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## Preface

The discovery of RNA interference (RNAi) as a methodology for gene silencing has revolutionized biological research and has provided an invaluable avenue for therapeutics. Since its unearthing in 1998, RNAi has been utilized by researchers for assessing the biological function of genes and has been the focus of numerous clinical trials. An ongoing challenge within the field of RNAi is the design of effective double-stranded RNA (dsRNA) molecules for the targeted silencing of genes. Small interfering RNA (siRNA), typically composed of two complementary 19–27 bp RNA molecules homologous to the target gene, is the most common strategy utilized for enacting RNAi. Its function is based on the sequence-specific cleavage of mRNA by the RNA-induced silencing complex (RISC), which functions endogenously to mediate gene regulation by cellular microRNA (miRNA). In addition to chemically synthesized siRNA, a number of methodologies exist for utilizing the Dicer machinery to produce siRNA within the cell from precursor RNA molecules, the most common of which is short hairpin (sh) RNA. There is debate within the field about the most advantageous methods for designing RNAi molecules for gene silencing. However, several innovative new RNAi designs have been developed to improve siRNA performance. Guidelines and protocols are provided within this volume for the selection of siRNA targeting sequences, for the strategic incorporation of chemical modifications, and for advantageous structural modifications to the classic siRNA and shRNA design. Strategies are also described for specific applications such as immunostimulatory siRNA that may provide therapeutic benefit against viral infections in mammals, the simultaneous targeting of multiple siRNAs, and siRNA-mediated crop virus resistance. The design of RNAi for gene silencing in embryonic, invertebrate, and plant systems requires a variety of unique approaches, several of which are described towards the end of this volume.

The foundation of any RNAi design protocol, regardless of the application, is the selection of the sequence to be targeted within the mRNA molecule. At this stage in understanding of RNAi methodology, targeting sequences cannot be predicted with certainty of efficacy in gene silencing (also called “knockdown” efficiency). For this reason, a validated siRNA target is often the first choice in target sequence selection. The term “validated siRNAs” can refer to those that have anywhere from 50 to 100% knockdown; however, depending on the experimental parameters, less than complete knockdown may not be sufficient for the ablation of downstream functions. Furthermore, siRNA target sequences have not been validated for all genes, and this particularly applies to nonmammalian species with partially characterized genomes. In other cases, it could be desirable to design siRNAs to selectively target one splice form of an mRNA or a homolog of a closely related gene. For any of these scenarios, a validated siRNA for the gene of interest, even if available, may not have the appropriate specificity. Moreover, most validated targets are not thoroughly tested for the so-called off-target effects that occur, in part, due to the sequence-specific activation of immune responses or degradation of nontargeted mRNAs. The ability to select an effective targeting sequence that also is specific to its target is, therefore, at the forefront of siRNA design.

To improve the success rate for selecting effective targeting sequence, algorithms have been developed based on empirical criteria, structural modeling of the RISC complex, and RNA secondary structure predictions. Chapter 1 provides an overview of the most important factors to consider when selecting a target sequence and also describes the development and utility of algorithms for target sequence selection within a historical context. Particular attention is applied to an increased understanding of the role of thermodynamics in selecting sequences that are effective in terms of the overall stability of the duplex, as well as the local internal stability, the incorporation of asymmetry that can facilitate the loading of the appropriate strand into the RISC, and the avoidance of off-target recognition of nontargeted mRNA sequences. A protocol is provided for incorporating each of these factors into the design of either siRNA or shRNA.

In Chapter 2, several analytical methodologies are considered for selecting functional siRNA target sequences using statistical modeling to predict the probability that a candidate siRNA sequence will be effective. These methodologies are evaluated by application to recently reported effective and ineffective siRNA sequences for a number of genes, and new methods for target selection are proposed. An additional newly emerging algorithm for siRNA target selection, siDirect 2.0, is described in Chapter 3. This algorithm is designed with regard to reducing off-target effects that can complicate experimental interpretation. Rationales and instructions are provided for using a Web-based server to identify candidate siRNA target sequences with reduced likelihood of producing off-target effects.

Target site accessibility is another major hurdle in siRNA site selection that is particularly important for the design of siRNA against highly structured targets, such as RNA virus genomes. In Chapter 4, a high-throughput method is described for identifying accessible target sites within highly structured RNA by measuring oligonucleotide hybridization kinetics under non-denaturing conditions.

In addition to careful target sequence selection, siRNA efficacy and specificity can be enhanced through selective chemical modification of nucleotides within the duplex. Chemical modification can reduce immunogenicity, increase the stability of the dsRNA complex, and increase the duration of knockdown. Chapter 5 reviews the recent literature describing a variety of modifications that have been used effectively in siRNA design and provides guidelines for incorporation of these modifications for specific *in vitro* and *in vivo* applications. In Chapter 6, the detailed applications and advantages are described for strategically applying substitutions using one form of helix-destabilizing non-nucleotide analog, Unlocked Nucleobase Analogs (UNA). Protocols are provided for the *de novo* design of optimized siRNAs containing UNA and the evaluation of these molecules for knock-down efficiency, target specificity, and low immunogenicity.

The functionality of siRNA can also be further enhanced by structural modification to the dsDNA backbone, and in Chapters 7 and 8 design protocols are provided for two such variants of the standard siRNA duplex, asymmetric siRNA (asiRNA) and fork-like siRNA (fsiRNA). These variants are designed to impart additional favorable asymmetry to the siRNA duplex to assist in RISC guide strand selection. The asiRNA design (Chapter 7) utilizes a shortened 15–16 nt sense strand and an antisense strand whose 5' end is blunted and 3' end has an extended overhang. Methods are provided for designing asiRNAs and for assessing their silencing activity and specificity. The fsiRNA design (Chapter 8) comprises a strategic introduction of duplex-destabilizing nucleotide substitutions at the 3' end region of the sense strand that can enable the effective use of target sites with otherwise unfavorable sequence parameters. Nuclease sensitive sites within the fsiRNA, which are identified through rational design and experimental observation, are stabilized by introducing chemical modification.

The decision of which siRNA design strategy to choose depends on the application, and Chapter 9 provides an innovative protocol for designing dual-targeting siRNAs for applications that require the silencing of two genes simultaneously. In this design, both strands within the duplex are functional, and through a careful series of steps, each strand can be fashioned to mediate silencing activity through both siRNA-like RNA cleavage and miRNA-like translational repression mechanisms. Methods are provided for finding partially complementary dual-targeting siRNA candidates, for predicting their siRNA and miRNA activity, and for scoring and prioritizing the most promising candidates.

For the majority of RNAi experimental designs, the immunostimulatory responses that can occur as a side product of the dsRNA-mediated activation of Toll-like receptors and other RNA-sensing proteins within the cell are perceived as an undesirable off-target response. However, in specialized cases, it may be advantageous to promote immune activation during siRNA-mediated knockdown. As an example, immunostimulation may help to enhance the function of siRNAs that are antiviral or antitumorigenic. Chapter 10 reviews the current strategies for designing siRNA variants that have increased immunostimulatory potency. Methods are also provided for the assessment of immune activation by these bifunctional siRNAs.

As a cost-effective alternative to chemically synthesized siRNA, recent studies have shown that pools of siRNA molecules that target a single gene can be produced enzymatically (termed endoribonuclease-prepared siRNA, or esiRNA). The use of a pool of siRNAs has the additional advantage of diluting out the sequence-specific off-target effects of individual transfected siRNA clones. Chapter 11 provides guidelines for the *in silico* selection of an esiRNA target region that is most suitable for gene silencing.

As an additional alternative to chemically synthesized siRNA, a number of methodologies exist for utilizing the endogenous cellular machinery to produce siRNA within the cell from precursor molecules that are processed by the Dicer complex. Vector-expressed siRNA has particular advantage for difficult-to-transfect cells and for stable expression. Chapter 12 provides an overview of the history and application of shRNAs in mammalian systems, including a comprehensive outline of the options for shRNA effector molecule design, vector and promoter selection, and the simultaneous delivery of multiple shRNAs. In Chapter 13, methods are provided for the design and construction of multiple different forms of vector-expressed siRNA, including shRNAs and artificial miRNAs (amiRNAs) for the RNAi of single gene targets; and polycistrons, extended shRNAs (e-shRNAs), and long hairpin RNAs (lhRNAs) for the RNAi of multiple targets. Practical information is provided for the usage of viral vectors for expressing these molecules.

In Chapters 14 and 15, the design of two additional structural variants of shRNA is described. Chapter 14 describes a unique RNAi design termed bifunctional shRNA or bi-shRNA. The bi-shRNA technology harnesses both cleavage-dependent and cleavage-independent RISC loading pathways to enhance knockdown potency, thus providing increased potential for research and therapeutic applications. Methods are provided for the design and construction of bi-shRNA, the assessment of RNAi functionality, and the liposome-mediated delivery of bi-shRNA for *in vivo* RNAi studies. Chapter 15 describes an additional variant form of shRNA termed short shRNA, or sshRNA. Rather than being expressed from a vector, sshRNA is chemically synthesized and transfected, allowing for the incorporation of enhancing chemical modifications. Principles and procedures are provided for the design and production of right- and left-handed loop forms of sshRNA.

For mammalian oocytes and early embryos, the interferon response is nonfunctional, and therefore, long dsRNAs can be used for RNAi without concern about immunostimulatory off-target effects. Chapter 16 provides a review of the production and application of

long dsRNA in mammalian cells. Currently available vectors for dsRNA expression and approaches for oocyte-specific transgenic RNAi are outlined.

In Chapter 17, the use of short and long dsRNA is described for RNAi of invertebrate model systems and human disease vectors. Similar to mammalian oocytes, the interferon response is not a critical factor in invertebrate RNAi design. Procedures are described for designing and preparing long dsRNA from genomic or cDNA sources using a bioinformatic approach that can be applied to the design of genome-wide RNAi libraries. Procedures are also described for designing short dsRNA for invertebrates and human disease vectors, which could be valuable for very short exons; and shRNA, which is useful for RNAi in invertebrate oogenesis and for long-term knockdown in cell lines. The construction and use of one such vector for shRNA in silkworm cell lines is detailed in Chapter 18.

In plants, amiRNA, has been used successfully for creating siRNA-mediated resistance to crop viruses. Chapter 19 describes the principles behind the design and production of a multi-amiRNA vector that can confer resistance to *Wheat streak mosaic virus*. The design involves replacement of five arms of the polycistronic rice miR395 with sequences targeting the viral genome and has the advantage over other historic methods of dsRNA-mediated RNAi in plants in that the target sites can be screened against matches to the plant genome to reduce off-target knockdown. Another method of choice for siRNA production in plants mimics a plant-specific class of endogenous small RNA termed trans-acting small interfering RNAs (tasiRNAs). tasiRNAs are produced from *TAS* gene-derived transcripts after they are targeted by an miRNA to promote downregulation of genes in trans. In Chapter 20, steps are described for designing and implementing the use of an innovative method for triggering tasiRNA-type transitivity to produce secondary siRNAs for target gene silencing. Instructions are provided for adapting this procedure, termed miRNA-induced gene silencing (MIGS), to the gene silencing of more than one gene.

RNAi has great potential for both research and therapeutic applications. Each application for each model system has its own set of hurdles in ensuring that the siRNA effector molecules are efficient, specific, and appropriately sustainable. However, through careful design, many of these hurdles can be overcome, ultimately improving experimental outcome and providing greater therapeutic value. The present volume provides practical information to assist in the design of siRNA and upstream siRNA-generating molecules using a variety of strategies that are relevant to a diverse array of applications in RNAi. It is my hope that the topics covered in this book will provide a broad understanding of the issues in RNAi and how they can be overcome strategically through design. This book should be of assistance to researchers, educators, clinicians, and biotech companies interested in harnessing an understanding of the power of RNAi technology.

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