Gene Therapy (2009) 16, 827–829

www.nature.com/gt

New concepts for siRNA design: the beauty of asymmetry

Asymmetry in siRNA design

D Grimm

Gene Therapy (2009) **16,** 827–829; doi:10.1038/gt.2009.45; published online 30 April 2009

Sparked in 1998 by a seminal paper from later Nobel prize winners Andrew Fire and Craig Mello,[1] RNA interference (RNAi) has rapidly become a standard tool for targeted gene knockdown and laid the foundations for a burgeoning field of research.[2] Briefly, RNAi describes a pathway in eukaryotic cells in which short, double-stranded RNAs (dsRNAs) trigger sequence-specific gene silencing. RNAi's unprecedented success as a gene knockdown tool is due, in part, to its simplicity; all that is required is a ~21 nucleotide (nt) double-stranded RNA molecule engineered to match a sequence in the target gene.

The path of RNAi delivery into cells follows a consistent pattern. Synthesized small interfering RNA (siRNA)[3] or vector-encoded short hairpin RNA (shRNA) act as the RNAi trigger and when they arrive in the cell are incorporated into a multi-protein complex known as an RNA-induced silencing complex (RISC). A key RISC component is the Argonaute-2 protein, which uses the siRNA as a guide to find and cleave a matching sequence on the mRNA that codes for the target gene. The nicked mRNA is then degraded by RNases, therefore the target gene is not expressed and Argonaute-2 is recycled to propagate further RNAi.

In addition to being strikingly simple, RNAi is attractive to biologists and clinicians alike because of its supreme efficacy and specificity. The lists of physiologically or pathologically relevant genes already inhibited effectively with some form of RNAi are long and growing. In some cases, siRNA testing is now moving into Phase III clinical trials.[4] Despite these early achievements, there is emerging apprehension that the sense or passenger strand of the double-stranded siRNA molecule can cause dysregulation of gene expression. Particularly worrisome is that as few as six or seven nucleotides can suffice to trigger inappropriate silencing through miRNA-like mechanisms.[5] Efforts to improve siRNA design include modifying the phosphorylation status of the ends of the two siRNA strands[5] and establishing chemical modification schemes.[4]

In a study published last year in *Nature Biotechnology,* Sun *et al*.[6] looked at the concept of siRNA asymmetry and took it to a new level. Using β-catenin as target, the authors investigated the minimal lengths of siRNA strands and found that silencing required at least 19 nt of target complementarity, validating data from earlier studies. They then asked whether this strict minimal length requirement applied only to the guide strand of the siRNA or to the guide and passenger strands. They designed a set of asymmetric siRNAs that maintained a 19-nt guide strand (with 2-nt overhangs) while cutting the passenger strand in a stepwise manner down to 12 nt. Unexpectedly, pairing with a 15-nt passenger strand was very efficient and mediated silencing at doses as low as 100 pM. The reverse design that involved truncating guide strands was inactive, which validated the need for a 19-nt guide strand. The activity suggested that asymmetric interfering RNAs, termed aiRNAs and characterized by long guide and short passenger strands, represent a novel and efficient scaffold structure for RNAi duplexes (Figure 1).

An important next question was whether these would still engage the canonical RNAi pathway. It was found that cleavage of the target mRNA occurred at a distinct spot, between nt 10 and 11 from the 5′ end of the aiRNA guide strand, as one would predict for a standard siRNA. Moreover, immunoprecipitation studies revealed a durable association of aiRNA with Argonaute-2/RISC proteins, suggesting efficient recruitment of the RISC complex.

To better assess the efficacy of their new RNAi trigger, Sun *et al*. utilized a small set of further targets to compare aiRNAs with standard siRNAs. Impressively, most aiRNAs at least matched the efficiency of the conventional siRNAs, and were even superior in some cases.

Finally, Sun *et al*. addressed the pivotal issue of unwanted off-targeting activity from their aiRNAs using two distinct strategies. Gene array data from cells transfected with aiRNAs or siRNAs showed that the latter deregulated roughly twice as many off-target genes. Compared with aiRNA, more siRNA off-targets also showed partial homology to the siRNA sense strand. They also found that both siRNA strands were highly active against artificial sense and anti-sense targets, but only the aiRNA guide strand gave functional silencing. Together, this supported the idea that asymmetric aiRNAs, because of their short 15-nt passenger strand, alter bias in favor of the guide strand and are thus less prone to causing off-target silencing than standard siRNAs.

At least two aspects make the study by Sun *et al*. particularly noteworthy. First, it prompts intriguing questions regarding mechanisms for processing asymmetric small double-stranded RNA. Though data in the paper relating to this key point are sparse, more insight comes from a recent similar study that also described sequentially truncated asymmetric siRNAs. Like Sun *et al*., Chu and Rana[7] identified a short (16-nt) passenger strand as capable of mediating RNAi. Two further findings, however, markedly contrast this study with the work by the Sun group. First, Chu and Rana noted that deletions were also tolerated by the guide strand so efficient RNAi can be created with a 16-nt guide/19-nt passenger strand (that is, the reverse of aiRNA). Second, silencing was even better when both strands were only 16 nt long (an inactive scaffold for Sun *et al*.) (Figure 1). Further analyses implied that the increased activity from the 16-nt siRNA resulted from its capacity to program higher concentrations of RISC. The discrepancies between these two papers clearly suggest that more studies are needed on the detailed

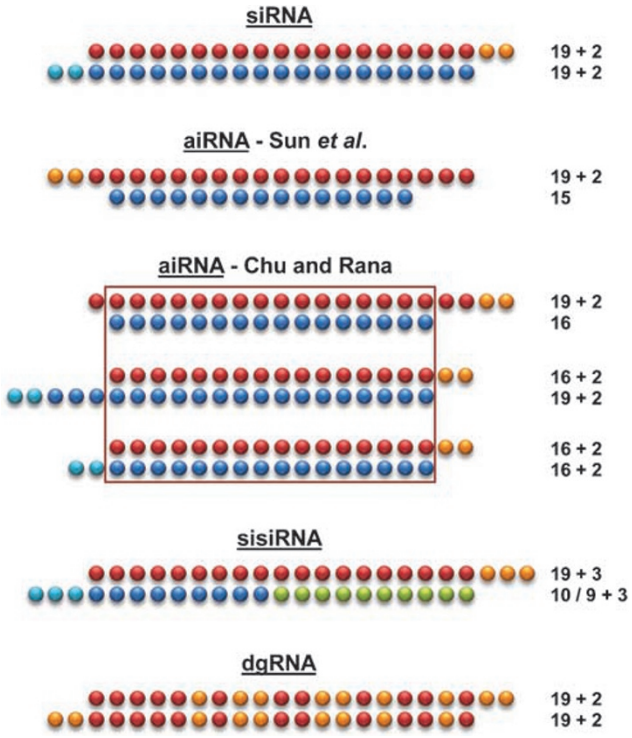

Figure 1 Shown (from top to bottom) are a canonical small interfering RNA (siRNA) (antisense or guide strand in red (5′-3′ orientation), sense or passenger strand in blue (3′-5′ orientation), unpaired nucleotides (nt) in orange), asymmetric interfering RNAs (aiRNAs) (according to Sun *et al*. or Chu and Rana, respectively),[6,7] sisiRNA (small internally segmented interfering RNA),[8] or an example of a double-guide siRNA (dgRNA).[9] The numbers depict nt in the guide and passenger strand (paired+unpaired).

mechanisms by which minimal asymmetric or symmetric siRNAs are incorporated into RISC and trigger RNAi.

Also interesting in this respect are data from another group, which imply that an intact sense strand might not be required for efficient RNAi at all. Instead, Bramsen *et al*.[8] reported pairing of an intact guide with two shorter (10/12 nt) passenger strands. The resulting tri-molecule construct, named sisiRNA (small internally segmented interfering RNA, Figure 1) remained capable of gene silencing, and furthermore accommodated heavily chemically modified guide strands not tolerated by standard siRNAs. By eliminating the passenger strand altogether, Hossbach *et al*.[9] took the technique to the other extreme. They synthesized siRNA duplexes of only target-complementary sequences, thus technically consisting of two guide strands. The general usefulness of this strategy remained an open question, but the crucial benefit from all these approaches was the decline of unwarranted off-targeting incidences from the passenger strand.

The second very notable aspect of the Sun *et al*. work is the potential improvement in aiRNA strand specificity and this has broad implications for therapeutic use of RNAi. Their evidence that aiRNA truly yields strand-specific RNAi is preliminary, but the general concept of minimizing off-targeting from the passenger strand by reducing it to a threshold of 15 or 16 nt (consistent between the Sun and Chu papers) is so strikingly simple and convincing that more detailed studies of these new RNAi triggers should be awaited with interest. One very revealing study would be a comprehensive comparison of aiRNA efficacies and specificities with the alternatives described above.

Of course, towards clinical implementation, this novel design will have to be tested and optimized rigorously. One parameter must be the algorithm for aiRNA design. Amongst the four targets studied, aiRNAs were either ~1.5 up to ~10-fold more efficient than the matching siRNAs, or slightly worse.[6] This suggests that aiRNA design requires improvement by testing more targets and RNAi triggers. A second parameter will be aiRNA testing in an animal context, in which these agents are likely to face the same challenges that initially hampered *in vivo* use of conventional siRNAs. Particular issues will be serum stability, targeted delivery and pharmacokinetics. It will be interesting to see whether asymmetric aiRNA triggers improve their RNAi potential after ribonucleotide modifications in one strand or the other. A third parameter will be validating the idea that aiRNAs are a safer class of RNAi drugs because there is less off-targeting. Beyond passenger strand effects, it will be essential to assess miRNA-like off-target effects on a proteomics level. Likewise, it will be crucial to explore the risks of toll-like receptor activation by aiRNAs. In fact, the possibility cannot be discounted at this point that aiRNAs, with their unique structure (partially ssRNA, partially double-stranded RNA), might be particularly good candidates for toll-like receptor activation, as suggested by earlier *in vitro* findings with similar molecules[10] (5′ tri-phosphorylation may also be required).

Despite the remaining questions and challenges, there is no doubt that the asymmetric aiRNA scaffold is another hugely promising entry into the growing repertoire of ingeniously and substantially improved RNAi drugs. An exciting direction might be to use the concept of siRNA strand asymmetry in vector-based RNAi triggers, such as short hairpin RNAs, to increase their strand specificity and overall safety. In parallel, novel computational tools may need to be developed, which include 15/16-nt complexes that yield short siRNAs with potentially greater potency. We anticipate the further optimization and evaluation of aiRNAs and other novel RNAi compounds in cell and animal models, and it is our hope that we can eventually also witness the clinical implementation of these exciting prospective therapeutics. ■

D Grimm is at the University of Heidelberg, Cluster of Excellence CellNetworks, BIOQUANT, Im Neuenheimer Feld 267, D-69120 Heidelberg, Germany.
E-mail: dirk.grimm@bioquant.uni-heidelberg.de

1 Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-

stranded RNA in *Caenorhabditis elegans*. *Nature* 1998; **391**: 806–811.
2 Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. *Nat Rev Gen* 2007; **8**: 173–184.
3 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; **411**: 494–498.
4 Behlke MA. Chemical modification of siRNAs for *in vivo* use. *Oligonucleotides* 2008; **18**: 305–320.
5 Pei Y, Tuschl T. On the art of identifying effective and specific siRNAs. *Nat Meth* 2006; **3**: 670–676.
6 Sun X, Rogoff HA, Chiang JL. Asymmetric RNA duplexes mediate RNA interference in mammalian cells. *Nat Biotechnol* 2008; **26**: 1379–1382.
7 Chu C-Y, Rana TM. Potent RNAi by short RNA triggers. *RNA* 2008; **14**: 1714–1719.
8 Bramsen JB, Laursen MB, Damgaard CK, Lena SW, Babu BR, Wengel J *et al*. Improved silencing properties using small internally segmented interfering RNAs. *Nucleic Acids Res* 2007; **35**: 5886–5897.
9 Hossbach M, Gruber J, Osborn M, Weber K, Tuschl T. Gene silencing with siRNA duplexes composed of target-mNA-complementary and partially palindromic or partially complementary single-stranded siRNAs. *RNA Biology* 2006; **3**: 82–89.
10 Zheng X, Bevilacqua PC. Activation of the protein kinase PKR by short double-stranded RNAs with single-stranded tails. *RNA* 2004; **10**: 1934–1945.