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Kenneth A. Howard *Editor*

RNA Interference from Biology to Therapeutics

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RNA Interference from Biology to Therapeutics

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I dedicate this book to my Mother,
Joan Howard.

Preface

The 2006 Nobel Prize in Medicine or Physiology was awarded to Andrew Fire and David Mello for their 1998 discovery of double-stranded RNA-mediated gene silencing by the process of RNA interference (RNAi). The capability to control and study cellular gene expression has opened up completely new research areas, shed exciting new light on existing fields, and provided researches with an unprecedented tool for investigating functional genomics and the potential to harness the RNAi mechanism as a potent therapeutic. This has resulted in an explosion of activity in both academia and industry.

Understanding the molecular mechanisms of RNAi is crucial for its transformation into a therapeutic modality. This dependency is the focus of "RNA Interference from Biology to Therapeutics", a concept applied to a Controlled Release Society (CRS) Educational Workshop at the 2009 CRS Annual meeting organised by the Editor.

The volume is structured to introduce the reader to the biological principles of RNAi followed by therapeutic delivery and disease treatment; however, integration of these aspects is a common thread running throughout. Education was an important consideration in the book preparation; therefore, the text provides sufficient background of the subject matter to allow utilisation as a learning tool for students.

The opening chapter gives an overview of RNAi pathways and the ground rules for therapeutic exploitation using synthetic small interfering RNA (siRNA) and vector-based approaches highlighted in subsequent chapters. SiRNA design towards RNAi pathway engagement is continued into chapter 2, focused on the development of Dicer-substrate therapeutics. The rapid emergence of the microRNA (miRNA) field, fuelled by its inherent role in regulation of cellular processes in normal and disease states, is highlighted in chapters 3, 13, and 14 dedicated to this subject. Chapter 3, for example, describes miRNA biology in tissue development and repair. In keeping with the "biology-therapeutic" link, the second part of this chapter describes its therapeutic exploitation in tissue regenerative medicine.

The clinical translation of RNAi therapeutics is dependent on enabling technologies to overcome both extracellular and intracellular delivery requirements; this is the focus of chapters 4–10. A number of delivery solutions and RNAi applications

are covered that include nanoparticle-based systems composed of polymer, lipid, or exosomes. Systemic and mucosal routes of administration are addressed as well as stealth and targeting strategies.

Chapters 11–14 focus on RNAi treatment for specific disease types. Chapters 11 and 12 discuss target site selection within the viral RNA genome, viral escape, and solutions such as targeting cellular host factors in the treatment of HIV and influenza. Chapters 13 and 14 describe the application of miRNA in cancer such as deregulated miRNA expression for the identification of novel diagnostic and prognostic biomarkers and novel therapeutic targets as well as a description of miRNA-based anticancer therapies.

The clinical translation of RNAi-based treatments is an ultimate goal. Chapter 15 describes Alnylam's clinical development of a siRNA therapeutic for Respiratory Synitial Virus (RSV) lung infections. Preclinical steps including siRNA screen, antiviral efficacy, toxicology, and immune studies are presented before an overview of recent Phase 1 and 2 clinical trials.

This book is highly relevant for experts in, or at the interface of, RNAi, delivery science, and medicine from a personal field perspective as well as opening up new interdisciplinary research possibilities. An attractive feature is the "Future Perspectives" section ending each chapter that gives global experts the opportunity to express a personal view on where the field is going, offering potential new research directives to the reader.

"*RNA Interference from Biology to Therapeutics*" is a comprehensive and truly unique text for those involved or interested in this extremely exciting, important, and high impact field.

Aarhus, Denmark Kenneth A. Howard

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I would like to express my sincere gratitude to all the authors for the time and effort they have given in the preparation of the excellent chapters contained in the book. I really appreciate your willingness to take on board editorial suggestions. I am grateful to the CRS and especially Series Editor Michael Rathbone for giving me the opportunity to put together a book that I believe was needed in the field. Cheers Mike My thanks go to Springer, Carolyn Honour, and Renata Hutter, and Renata, thank you for your great work and always being so polite.

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Chapter 1
RNA Interference Pathways and Therapeutic Exploitation

Jesper B. Bramsen and Thomas B. Hansen

Abstract RNA interference (RNAi) is currently the method of choice to experimentally silence endogenous gene expression for functional genomics studies, while ongoing clinical trials point to its great therapeutic potential. Harnessing endogenous RNAi pathways to effectuate gene silencing by introducing artificial RNAi substrates or inhibitors translates into effective silencing efficiencies with high predictability and reliability but also has the potential to disturb endogenous gene regulation by the native inhabitants of the RNAi pathway, the microRNAs (miRNAs). A wealth of RNAi strategies have been developed over the last decade to produce optimal experimental triggers of RNAi entering at all levels in the RNAi pathway. Here we provide an overview of RNAi silencing pathways and its transformation into a therapeutic mode using vector-based approaches and chemically optimized, small interfering RNAs (siRNAs).

1.1 Entering the Age of RNA Interference

Nobel Prizes are rarely awarded based on discoveries <10 years old. Yet, in 2006, Craig Mellow and Andrew Fire were awarded the Nobel Prize in medicine or physiology for their 1998 discovery of double-stranded RNA-mediated gene silencing by a process of RNA interference (RNAi) [1]. Here a decade later, the impact of this discovery has been immense; RNAi has opened up completely new research areas, shed exciting new light on existing research fields, and provided researches with an unprecedented powerful tool for functional genomics investigations and potential diagnostic and therapeutic agents.

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Fire and Mellow showed that the introduction of exogenous long double-stranded RNA (dsRNA) into the nematode worm *Caenorhabditis elegans* triggers potent silencing of genes that share perfect sequence complementary to the introduced dsRNA, a silencing effect far superior to what was typically seen using single-stranded antisense RNA, the prevalent gene silencing tool at the time.

Several important observations quickly followed; the exogenous long dsRNA was found to be enzymatically processed into shorter 20–25-base pair (bp) dsRNAs acknowledged as the true effectuators of RNAi [2–4]. The subsequent observation that exogenous, synthetic 21-bp dsRNAs could trigger highly effective and sequence-specific gene silencing upon introduction in the cytoplasm in almost all investigated eukaryotic organisms provided researches with an extremely potent gene silencing tool [5]. These small dsRNA species, coined small interfering RNAs (siRNAs), marked the birth of a completely new silencing mechanism and the entry into the RNAi era of molecular biology and medicine. Besides being established as an ubiquitous tool in basic research, the number of RNAi-based preclinical and clinical trials has increased rapidly over recent years providing optimism for successful clinical translation [6, 7], a high achievement for such a fledgling technology.

1.2 RNAi Pathways

1.2.1 The Natural Biological Functions of RNAi Interference

As presented throughout this chapter, the unprecedented power of RNAi as a gene silencing tool originates from harnessing natural gene silencing pathways to effectuate efficient degradation of any chosen target mRNA. All key enzymes in the RNAi pathway are broadly conserved throughout the eukaryotic clade, suggesting the existence of a minimized version of the RNAi machinery in the last common ancestor of eukaryotes [8]. Here, RNAi is hypothesized to have served primarily as an ancient defense mechanism that functions *in cis* to endonucleolytically cleave and degrade exogenous long cytoplasmic dsRNA originating from viral infection [9–11], aberrantly expressed transgenes [10, 12–14], mobile genetic elements [15, 16], or aberrant processing of endogenous mRNA [17]. In higher eukaryotes, however, the RNAi machinery has evolved to be a key aspect in the general regulation of endogenous gene expression. Here, a class of endogenous 20–25 nucleotide single-stranded RNAs, known as microRNAs (miRNAs), are found to regulate the stability and translation of mRNAs *in trans* upon directly base pairing to their 3′untranslated regions (3′UTRs). The first example of a miRNA, a noncoding gene termed *lin-4* encoding a small RNA species of ~22 nt, was identified in the nematode worm *C. elegans* in 1993 [18]. It was hypothesized that *lin-4* represses translation of the *lin-14* gene by base pairing partially with putative target sites positioned in the 3′UTR of the *lin-14* mRNA. It took almost 7 years before the second miRNA, *let-7*, was shown to regulate the *lin-41* mRNA through a similar mechanism. Interestingly, *let-7* was found to be evolutionary conserved in a wide range of animal species indicating a more general role of miRNA in gene regulation [19–21].

Accordingly, thousands of miRNAs have since been identified, a task greatly enhanced by next generation sequencing techniques (see http://www.mirbase.org), and the RNAi machinery has indeed proved widespread and important in controlling gene expression in nearly all aspects of cellular metabolism.

1.2.2 RNAi Pathways: Step by Step

Considering the essential roles for RNAi pathways in organism homeostasis, it is clear that the development of safe RNAi-based therapeutics requires a thorough understanding of the RNAi pathways by which eukaryotic organisms regulate their transcriptome (Fig. 1.1). In essence, any artificial RNAi trigger employed, either introduced into the cytoplasm as synthetic siRNAs or transcribed in the cell nucleus from artificial RNAi vectors, will have to be carefully engineered to structurally mimic endogenous RNA intermediates in the RNAi pathway in order not to interfere with normal miRNA function. This section will, therefore, highlight key aspects of the maturation and function of miRNAs in order to establish the ground rules under which RNAi-harnessing approaches must comply.

Overall, the RNAi pathway has the potential to respond to a variety of RNA substrates of both endogenous origin, that is, primary miRNA precursors (pri-miRNAs) transcribed in the cell nucleus and exogenous origin such as cytoplasmic, foreign dsRNAs, e.g., viral RNA, siRNAs, etc. The terms "miRNA pathway" and "siRNA pathway" are often encountered in the literature to distinguish RNA substrates of endogenous and exogenous origin, however, in essence all mature RNAi triggers (miRNA and siRNA produced by the RNAi proteins from nuclear miRNA precursor and cytoplasmic dsRNA, respectively) are functionally identical once incorporated into the effector protein complex RISC in the cytoplasm [22, 23]. Concurrently, multiple entry routes into the RNAi pathway can be exploited for gene silencing therapeutics.

Focusing first on endogenous RNAi substrates, the majority of miRNAs are transcribed as primary transcripts (pri-miRNA) in the nucleus by RNA polymerase II and are situated either intronically in coding mRNA or as separate transcription units [24, 25]. The pri-miRNA is hairpin structured and is initially recognized by the bipartite complex, the microprocessor, composed of the endonucleolytic enzyme Drosha and its cofactor DGCR8 [26, 27]. The microprocessor crops the hairpin structure out of its flanking sequence, resulting in a 50–70-nt hairpin RNA, termed the precursor miRNA (pre-miRNA). Drosha is composed of dual RNase III domains responsible for the endonucleolytic cleavage, and the resulting product consists of a 5′ phosphate and 3′ hydroxyl group. Moreover, from a structural point of view, the Drosha-processed pre-miRNA typically has two unpaired nucleotides in the 3′ end, referred to as a 2-nt 3′ overhang. Basically, microprocessor products (with a few exceptions) serve as *bona fide* substrates in the downstream biogenesis pathway; therefore, the specific pri-miRNA recognition by the microprocessor is a defining step in miRNA biogenesis although the exact determinants of microprocessor recognition are still debated.

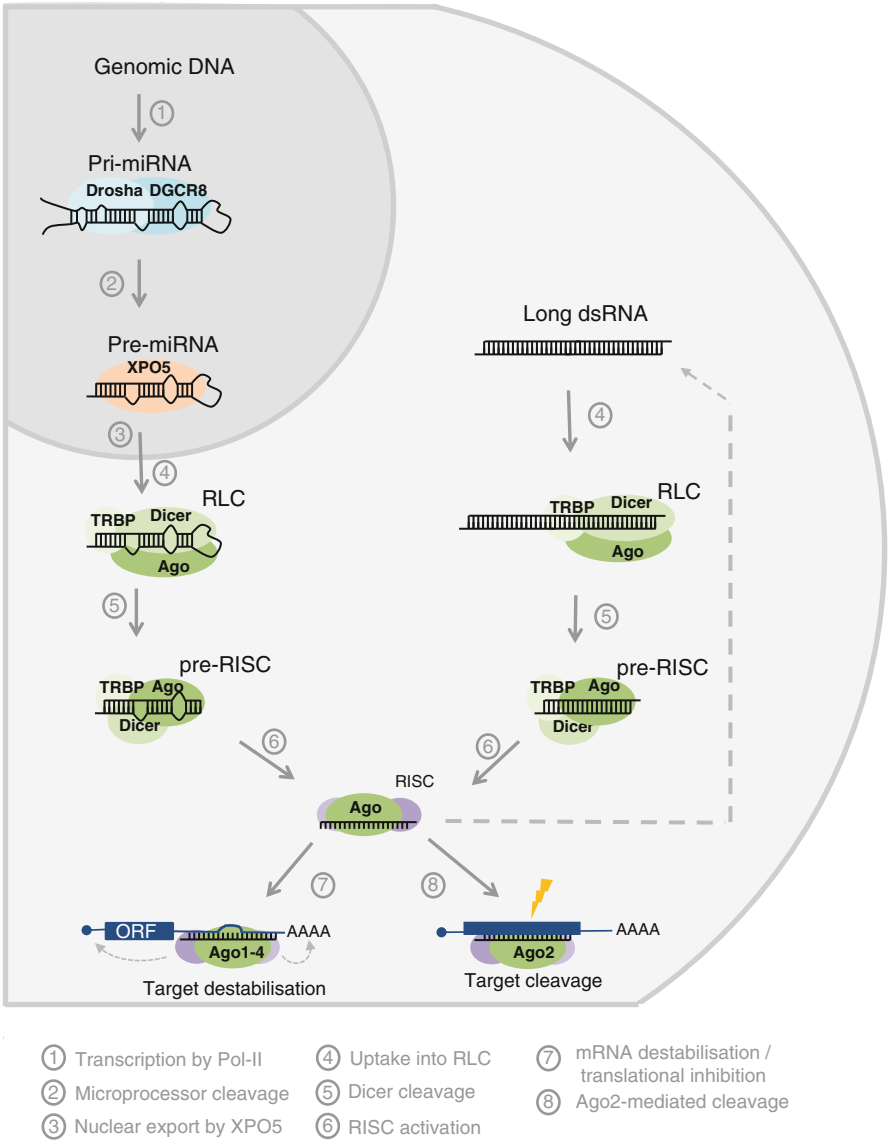

Fig. 1.1 Overview of the RNAi pathways. Natural miRNAs are transcribed by pol II as part of long pri-miRNAs ①, which are processed in the nucleus (*gray circle*) by the microprocessor (containing DGCR8 and Drosha) into pre-miRNAs ②. These are subsequently exported to the cytoplasm by XPO5 ③ and loaded into a RISC-loading complex (RLC) containing, e.g., Dicer and TRBP ④. In the cytoplasm, Dicer cleaves the pre-miRNA (*left*) and cytoplasmic long dsRNA (*right*) into a miRNA or siRNA duplex, respectively ⑤, thereby generating pre-RISC in which one strand is selectively loaded into active RISC ⑥. RISC can now direct mRNA destabilization/translational inhibition of target sharing only partial sequence complementarity to the guiding strand ⑦ (typical for miRNAs) or RNA cleavage of targets sharing perfect sequence complementarity to guide strand ⑧ (typical for siRNAs). SiRNAs generated from cytoplasmic long dsRNA can, due to their perfect sequence identity, target their original source, e.g., viral dsRNA for degradation (*dashed line*). Refer to text for more detailed description

With the initial step in miRNA biogenesis being compartmentalized to the nucleus, a translocation of the pre-miRNA across the nuclear membrane through the nucleopore complex (NPC) is a required step in the pathway. Pre-miRNA export is dependent on Exportin-5 (XPO5) [28], which is believed to be the rate-limiting step in miRNA biogenesis [29]. XPO5 has high affinity toward hairpin-like RNA with a structured stem exceeding 18 bp in length and with 3′ rather than 5′ overhangs [30], consistent with microprocessor products serving as canonical XPO5 substrates.

After export to the cytoplasm, the pre-miRNA is subjected to further processing by the so-called RISC-loading complex (RLC) [31–33] in which Dicer, another RNase III enzyme, cleaves off the pre-miRNA loop to produce a 21–22-nt miRNA duplex [3]. The pre-miRNA structure is, in part, recognized by the terminal 2-nt 3′ overhang, and consequently Dicer is typically regarded as an exonuclease. Like Drosha, Dicer-cleavage produces 2-nt 3′ overhangs; thus, the resulting miRNA duplex displays overhangs at both termini. Here, at the levels of Dicer cleavage, the miRNA and siRNA pathways meet (Fig. 1.1); in addition to processing pre-miRNA, Dicer also consecutively cleaves long dsRNA, such as foreign cytoplasmic dsRNA, from the terminus into smaller 21–22-nt siRNA duplexes [34]. Subsequently, miRNA and siRNA duplexes are incorporated into a pre-RISC [35], a complex composed primarily of one of four mammalian Argonaute proteins (Ago 1–4) as the core component. One strand in the miRNA or siRNA duplex (the passenger, non-guiding strand) is subsequently removed during RISC activation, resulting in single-stranded RNA in association with the activated RISC (the guide strand). This process is referred to as strand selection. The selection and incorporation of guide strand is at least, in part, determined by the molecular thermodynamics of the miRNA/siRNA duplex [36, 37]: Here, the least stable 5′ end in terms of base pairing strength is predominantly observed as the guiding strand. An examination of RISC activation in more detail reveals two distinct mechanisms: a RISC-mediated cleavage of the passenger strand, restricted to near-perfect RNA duplexes, such as siRNAs [38, 39], or a helicase-dependent mechanism responsible for unwinding the duplex [38, 40]; in both cases, strand selection is complying with the thermodynamics-based rules above.

Once incorporated into RISC, the single-stranded miRNA base pairs with target sites typically positioned in the 3′UTRs of mRNA, whereas siRNA target sites are located according to their origin or original design, typically within the open reading frame (ORF). Two possible effector mechanisms occur depending solely on the nature of Argonaute protein found in RISC and the level of complementarity between the guiding strand and the target mRNA: (1) in case of full complementary between guide strand and target, which is typical to siRNAs guide strands; Ago2 catalyzes the endonucleolytic cleavage, termed slicing, of target mRNAs, at the position located opposite the 9–10th nucleotide position counting from the guide strand 5′ end [41, 42]. This subjects the target mRNA to rapid exonucleolytic decay [22, 43] that is the intended and prevalent process in most typical siRNA-mediated mRNA “knockdown” experiments. (2) In case of more imperfect base pairing, essentially requiring only complementarity between the seed sequence of the miRNA (position 2–7/8) and the 3′UTR (seed match), cleavage is not observed, and

RISC instead destabilizes mRNA or represses its translation [23, 44, 45] in a process much less potent than siRNA-mediated slicing. Of the four closely related Ago proteins, Ago1–4, only Argonaute2 has endonucleolytic mRNA cleavage potential, whereas the three other closely related Ago proteins (Ago1, 3, and 4) are devoid of endonucleolytic activity [41]. The vast majority of characterized miRNA-mediated effects are facilitated by seed match target sites in the mRNA 3′UTR leading to mRNA destabilization or translational repression by mechanisms that are still debated but likely involve recruitment of a deadenylation complex (CAF1:CCR4:NOT1) and mRNA destabilization through deadenylation. Due to their functional identity, however, siRNAs also behave as miRNAs to destabilize hundreds of RNAs in addition to the intended target upon base pairing of the siRNA seed region to 3′UTRs, a process known as off-targeting (discussed in Sect. 1.4.6).

1.3 Harnessing RNAi Pathways for Gene Silencing Therapeutics

1.3.1 The Benefits of RNAi-Based Therapeutics

The breakthrough for RNAi as a potential human therapeutic was the observation of potent, sequence-specific, and seemingly safe knockdown of endogenous gene function in cell culture upon introduction of synthetic 21mer siRNAs with perfect sequence complementarity toward its target [5]. The importance of this observation for RNAi therapeutics cannot be underestimated, and synthetic siRNAs are the preferred gene silencing tool in vitro, a success that emanates from their general consistency, high efficiency, and ease of use. Today, it is a simple matter to order designed commercial synthetic siRNAs to obtain the desired gene knockdown in short-term cell culture experiments using commercial transfection reagents. Hitherto, synthetic siRNAs have provided the bedrock for the successful RNAi-based therapeutics: The first knockdown of an endogenous gene, apolipoprotein B (ApoB) using a clinically relevant formulation and administration route, was observed in mouse livers after standard intravenous injections of a chemically modified, but naked (non-formulated), siRNA in 2004 [46]. Also, the first successful knockdown of a cancer target gene in humans, the M2 subunit of ribonucleotide reductase (RRM2), was achieved in a clinical phase I trial in tumors from melanoma patients upon systemic delivery of siRNA nanoparticles [7] (for more details on ongoing clinical trials, refer to http://clinicaltrials.gov).

There are several contributing components to the great success of RNAi-based gene silencing as compared to the competing antisense oligonucleotide (ASOs) technology developed in the 1970–1980s [47]. Similarly to ASO technology, the high predictability and specificity of nucleic acid base pairing provides fully programmable and specific gene silencing which in practice renders all genes “druggable”: Researchers need only to produce short dsRNA species with a structure recognizable to RNAi proteins and perfect sequence complementarity to a particular mRNA target

to achieve gene knockdown. Yet, RNAi-based silencing strategies hold additional benefits; exogenous RNAi substrates, such as synthetic siRNAs, are rapidly incorporated into RNAi protein complexes to protect them from nuclease degradation [48, 49] and, very importantly, to orchestrate their transport to their target mRNA in the cytoplasm, thereby effectuating multiple rounds of target cleavage [10]. Furthermore, the structural mimicry of endogenous miRNA species by artificial siRNA triggers allows them, at least in theory, to remain undetected to cellular sensors of foreign dsRNA, thereby preventing triggering of innate and adaptive immune responses.

1.3.2 Therapeutic Entry into the RNAi Pathways

Despite the overwhelming popularity of synthetic siRNAs, a wealth of strategies for harnessing endogenous RNAi pathway as a gene silencing tool have been developed to best suit the given experimental system or organism. Collectively, these strategies exploit the fact that protein complexes in the RNAi pathways respond to the structure of their RNA substrates rather than their sequences and that miRNA pathways and siRNA pathways intersect in the cytoplasm at the level of RISC loading and shares degradation pathways (Fig. 1.1). Therefore, any desired guide strand can be loaded into Ago2-RISC to effectuate mRNA target cleavage once embedded within a suitably structured RNAi substrate mimic, e.g., primary miRNA transcripts, precursor miRNAs, miRNA duplexes, dsRNA shorter than 30 bp, or most popularly 21mer siRNAs (Fig. 1.2).

As discussed in greater detail in Sect. 1.4, most experimenters typically use synthetic 21mer siRNAs as triggers of RNAi: These have perfect structural identity to natural Dicer cleavage products (typically two 21-nt RNA strands annealed to form a 19-bp dsRNA duplex stem and 2-nt 3′ overhangs at both ends) and are upon introduction into the cell cytoplasm loaded into RISC by RLC to facilitate transient gene knockdown. The siRNA sequence is typically designed to target the mRNA ORF, and silencing effect persists for 2–7 days in typical cell-cultured siRNA. As described in Sect. 1.4, numerous successful siRNA designs utilizing shorter of longer RNA backbones and chemical modification have been developed to enhance silencing potency, specificity, and longevity. Still, the lack of efficient means to achieve cytoplasmic delivery in vivo is the major bottleneck for therapeutic applications, and reports of siRNA immunogenicity and off-targeting also need immediate addressing.

As described in Sect. 1.5, RNAi triggers may also be encoded by RNAi plasmids or viral vectors, which are introduced into the cell nucleus and here utilize the cellular transcription apparatus to ensure a continuous production of the intended RNAi substrates. This potentially allows for long-lasting RNAi and can also ensure efficient delivery of RNAi constructs into cells that are otherwise difficult to target. Especially viral vectors hold therapeutic potential: Whereas retro- and lentiviral vectors may have limited use in therapeutics due to their integration into the host genome (i.e., risk of insertional mutagenesis), the nonintegrating viral vectors such as adeno- and adeno-associated viral vectors may hold greater therapeutic potential [50, 51].

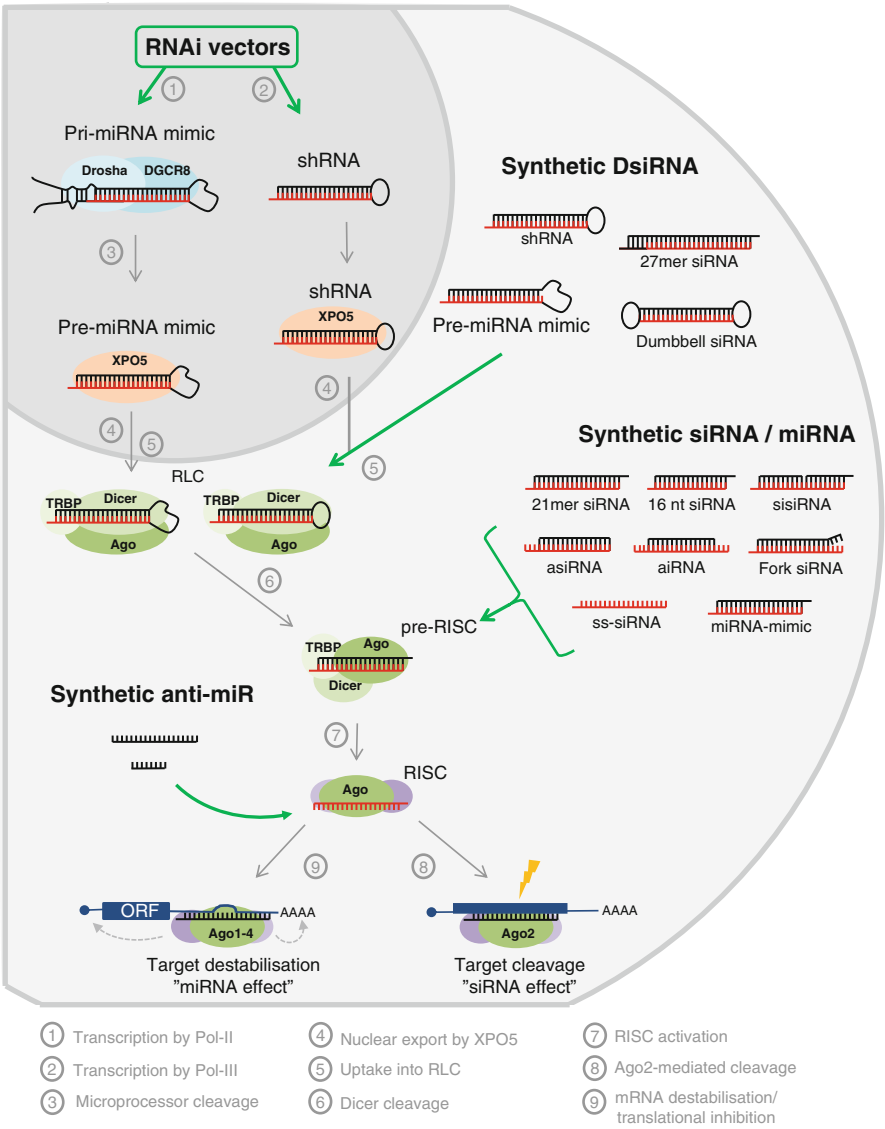

Fig. 1.2 Harnessing endogenous RNAi pathways for gene silencing therapeutics. Artificial triggers of RNAi can be introduced via RNAi vectors that are transcribed in the nucleus; pol III-transcribed cassettes will typically encode shRNAs that are directly transported to the cytoplasm and loaded into RLC (*right side*). Alternatively, RNAi triggers are delivered as pol II-transcribed pri-miRNA mimics, which are cleaved by the microprocessor prior to loading into RLC in the cytoplasm. Synthetic triggers of RNAi are instead introduced directly into the cell cytoplasm; Dicer substrate siRNAs (DsiRNAs), either in the form of shRNAs, pre-miRNA mimics, or 27mer dsRNA, can enter the RLC and are subsequently loaded into pre-RISC upon Dicer cleavage. Various design versions of synthetic 21–23mer siRNAs, and notably also miRNA mimics, are instead loaded into pre-RISC without Dicer cleavage and can subsequently guide Ago2-mediated target cleavage (referred to as on-targeting for siRNAs) or Ago-mediated RNA destabilization/translational inhibition of targets sharing perfect seed matching (the natural miRNA function which is referred to as of-targeting for siRNAs). Endogenous miRNAs can also be inhibited by introducing 12–25-nt chemically engineered antisense oligonucleotides, named anti-miRs or antagomirs, into the cell cytoplasm where they bind and inhibit the single-stranded miRNAs loaded into active RISC. Refer to text for more detailed description

In addition to silencing mRNA by custom siRNAs, recent RNAi strategies have focused on modulating the levels of endogenous miRNAs as these are often found to be up- or downregulated in human diseases such as cancer [52]. The so-called miRNA replacement therapy aims to introduce mimics of natural miRNAs into the RNAi pathway [53]. Here they will exert the functions of their natural counterpart to simultaneously target multiple mRNAs through seed-mediated base pairing to their 3′UTR (rather than cleaving a single target as intended for siRNAs). miRNA mimics are very similar to siRNAs and can be introduced as synthetic dsRNA molecules or via DNA vectors (described in the following sections). In practice, synthetic miRNA mimics are often designed as perfectly base-pairing siRNA-like molecules where the guiding strand is identical to a given endogenous miRNA. Therefore, delivery and chemical optimization strategies are basically similar to siRNA design; however, modifications that reduce siRNA off-targeting (i.e., miRNA effects) should obviously not be applied in miRNA mimic design.

Finally, endogenous miRNA function can be inhibited by introducing 12–25-nt single-stranded oligonucleotides designed antisense to the given miRNA. Upon introduction into the cell cytoplasm, such molecules, referred to as anti-miRs or antagomirs [54, 55], will bind to RISC-loaded miRNAs and block their natural functions. Being single-stranded, anti-miR typically needs extensive chemical modification both to resist degradation by nucleases and also to enhance their binding affinity to target miRNAs (e.g., by locked nucleic acids (LNA) [56]), to trap the RISC-loaded miRNAs in a nonfunctional state or even promote their degradation [57]. Significant progress has already been made using anti-miR designs in primates [56, 58]. As an alternative, the so-called miRNA sponges transcribed from DNA vectors introduced into the cell nucleus have allowed long-lasting inhibition of endogenous miRNA function [59]. In practice, such sponges are mimicking the structure of natural miRNA targets as RNA transcripts expressed from strong pol II promoters designed to contain multiple binding sites, often heptameric sequences complementary to the target miRNA seed. Notably, emerging evidence suggests that the unknown function of human pseudogenes or long noncoding RNAs may indeed be to function as natural sponges for endogenous miRNAs [60].

1.4 The Application of Synthetic siRNA as RNAi-Based Therapeutics

Synthetic siRNAs are by far the most widely used triggers of RNAi in cell culture in which they are introduced into the cell cytoplasm and directly loaded into RISC. Although efficient transfection can be achieved in most cell culture systems by the use of commercial transfection reagents, in vivo delivery of synthetic siRNAs into the target cells is far more challenging. Synthetic siRNA suffers from a number of drawbacks compared to most small-molecule drugs; namely, they are macromolecules (~14,000 Da) and hydrophilic due to their anionic phosphodiester backbone that restricts entry across the cellular membrane required for target interaction.

Furthermore, they are susceptible to serum nucleases and have poor pharmacokinetic properties as a consequence of e.g., rapid renal clearance upon intravenous injection [61]. Moreover, siRNA silencing specificity and safety has been challenged by the general finding of siRNA off-targeting [62, 63] and potential immunogenicity [64, 65] resulting in the realization that further clinical progress will require greater investments than initially envisaged to overcome these issues. Encouragingly, this process is well underway; synthetic siRNA technology is now dramatically improved by chemical modification that will most likely fulfill its potential if allowed to mature in a similar manner to development and the emerging success of ASO technology [66–68].

1.4.1 Synthetic siRNA Types

The most simple and popular siRNA design today mimics natural Dicer cleavage products and comprises a 21-nt guiding strand antisense to a given RNA target and a complementary passenger strand annealed to form a siRNA duplex with a 19-bp dsRNA stem and 2-nt 3′ overhangs at both ends [4, 5]. Other siRNA designs mimic Dicer substrates to enhance incorporation into RNAi pathways [69] and siRNA potency [referred to as Dicer substrate siRNAs (DsiRNAs)] [70]; especially synthetic 27mer DsiRNAs [71–75] can have very high activity; yet, concerns of unpredictable efficiency and potential immunogenicity [76] need to be addressed [73–75]. A variety of alternative siRNA designs exhibiting different architectures have been developed such as Dicer-independent short shRNAs with RNA stems ≤19 bp [77, 78], blunt 19-bp siRNAs [79, 80], blunt fork-siRNAs [82, 83], single-stranded siRNAs (ss-siRNAs) [84–86], dumbbell-shaped circular siRNAs [87], asymmetric siRNAs (aiRNA) and asymmetric shorter-duplex siRNA (asiRNA) harboring a shortened SS [78, 88, 89], bulge-siRNA [90], and sisiRNAs [91] (Fig. 1.2). Some of these aim to enhance siRNA potency and specificity by ensuring the preferential loading of the guide strand in RISC and/or rendering passenger strand nonfunctional. Examples include the asymmetric siRNAs (asiRNAs) which utilize a 5′ end truncated passenger strand [78, 88] and the sisiRNAs which utilize two short 10–12-nt passenger strands, all of which cannot contribute to siRNA off-targeting [91]. Also, fork-siRNA contains mismatched bases in the 3′ end of the passenger strand which enhance loading of the siRNA guide strands due to a lower thermodynamic stability of its 5′ duplex end. Finally, the use of asymmetric siRNA overhangs also ensures preferential guide strand loading and improved siRNA activity [92]. Other designs aim to enhance siRNA nuclease resistance to prepare unmodified siRNA for usage in vivo: Blunt 19-bp siRNAs and dumbbell-shaped circular siRNAs are both reported to be more resistant to nuclease degradation, even when unmodified, as they contain no free 3′ overhang [80, 87]. Recently, siRNAs has also been incorporated in larger nucleic acid structures with the prospect of enhancing delivery and bioavailability [93, 94].

1.4.2 Maximizing siRNA Activity

Maximizing siRNA silencing activity minimizes the siRNA dose required for efficient gene silencing which is important when considering the difficulties of siRNA delivery in vivo. The siRNA sequence is a primary determinant of siRNA activity [36, 95, 96], and large screens have identified several design rules [97–103] such as a low GC-content of 30–50% [99, 104], especially in the guide strand seed region [99, 102] and preferences for low internal thermostability [99, 101, 102]. Importantly, siRNA sequences should be chosen to render the siRNA duplex thermodynamically asymmetric as the siRNA strand having the 5′ end engaged in the thermodynamically least stable part of the duplex will, as noted above, be preferentially utilized as guiding strand in RISC [36, 37]. Furthermore, siRNA target sites should be accessible and not hidden in stable secondary structures [105–109] nor occupied by RNA-binding proteins [110], and in effect, efficient target sites are often found in AU-rich regions [111]. SiRNA chemical modification screens show that careful chemical engineering of siRNA can enhance their activity beyond unmodified siRNAs [112]; a few examples of dramatic potency improvements have been reported [148]; yet, typically, only modest (less than twofold) improvements in siRNA potency are seen, for example, upon moderate modification of siRNA strand 3′ ends [112–118]. Instead, modifications that favor loading of the guide strand into Ago2-RISC seem to be a reliable, sequence-unspecific strategy to enhance siRNA potency either by introducing optimal siRNA thermodynamic asymmetry using stabilizing modifications (e.g., LNA [119], 2-thiouracil [120], 2′-F) in the passenger strand 5′ end or by introducing destabilizing modifications (such as OXE, ethylamino, UNA, dihydrouracil, or PS [112, 119, 121]) in its 3′ end. Furthermore, chemically modified 3′ overhangs that are favored and unfavored during strand selection by RISC have been identified and can easily be incorporated into the guide and passenger strands of the siRNA, respectively [92, 112]. As a note of caution, the industry standard and popular 2-nt DNA overhang dTdT has been suggested to reduce silencing longevity significantly [122].

1.4.3 Motivation for Chemically Modified siRNA

Unmodified synthetic siRNAs seem most suited for short-term gene silencing experiments in cell culture, where adverse side effects such as siRNA immunogenicity and off-targeting (see below) go unnoticed or are disregarded. Higher standards for siRNA performance and safety are required to establish siRNA-based therapeutics. These concerns can be resolved by chemical modification [81, 91, 112, 123–126]. Unmodified, naked siRNAs are highly labile in biological fluids due to their degradation by ribonucleases, and their poor pharmacokinetic properties in vivo reduce intracellular delivery [46, 61, 127]. Furthermore, siRNAs may be immunogenic [64, 65, 128–130] and inherently trigger non-intended off-target regulation of genes harboring seed matches of either siRNA strand (i.e., each siRNA

strand has the potential to function as a miRNA, thereby potentially affecting hundreds of mRNAs) [62]. A wealth of modification types have been tested to circumvent these shortcomings including modification of the phosphodiester backbone (e.g., by phosphorothioate linkages (PS) [131–138]), substitution of the ribose 2′-OH group [e.g., by 2`-*O*-Methyl (OMe), 2′-Flouro (F), 2′-Methoxymethyl (MOE)], or using bridged nucleic acids (such as LNA [139], carbocyclic-LNA [112, 140], ENA [141], carbocyclic-ENA [112, 140], and oxetane (OXE) [112, 142]), substitution of the ribose unit with six carbon sugars (such as ANA, HNA, 2′-F-ANA, and CeNA [112–117]), by disrupting the ribose ring structure (such as in unlocked nucleic acids (UNAs) [124, 143]), or modification of nucleoside bases (5-iodo-, 2-thio- and pseudouracil [120, 131, 144]) (for a more comprehensive review of chemical modification types, see [126]). Modifications, however, must be compatible with siRNA function, and positional tolerances for siRNA modifications have been well established by empirical testing; by rule of thumb, the guide strands 5′ phosphate, 5′ end (seed region), and central positions are particularly sensitive, especially to several or bulky modifications. In contrast, the entire length of the passenger strand, the 3′ end, and overhang of the guide strand are fairly tolerant and can be chemically modified to enhance siRNA performance [112, 126, 131–136, 141].

1.4.4 Improving siRNA Nuclease Resistance

Most unmodified siRNAs are degraded by ribonucleases within minutes in mammalian serum [46, 112, 131, 135, 145, 146], and enhancing siRNA stability has long been considered essential for siRNA function in vivo. Indeed, extensive chemical stabilization was found essential for successful silencing in mouse livers upon low-pressure intravenous injection of naked siRNA, a strategy relevant to siRNA therapeutics [147]. Yet, only few extensively or fully modified siRNAs are reported to be both highly stable and potent [147–149] as extensive chemical modification of siRNAs will typically reduce their activity [112]. Instead, moderate modification levels using phosphorothioates [131–136], thermostabilization of the siRNA duplex stem by LNA [112, 119, 135, 136, 150], OMe or 4′ thioribose [151], or combinations of these modifications have been successful in creating stable and potent siRNA for applications in vivo [46, 152, 153].

Recently, suggestions to modify only nuclease hypersensitive positions in the siRNA were put forward to limit modification levels and preserve silencing [154, 155]. As modification of siRNA 3′ overhangs are very well tolerated by the RNAi machinery, numerous modification types will provide 3′ exonuclease resistance and modestly enhance siRNA stability [112, 131–136]. As most dsRNA-specific endoribonucleases are preferentially recognizing UpA, UpG, and CpA dinucleotide motifs [154–157], further siRNA stabilization by modifying these vulnerable positions, for example, by 2′-OMe, is a straightforward approach to significantly enhance stability [154, 155].

It is worth noting that the benefits of enhancing siRNA nuclease resistance seem primarily to originate from effects during siRNA delivery prior to siRNA

internalization or enhanced interaction with the intracellular RNAi machinery [158]. Once inside the cytoplasm, siRNAs are more stable, some likely protected by RISC incorporation [159], and silencing can persist for 30–90 days in slowly or nondividing cells [158, 160]. Therefore, chemical modification of siRNAs to enhance nuclease resistance is primarily needed in applications using "naked" siRNA and less when using shielding delivery agents such as nanoparticles.

1.4.5 Reducing siRNA Immunogenicity

Synthetic siRNAs were initially considered to be non-immunogenic in mammalian cells, due to their structural mimicry of endogenous Dicer cleavage products [5]. However, with increasing investigations, siRNAs are now reported to induce innate immune responses through mechanisms dependent on cell type, delivery route, siRNA structure, and sequence [76]. Specific single-stranded sequence motifs such as GUCCUUCAA [64], UGUGU [129], UGGC [161], and GU [162] have been reported to render siRNA immunogenic. These are recognized by Toll-like receptors 7 and 8 (TLR7/8), transmembrane receptors found in the endosomes of immune cell populations [163]. These responses can be potentiated with the use of transfection agents that facilitate endosomal delivery. Recently, uridine content has been reported to correlate with TLR7/8 activation [164], thereby severely complicating siRNA design. Encouragingly, TLR7/8 activation may be largely avoided by chemically modifying or shielding immune-stimulatory sequences: Several modification types, especially 2′-modified nucleotides (DNA, 2′-OMe, 2′-F, LNA), can abrogate siRNA immunogenicity [130, 147, 165, 166], and modification of uridines only with either 2′-F or 2′-OMe [167] or DNA [168] may be sufficient. In particular, 2′-OMe modification of the passenger strand has been proposed as a universal approach to avoid TLR7 activation [169].

siRNA duplex length also seems to affect siRNA immunogenicity; several studies suggest that 21mer siRNAs are not immunogenic in several cell lines even at high concentrations, whereas 25mer (or longer) siRNAs trigger concentration-dependent immunogenicity in HeLa S3, DU145, and MCF7 cells, but not in HeLa and HEK293 cells. Consequently, it has been suggested to avoid non-modified 27–29mer DsiRNA design for siRNA applications, at least in vivo [76]. siRNA sequence length is sequence-independent monitored by the transmembrane TLR3 (among others) expressed on the surface and in endosomes in the dendritic subpopulation of the leukocytes but also in many primary cell types and popular cell lines [76, 170]. Notably, TLR3 activation by the popular 21mer siRNA has been reported upon intraocular injection in mice regardless of their sequence and 2′-OMe modification, whereas a shorter 19mer siRNA design was safe [171]. Another sensor of siRNA length is protein kinase R (PKR), present in all cells and stimulated by cytoplasmic dsRNA longer than 30 bp [172]. 25–30mer siRNAs did not activate PKR in HEK293 cells [71]; yet, canonical 21mer siRNAs have been shown to bind or trigger modest PKR activation in murine microglial N9 cells [173], T98G cells [174–176], and HeLa cells [175]; therefore, the impact of PKR