNA [1,2]. Long dsRNA is processed to 21–23 bp short interfering RNA (siRNA) with 2 nt 3′ overhangs by the RNAseIII-like protein Dicer [3]. These cleavage products are subsequently incorporated into the RNA-induced silencing complex (RISC) [4]. Delivery of chemically synthesized short interfering RNAs, mimicking Dicer cleavage substrates, results in sequence-specific, robust silencing of the expression of the corresponding endogenous gene [5], thus bypassing the non-specific inhibitory mechanisms elicited by longer dsRNA in mammalian cells [6]. RNAi can also be induced by endogenous expression of short hairpin RNAs (shRNAs) [7]. shRNAs are structurally related to a highly conserved class of small RNAs known as microRNAs (miRNAs) that mediate RNAi through a translational inihibition mechanism involving imperfect complementarity to sites in the 3′ UTR of target genes [8]. miRNAs are transcribed as precursors that are first processed in the nucleus by the RNaseIII protein Drosha in the Microprocessor complex [9–11]. The product of Drosha-mediated processing, pre-miRNA, is exported to the cytoplasm by Exportin 5 [12], for further processing by Dicer to the mature miRNA [13]. One of the strands is incorporated into a RISC-like silencing complex [14].

RNAi has recently become the method of choice for mammalian cell genetic analysis and has the potential to serve as a therapeutic treatment for a variety of acquired and hereditary diseases [15]. In this review, we will describe the various methodologies for eliciting RNAi by either synthetic or expressed RNAi effector molecules.

2. Synthetic siRNA-mediated RNAi

2.1. Enzymatically generated siRNA

The most cost effective and quickest method for siRNA synthesis is T7 phage RNA polymerase mediated in vitro transcription from short double-stranded oligo cassettes containing the promoter sequence immediately upstream of the siRNA strand template sequence to be transcribed [16,17]. The siRNA strands are synthesized in separate reactions and hybridized before purification. Once the template oligos are available, template preparation (annealing), in vitro transcription, siRNA annealing, and purification can be completed within 24 h. The siRNAs synthesized by this method frequently contain a GGG leader sequence (deriving from the promoter) as well as a 5′ triphosphate group [18]. The hybridized siRNA thus needs to be processed by T1 ribonuclease to remove the single stranded 5′ GGG overhang. If the siRNAs are transcribed with UU 3′ overhangs, T1 processing may be incomplete due to the potential formation of two G:U wobble base pairs with the G's of the 5′ leader sequence. Incomplete processing will result in retention of transcripts with 5′ triphosphate groups, which triggers non-specific inhibition of gene expression through the interferon pathway [18]. Although we have shown that the interferon response and RNAi are independent pathways [18], these siRNAs should be used with great caution in applications related to viral infection or innate immune responses. This approach may therefore be more appropriate for initial screening of target sites prior to validation of any results by chemically

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synthesized siRNAs. Replacement of the 3′ UU residues with 3′AA that cannot form wobble base pairs with the 5′GG improves processing and reduces the potential for interferon induction [18]. Changing the siRNA from 19 + UU to 21 + AA, with a 21 nt target complementary duplex region was also associated with enhanced RNAi activity.

Different siRNA sequences display widely differing efficacies, requiring screening of multiple sequences [19–21]. One way to get around this problem is by application of a pool of enzymatically generated siRNAs. Dicer, an RNase III family enzyme, cleaves in vitro transcribed long dsRNA into a pool of siRNAs suitable for gene silencing [22]. Therefore, several groups have produced a recombinant version of Dicer and used it to digest in vitro transcribed dsRNAs into a complex pool of siRNAs (d-siRNA) [23]. Nearly every pool of d-siRNAs is capable of eliciting specific gene silencing. This approach eliminates the need to identify an individual effective siRNA and has proven to be useful for transiently silencing many endogenous genes in several types of cells. Although the method is efficacious, cost effective, and relatively quick, there are some potential problems. Any residual unprocessed long dsRNA will activate RNA-dependent protein kinase (PKR), resulting in non-specific translational inhibition [6]. Gel purification of 21–23mer siRNAs from unprocessed long dsRNAs and partially processed products is therefore essential before transfection of siRNAs into cells. Additional concerns associated with the use of pools of siRNAs are the potential increased off-target effects occasionally observed with siRNA [24]. Competition from less efficacious siRNAs in a pool may also reduce the overall efficacy compared to utilization of one optimal siRNA sequence. A further advantage of utilizing one siRNA of known sequence is that any observed phenotypes of the siRNA can be verified through the application of a second siRNA species targeting the same gene. The siRNA pool approach may require additional confirmation through the use of target-specific and mismatched siRNA of defined sequence to verify the sequence specificity of the observed phenotype.

2.2. Chemically synthesized siRNAs

Chemically synthesized siRNAs represent the gold standard for RNAi applications. They are of a uniform composition and can be synthesized at higher amounts and with a wider range of chemical modifications than by other methods [25–27]. The disadvantages include higher cost and increased synthesis time. Initial studies in *Drosophila melanogaster* embryo lysates concluded that 21 nt siRNAs with 2 nt 3′ overhangs were the most efficient triggers of sequence-specific mRNA degradation [28], and most subsequent studies have therefore employed this format. During investigation of cellular interferon induction caused by in vitro transcribed siRNAs, we observed that some siRNAs of length 25–27 appeared to have greater potency than synthetic 21mer siRNAs directed to the same target site [18]. Synthetic RNA duplexes of varying length containing 3′-overhangs, 5′-overhangs, or blunt ends, were tested for their relative potency in several reporter systems [29]. Using duplex RNA at several concentrations, we observed that potency increased with length up to a duplex length of 27 bp. Increased potency was observed even for siRNAs with 5′ overhangs or blunt ends [29]. Reduced efficacy was observed for siRNA with longer than 27 bp stems, which also exhibited slower in vitro Dicing kinetics. Importantly, the 27mers do not induce interferon or activate PKR. Hannon and colleagues [30] also found synthetic shRNAs with 29-base-pair stems and 2-nucleotide 3′ overhangs to be more potent inducers of RNAi than shorter hairpins. Maximal inhibition of target genes was achieved at lower concentrations and silencing persisted longer. The improved efficacies of the longer forms of siRNA, termed "disRNAs" or "Dicer-substrate siRNAs", is postulated to result from their recognition and cleavage by Dicer, followed by their subsequently more efficient incorporation into the RISC complex. This interpretation is supported by observations that Drosophila Dicer is not only instrumental in handing over siRNA to nascent RISC, but is itself a component of the latter [31,32]. Providing the RNAi machinery with a Dicer substrate therefore presumably results in more efficient incorporation of the active 21mer into RISC. DisRNAs have subsequently been successfully employed by others [33].

Further investigation determined that the efficacy of dsiRNAs varies with the target (Kim et al., unpublished data). One reason for this is that multiple 21mer siRNAs of potentially highly variable activity can result from the same 27mer after processing by Dicer. We investigated this possibility by electrospray ionization mass spectrometry (ESI MS) analysis of in vitro diced dsiRNA. As expected, multiple 21–22mer products were generated from the dicing reactions. Optimal design of Dicer substrate siRNAs thus requires the ability to either predict the resulting 21mer(s) or direct cleavage to generate only a desired 21mer. The natural role of Dicer in cells appears to be finalizing the processing of miRNA [13], a highly conserved class of small RNAs that function in the regulation of expression of a wide range of genes at the translational level [34]. The substrates of Dicer, pre-miRNA, are bulged stem-loop structures with 2 nt 3′ overhangs, and recent reports suggest that the overhangs in the open end of the stem in such structures are bound by Dicer and determine the direction of processing [30]. The miRNA strand harboring the 3′ overhang is utilized more frequently than the top strand [35]. In an attempt to introduce a similar directionality into disRNA, we developed a format of 27mer (25/27R) in which the top strand is 25mer, and the bottom strand a 27mer with 2 nt overhangs in 3′ end. This reduced the complexity of dicing products but did not result in a single product. Further investigations determined that incorporation of DNA nucleotides in the 3′ end of the top strand (near the blunt end of the duplex) resulted in processing proceeding exclusively from the overhang end, producing a single primary 21mer siRNA of predictable sequence (Fig. 1). It is therefore possible to design a disRNA that is processed by Dicer to yield a specific, desired 21mer species. The same 21mer could be generated from a disRNA of slightly different sequence in which the top strand is 27mer with 2 nt overhangs in the 3′ end, while the bottom strand is a 25mer (27/25L), and the DNA nucleotides were introduced in the bottom strand near the blunt end. The 25/27R is processed to the 3′ end (right direction) and produces one major 21mer (R form). The 27/25mer is processed in the left direction and produces the same 21mer (L form). Interestingly, the "R" versions of the asymmetric 27mers were consistently more efficacious than the "L" versions (Fig. 1). This difference in potency of disRNA producing the same 21mer duplex suggested to us the possibility that Dicer processing may introduce some asymmetry in strand incorporation into RISC through preferential binding to the 3′ overhang. RISC-mediated