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Cooperativity in Mammalian RNA Silencing

A Dissertation Presented

By

JENNIFER A. BRODERICK

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

July 26, 2011

NEUROSCIENCE PROGRAM

COOPERATIVITY IN MAMMALIAN RNA SILENCING

A Dissertation Presented
By

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My journey here was filled with the generous companionship of many to whom I owe a debt of gratitude. From his first passionate lecture that left my mind vivified and sold me on an adventure in his lab, Phil Zamore has been unyielding in his support for this project. His uncompromising intellectual guidance pushed my abilities toward the highest standards of pedagogy and research and saved me from falling victim to the easy route. My co-mentor, Neil Aronin, inspired and provoked reflection along the way and crystallized the path to therapeutic application of RNA silencing in patients with Huntington's disease. There is no match for the exceptional intellectual environment created by their co-mentorship of my thesis research.

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–JAB

August, 2011

Copyright Information

Chapter II

Argonaute protein identity and pairing geometry determine cooperativity in mammalian RNA silencing.

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Appendix

microRNA Therapeutics.

Broderick JA and Zamore PD. Gene Therapy, (28 April 2011); doi:10.1038.

Abstract

Argonaute proteins are the core component of an RNA silencing complex. The human genome encodes four Argonaute paralogs –Ago1, Ago2, Ago3 and Ago4– proteins that are guided to target mRNAs by microRNAs. More than 500 miRNAs are conserved between mammals, and each microRNA can repress hundreds of genes, regulating almost every cellular process. We still do not fully understand the molecular mechanisms by which miRNAs regulate gene expression. Although we understand many aspects of microRNA biogenesis and formation of the RNA-induced silencing complex, much less is known about the subsequent steps leading to target mRNA regulation.

Mammalian microRNAs rarely have complete complementarity to their target mRNAs so, instead of endonucleolytic cleavage by Ago2, microRNAs destabilize or repress translation of target mRNAs. Here I explored the functional limits of Argonaute proteins bound to their targets directly and indirectly through microRNAs in mammalian cells. I revealed the different abilities for Argonaute proteins bound at multiple sites in a target to generate cooperativity in silencing based on the extent of pairing between the microRNA and target mRNA. Further, I harnessed the endogenous microRNA silencing mechanism to repress an mRNA that is not a direct target of the microRNA by tethering the RNA-induced silencing complex to the 3' UTR of an mRNA. This strategy allows tissue-specific gene silencing due to the limited endogenous expression profile of the recruited microRNA. Efforts made herein further our

mechanistic knowledge of microRNA-induced gene silencing in mammalian cells and advance microRNA-based strategies toward treating human disease.

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Chapter I: Introduction

Introduction

The discovery that small RNAs can silence gene expression through RNA interference identified a fundamentally important regulatory mechanism for biology (Fire et al., 1998). Its application has had a huge impact on basic research and it is anticipated that mammalian RNA silencing will allow discoveries and solutions for unmet challenges in human health. Although much is known about the mechanism of RNA interference in model organisms and some RNAi-based human therapeutics are in development, we are still ignorant of many details specific to its function in mammalian cells (Ghildiyal and Zamore, 2009; Joshua-Tor and Hannon, 2010; Vaishnaw et al., 2010). This thesis project was undertaken with the hope that we could advance our understanding of mammalian microRNA silencing, while restricting our methods so as to remain true to physiological conditions.

Argonaute proteins are the core of the RNA induced silencing complex (RISC) and provide an anchor for the small RNA that guides a RISC to its target mRNA and prevents protein expression. A member of the Argonaute family of proteins functions in all RNA silencing effector complexes (Tabara et al., 1999; Tabara et al., 2002; Hammond et al., 2001; Hutvagner et al., 2001; Catalanotto et al., 2002; Martinez et al., 2002; Mourelatos et al., 2002; Djikeng et al., 2003; Shi et al., 2004). The human genome encodes four Argonaute paralogs, Ago1, Ago2, Ago3 and Ago4. Argonaute proteins contain a P-element induced wimpy testis (PIWI) domain that is structurally homologous to RNase H from bacteria, and

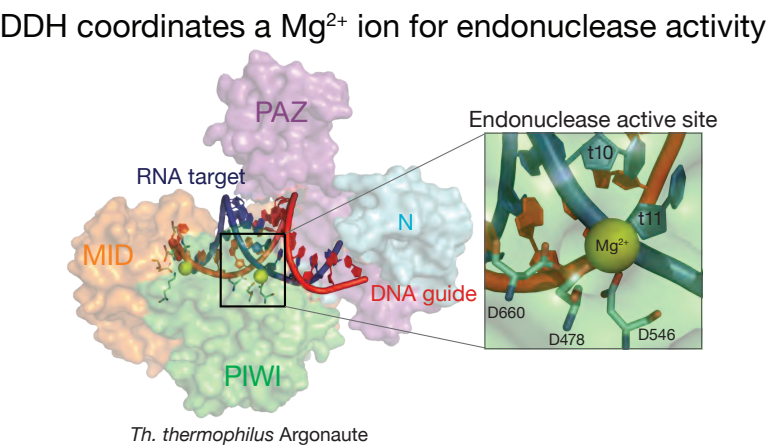
typically cleave their target RNAs after the nucleotide paired to the tenth base of the small RNA guide (Elbashir et al., 2001a; Elbashir et al., 2001b; Tolia and Joshua-Tor, 2007). Cleavage requires three key amino acids—D, D, H—that form a magnesium-binding catalytic triad that promotes nucleophilic attack by hydroxide on the phosphodiester bond (Figure 1.1, 1.2) (Kanaya et al., 1996; Haruki et al., 2000; Martinez and Tuschl, 2004; Schwarz et al., 2004; Song et al., 2004; Rivas et al., 2005).

hAgo1	PVIFLGAD [*] VT	HPPAGDGKKP	SITAVVGSMD	AHPSRYCATV	RVQRPRQEI-	-----I	EDLSYMVREL	LIQFYKSTRF	KPTRIIFYR [*] D	GVPEGQLPQI
hAgo3	PVIFLGAD ^{VT}	HPPAGDGKKP	SIAAVVGSMD	AHPSRYCATV	RVQRPRQEI-	-----I	QDLASVMREL	LIQFYKSTRF	KPTRIIFYR ^D	GVSEGQFRQV
hAgo2	PVIFLGAD ^{VT}	HPPAGDGKKP	SIAAVVGSMD	AHPNRYCATV	RVQQHRQEI-	-----I	QDLAAMVREL	LIQFYKSTRF	KPTRIIFYR ^D	GVSEGQFQQV
hAgo4	PVIFLGAD ^{VT}	HPPAGDGKKP	SIAAVVGSMD	GHPSRYCATV	RVQTSRQEIS	QELLYSQEVI	QDLTNMVREL	LIQFYKSTRF	KPTRIIYYRG	GVSEGQMKQV

hAgo1	LHYELLAIRD	ACIKLEKDYQ	PGITYIVVQK	RHHTRLFCD	KNERIGKSGN	IPAGTTVDIN	ITHPFEEDFY	LCSHAGIQGT	SRPSHYVVLW	DDNRFDADEL
hAgo3	LYYELLAIRE	ACISLEKDYQ	PGITYIVVQK	RHHTRLFCD	RTERVGRSGN	IPAGTTVDTD	ITHPYEEDFY	LCSHAGIQGT	SRPSHYHVLW	DDNCFDADEL
hAgo2	LHHELLAIRE	ACIKLEKDYQ	PGITFIIVVQK	RHHTRLFCD	KNERVGRSGN	IPAGTTVDTK	ITHPTFEEDFY	LCSHAGIQGT	SRPSHYHVLW	DDNRFSSDEL
hAgo4	AWPELIAIRK	ACISLEEDYR	PGITYIVVQK	RHHTRLFCD	KTERVGRSGN	VPAGTTVDST	ITHPSEEDFY	LCSHAGIQGT	SRPSHYQVLW	DDNCFDADEL

hAgo1	QILTYQLCHT	YVRCTRSVSI	PAPAYYARLV [*]	AFRARYHLVD	KEHDSGEGSH	ISGQSNGRDP	QALAKAVQVH	QDTLRMTMYFA
hAgo3	QLLTYYQLCHT	YVRCTRSVSI	PAPAYYAH ^{LV}	AFRARYHLVD	KEHDSAEGSH	VSGQSNGRDP	QALAKAVQIH	QDTLRMTMYFA
hAgo2	QILTYQLCHT	YVRCTRSVSI	PAPAYYAH ^{LV}	AFRARYHLVD	KEHDSAEGSH	TSGQSNGRDH	QALAKAVQVH	QDTLRMTMYFA
hAgo4	QLLTYYQLCHT	YVRCTRSVSI	PAPAYYARLV	AFRARYHLVD	KDHDSAEGSH	VSGQSNGRDP	QALAKAVQIH	HDQTHTMYFA

Figure 1.1: Conservation of the amino acid sequence of the PIWI domain of mammalian Argonautes.



Adapted from Cenik and Zamore.

Figure 1.2: Coordination of a magnesium ion for endonuclease activity.

Of the four human Argonautes, only Ago2 has the ability to catalyze site-specific, small RNA-directed endonucleolytic target cleavage (Liu et al., 2004; Song et al., 2004; Meister et al., 2004). Like Ago2, Ago3 contains an apparent catalytic triad, but unlike Ago2, it lacks endoribonuclease activity. For Ago1 and Ago4 there is no catalytic triad, explaining their lack of endoribonuclease activity (Meister et al., 2004; Rivas et al., 2005; Azuma-Mukai et al., 2008). Extensive, but not complete, complementarity between a small RNA guide and an mRNA is required for Argonaute-catalyzed target cleavage (Hutvagner and Zamore, 2002; Schwarz et al., 2002; Haley and Zamore, 2004; Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005). In contrast, small RNAs with only partial complementarity to their target mRNAs, especially those bearing mismatches near the cleavage site, cannot direct endonucleolytic cleavage of their target (Holen et al., 2002), but instead reduce the stability of the target mRNA (Guo et al., 2010) and, in some conditions, cause translational repression (Doench et al., 2003; Doench and Sharp, 2004).

Since the identification of Ago2 as the endoribonucleolytic component of mammalian RISC, there has been little advancement in our understanding of the function of the individual Argonaute proteins (Liu et al., 2004; Meister et al., 2004). Does Ago2 require nuclease activity as part of its normal cellular duties? How is functional specificity established for the different Argonaute family members? Argonaute expression patterns in human tissues overlap and most cultured mammalian cell lines express all four proteins in different proportions (Sasaki et al., 2003; Meister et al., 2004). Disruption of the mouse Ago2 gene produced an embryonic-lethal phenotype, while other Argonautes are dispensable for mammalian development, suggesting that Argonaute proteins

are not redundant, but might be functionally specialized (Liu et al., 2004; Schmitter et al., 2006).

Argonaute family of proteins

The Argonaute family of proteins is conserved in plant, animal and fungi kingdoms. Argonaute proteins exist in eubacteria and archaea, hence, small nucleic acids may have guided regulation since cellular life began. Subsequent diversification of the small RNA guides allowed Argonautes to acquire specialized roles. Phylogenetic analysis separates the Argonautes into three clades, Ago-like, PIWI, and worm Argonautes (WAGO), that reflect their function in RNA silencing pathways. The Argonaute family is defined by the presence of both the Piwi Argonaute Zwiller (PAZ) domain, that binds to the 3' end of the small RNA, and Piwi domains (Carmell et al., 2002; Ma et al., 2004). Ago-like proteins transcriptionally and post-transcriptionally regulate targets using microRNAs (miRNAs) and small interfering RNAs (siRNAs) as guides in plants, animals and fission yeast (Tolia and Joshua-Tor, 2007). PIWI-like proteins are specific to animals and they function by using PIWI-interacting RNAs (piRNAs) as guides to silence transposons in germ cells and ovarian follicle tissues (Aravin et al., 2007; Brennecke et al., 2007; Brennecke et al., 2008; Li et al., 2009; Malone et al., 2009). Although thought to be specific to gonadal cells, PIWI mRNA is also expressed in brain and kidney (Sharma et al., 2001). Identification of piRNAs in dendrites of mouse hippocampus showed that they function to repress translation of mRNA targets that regulate dendritic spine formation (Lee et al., 2011). The WAGO clade contains 18 Argonaute proteins that are specific to *C. elegans*. WAGO proteins use secondary small RNAs containing 5' - triphosphates that are synthesized by an RNA-dependent RNA polymerase from

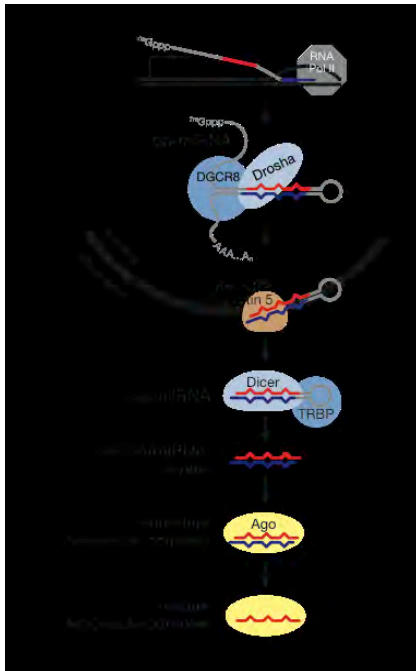


Figure 1.3: microRNA biogenesis pathway.

RNA templates. WAGO protein function is not completely understood, however, WAGO proteins silence endogenous mRNA targets when guided by exogenous dsRNA (Tabara et al., 1999; Yigit et al., 2006). WAGO proteins also regulate chromosome structure and segregation (Claycomb et al., 2009; Conine et al., 2010; Gu et al., 2009).

microRNA biogenesis

miRNAs are 21–23 nt long RNAs that direct Argonaute proteins to bind to and repress complementary mRNA targets. More than 500 miRNAs are conserved between mammals, and each miRNA can repress hundreds of genes, regulating almost every cellular process (Bartel, 2009; Chiang et al., 2010). Individual miRNAs are often produced only in specific cell types or developmental stages (Landgraf et al., 2007). miRNAs are transcribed from their own genes by RNA polymerase II (Bracht et al., 2004; Lee et al., 2004). Consequently, miRNA primary transcripts (pri-miRNAs) begin with 5′ 7-methylguanosine caps and end with 3′ poly(A) tails (Figure 1.3). The pre-miRNA, a ~65 nt stem-loop structure that contains the miRNA and its corresponding miRNA* within its stem, resides within the pri-miRNA. Cleavage of the pri-miRNA by the ribonuclease III (RNase III) enzyme, Drosha, releases the pre-miRNA stem-loop, which bears the 2 nt 3′ overhanging ends characteristic of cleavage by RNase III enzymes (Lee et al., 2003; Han et al., 2006). The pre-miRNA is then exported to the cytoplasm, where its loop is

removed by a second, RNase III enzyme, Dicer, that specifically recognizes the pre-miRNA structure, including its 2 nt 3' overhanging end, and cuts both strands (Han et al., 2004; Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004; Zeng and Cullen, 2004). The resulting miRNA/miRNA* (miRNA-5p/miRNA-3p) duplex is then loaded into the RISC loading complex (RLC) which, in humans, consists of Argonaute, Dicer, and TAR-RNA binding protein (TRBP), in a process facilitated by hydrolysis of ATP and central mismatches (Yoda et al., 2010). The requirement for Dicer in the RLC has been called into question because immunodepletion of human Dicer supports RNAi in vitro and in Dicer null mouse ES cells (Martinez et al., 2002; Kanellopoulou et al., 2005; Murchison et al., 2005). Following loading, one strand of the miRNA duplex departs from the Argonaute protein by an unknown cleavage-independent mechanism where release of the discarded strand is promoted by mismatches in the seed and 3' middle region of the miRNA/miRNA* duplex (Yoda et al., 2010). The result is a mature, active miRNA:protein complex called miRISC.

Although the canonical miRNA biogenesis pathway requires two RNase III enzymes, Drosha and Dicer, there is an alternative mechanism that does not require Drosha recognition and cleavage. Mirtrons are a subclass of pre-miRNAs that are excised from intron lariats from their pri-miRNAs by the spliceosome then, after debranching, fold into Dicer substrates (Okamura et al., 2007; Ruby et al., 2007; Berezikov et al., 2007; Babiarz et al., 2008; Chiang et al., 2010; Chong et al., 2010). Another interesting exception to the canonical biogenesis mechanism is Dicer-independent miR-451 that was discovered while investigating the requirement for Ago2 expression in embryonic mouse development (Cheloufi et al., 2010).

In small RNA deep-sequencing libraries from mice that express a catalytically inactive Ago2, mature miR-451 was not observed (Cheloufi et al., 2010). Analysis of the structure of the precursor to miR-451 showed that the stem of the pre-miRNA was only 17 nucleotides long and the mature miRNA strand sequence extended into the loop region and complementary arm of the hairpin. This arrangement of the hairpin is incompatible with Dicer processing of miRNAs. In mutant Ago2 cells, the mature miR-451 was not observed, but the 40 nt precursor, a product of Drosha, was detected by northern probes to both arms of the precursor miRNA.

The miR-451 precursor was detected in Ago2-immunoprecipitates, suggesting that Ago2 was processing pre-miR-451. Deep sequencing of small RNAs showed that Ago2 generated an intermediate 3'-hydroxyl end at the position consistent with endonucleolytic cleavage by Ago2 (Elbashir et al., 2001a). The authors predicted that the Ago2 product is most likely trimmed further by an unspecified exonuclease because deep sequencing revealed a distribution of 3' ends that contained non-templated uracils. Although no other known miRNAs are processed by this alternative biogenesis mechanism, the requirement for Ago2 to process miR-451 explains why animals have retained Ago2, the only catalytic Argonaute, despite the fact that very few miRNAs cleave their targets (Yekta et al., 2004; Davis et al., 2005).

Deciphering miRNA function

While RNA interference is a powerful tool to determine biological functions of each gene, artificially introducing or inhibiting miRNA function can provide clues to their function in normal cellular processes and human disease. The molecular function of an individual miRNA can be discovered by inhibiting it

and measuring the changes in the levels of each mRNA or protein in the cell or by evaluating other phenotypic changes such as developmental defects, cell proliferation, organ function or behavior.

Small RNAs in RISC are inhibited from regulating their targets when they

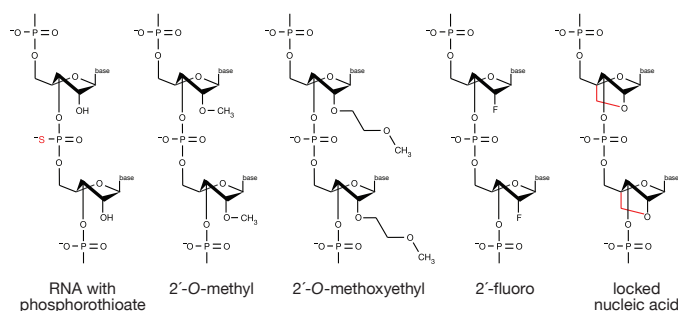


Figure 1.4: Chemical modifications of RNA.

bind with perfect complementarity to a competitor oligonucleotide that contains modifications to prevent its cleavage.

Antisense oligonucleotides (ASO) are single stranded DNA or RNA molecules that bind other nucleic acids by Watson-Crick base pairing. Chemical modification of the ribose backbone of ASOs prevents Ago2-mediated cleavage of the ASO and improves their in vitro and in vivo stability, as well as their in vivo delivery (Figure 1.4). 2'-O-methyl modified ASOs complementary to a miRNA bound to an Argonaute protein are effective miRNA inhibitors when introduced by lipid-mediated transfection into cultured human cells or by injection in whole nematodes. Antagomirs are synthetic ASOs that contain 2'-O-methyl-modified ribose sugars, terminal phosphorothioates and at the 3' end, a cholesterol group, which helps deliver the antagomir to cells. ASOs modified to contain 2', 4' methylene bridges, called locked nucleic acids (LNAs), are especially potent due to structural constraints forcing them into the C3'endo conformation. Restricting the conformation of the nucleotide imparts increased RNA:RNA melting temperature by 2.4° C per modification. The unique target

mRNA-binding properties of a miRNA bound to an Argonaute protein allow LNA modified ASOs to inhibit miRNAs when pairing only to the 8 nucleotides as nearly all the binding specificity comes from the seed sequence (Obad et al., 2011). Such tiny LNAs can inhibit miRNA families that share the same seed sequence. Unconjugated tiny LNAs, when delivered systemically to mice, showed uptake in normal cells and breast tumors where they inhibited the targeted miRNAs (Obad et al., 2011).

ASOs function, at least in part, as competitive inhibitors of miRNAs, suggesting that miRNA-binding RNA transcripts may also sequester and thereby inhibit specific miRNAs. Expressing such decoy transcripts could provide an alternative to using proprietary oligonucleotide chemistries and delivery formulations, enabling researchers to examine the consequence of inhibiting each known miRNA in a particular cell or model animal or plant (Ebert et al., 2007; Loya et al., 2009; Todesco et al., 2010). Moreover, miRNA-binding transcripts can be expressed from viral vectors, allowing the development of anti-miRNA gene therapy approaches. The first demonstration of such transcripts, miRNA decoys or sponges, inhibiting miRNA function preceded the discovery that plants naturally use such miRNA-binding transcripts to reduce the activity of specific miRNAs (Franco-Zorrilla et al., 2007).

microRNA stability

In addition to directing the mechanism of target silencing, the extent to which a target and miRNA pair has been shown to affect the stability of the miRNA. In 2002, it was shown that an endogenous miRNA could cleave multiple exogenous mRNA targets to which it paired perfectly (Hutvagner and Zamore, 2002). However, it was not known if a single miRISC, with partial pairing to its

target was able to direct regulation of multiple target mRNA molecules. Baccarini et al. (2011) tested the multiple-turnover hypothesis by inhibiting the biogenesis of a miRNA that targeted a stably expressed GFP reporter gene. Careful quantitation showed that the number of target transcripts a miRNA regulated was more than the number of molecules of miRNA, indicating that miRISC was capable of multiple-turnover. The target was not destroyed during silencing, due to the bulged miRNA: target pairing, and it continued to be silenced after blocking biogenesis of the miRNA. If miRISC was not multiple-turnover, then following the inhibition of biogenesis, an increase in target expression should have occurred. The decay rate of the miRNA was measured in the presence of a perfect target or a target that paired only to the 5' end of the miRNA or a bulged target. The decay rate was faster with the perfectly pairing target than for the bulged pairing target or the target that paired only to the 5' end of the miRNA (Baccarini et al., 2011). Their analysis supports a model in which the degree of complementarity between the target and the miRNA not only directs target silencing, but also influences the stability of the miRNA.

The first evidence for target-dependent destabilization of miRNAs was observed with the use of synthetic, chemically modified ASOs to inhibit miRNAs in mammalian cells (Krutzfeldt et al., 2005). At the same time the Zamore lab was investigating the enzyme Hen1 which, in plants and flies, methylates the 2' hydroxyl of the 3' terminal nucleotide of siRNAs (Park et al., 2002; Li et al., 2005; Yu et al., 2005; Horwich et al., 2007; Pelisson et al., 2007; Saito et al., 2007). Modification by Hen1 of miRNAs and siRNAs in flies occurs after loading in Ago2, but small RNAs that load in Ago1 are not modified by Hen1 (Horwich et al., 2007). When there is extensive complementarity to target, the 3' end of the

guide RNA in Ago2 must release from the PAZ domain of the Argonaute protein in order to pair with and cleave the target (Tomari and Zamore, 2005; Wang et al., 2008). For small RNAs in Ago2, Hen1 methylation at the 3' terminal nucleotide protects the small RNA from being uridylated and becoming a substrate for exonucleolytic degradation (Ameres et al., 2010; Ameres et al., 2011). The methylation of an siRNA in Ago2 protects its 3' end and allows multiple rounds of target cleavage by a single RISC. In contrast, human Argonautes are orthologs of fly Ago1 and small RNAs in human cells are not terminally methylated. miRNAs in mammalian Argonautes generally have less extensive pairing to their target mRNAs, this allows the 3' end of the miRNA to remain bound in the PAZ domain and prevents uridylation and exonucleolytic degradation of the miRNA, preserving its capacity to silence multiple target mRNAs (Wang et al., 2008; Ameres et al., 2010).

In worms, target-dependent destabilization of miRNAs has not been observed, but recent work indicates that the stability of a miRNA* strand can be *increased* in the presence of a complementary target (Chatterjee et al., 2011). The authors call this target-mediated miRNA protection (TMMP) and propose that this may be a post-loading proofreading mechanism to sort miRNAs into Argonautes because in worms preferential strand loading in RISC, driven by thermodynamic asymmetry of miRNAs, is not prevalent. Considered together, these mechanisms suggest an intimate dynamic between miRNAs and their target mRNAs.

Small RNA sorting into Argonaute proteins

Expression of four mammalian Argonaute paralogs might reflect a biological specialization. For example, each Argonaute protein could prefer to

load a specific type of small RNA trigger, such as a double stranded siRNA or a miRNA, thereby sorting the small RNAs, based on their structure or sequence, into a specific Argonaute protein.

Evidence supporting a small RNA sorting hypothesis stems from the discovery of sorting of small RNAs in other organisms. In *C. elegans*, miRNAs sort into Argonaute orthologs, Argonaute-like gene 1 and 2 proteins, ALG-1 and ALG-2, but siRNAs load into RNAi defective 1 protein, RDE-1. In *Drosophila*, each small RNA duplex or miRNA-miRNA*, is structurally interrogated by the Argonaute proteins, dAgo1 and dAgo2 before it is loaded in RISC. *Drosophila* Ago2 prefers to load a guide strand of the duplex that is more stably paired at its 5' end or has more central pairing to its partner strand, and *Drosophila* Ago1 prefers to load the strand of a miRNA/miRNA* whose 5' end contains central mismatches (Forstemann et al., 2007; Steiner et al., 2007; Tomari et al., 2007; Czech et al., 2009; Okamura et al., 2009; Ghildiyal et al., 2010). In *Arabidopsis thaliana*, sorting of trigger RNAs into Argonautes is directed by the identity of the 5' nucleotide (Mi et al., 2008; Montgomery et al., 2008; Takeda et al., 2008; Wu et al., 2009). The crystal structure of a MID domain from a eukaryotic Ago protein, human Ago2, in complex with nucleoside monophosphates (AMP, CMP, GMP, and UMP) mimicking the 5' end of miRNAs, shows that there are specific contacts made between the base of UMP or AMP and a rigid loop in the MID domain (Frank et al., 2010). The loop structure discriminates against CMP and GMP, but AMP and UMP bind with up to 30-fold higher affinity. Thus nucleotide specific interactions in the MID domain of eukaryotic Ago proteins explain the observed preference for U or A at the 5' end of miRNAs (Lewis et al., 2005).

Several studies looked for a mammalian Argonaute small RNA sorting mechanism. In Jurkat cells, an immortalized line of human T lymphocyte cells, the identity of miRNAs that immunoprecipitated with endogenous Ago2 and Ago3 overlapped, indicating that Argonaute proteins are not specialized for their interaction with small RNAs (Azuma-Mukai et al., 2008). The strongest, yet not convincing, evidence for functional specialization of the Argonaute proteins comes from inducible Argonaute knockout mouse embryonic stem cells. In mouse embryonic stem cells expressing a single exogenous Argonaute protein, some preference based on the structure of the exogenously supplied siRNA was detected in reporter assays: Ago1 and Ago2 were more effective than Ago3 and Ago4 at silencing a luciferase reporter when the siRNA was a perfectly pairing duplex, and all Argonautes could silence the luciferase reporter when the siRNA duplex contained central bulges (Su et al., 2009). Analysis of human RISC assembly using epitope-tagged Argonaute proteins ruled out a structurally-driven sorting mechanism for exogenous siRNAs containing miRNA/miRNA*-like pairing structures and revealed that they can load in any Argonaute protein. However, it did show that perfect siRNA duplexes in vitro could be loaded only into Ago2, because they require slicer activity to cleave the passenger strand (Matranga et al., 2005; Yoda et al., 2010).

Target pairing specialization

Since there is no rigid sorting mechanism for small RNAs into mammalian Argonaute proteins, perhaps the specialization of Argonautes is determined at the next step in the pathway, by sensing the structure of the miRNA:mRNA target pairing. The crystal structure of prokaryotic Argonaute protein from *Thermus thermophilus* with a DNA guide strand and an RNA target revealed molecular details that are consistent with known characteristics of miRNAs. For example, target recognition is directed by the seed sequence of a miRNA, nucleotides 2 to 8 from the 5' end of the guide strand (Figure 1.5) (Brennecke et al., 2005; Lewis et al., 2005; Bartel, 2009). This region of a miRNA was identified by computational clustering analysis of the target pairing requirement in evolutionarily conserved miRNAs and proven experimentally to direct target recognition and regulation by RISC (Lewis et al., 2003; Rajewsky and Socci, 2004; Lewis et al., 2005; Grimson et al., 2007; Baek et al., 2008; Selbach et al., 2008; Friedman et al., 2009).

The structural analyses of Argonaute protein from *T. thermophilus* with a guide RNA showed that the phosphodiester backbone contacts the MID/PIWI lobe of the protein, forcing the seed nucleotide bases 2 to 6 to face the exterior of the protein in a continuous A-form helical arrangement that allows hydrogen bonding to the target mRNA (Ma et al., 2005; Parker et al., 2005). The bases in the 3' region of the guide RNA were not pre-ordered. The asymmetry for ordered

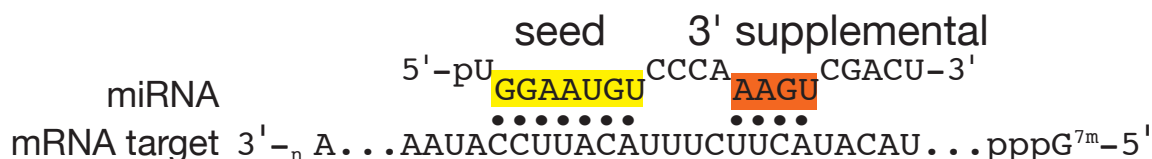


Figure 1.5: Seed region of a microRNA (yellow) and 3' supplemental pairing (orange).

arrangement of the bases in the structure is consistent with biochemical energetics of RISC function showing that 3' complementarity between target and guide RNA is not required for target cleavage, but does contribute to binding stability (Haley and Zamore, 2004; Ameres et al., 2007).

The pre-ordered arrangement of seed nucleotides 2-6 explains why a position 5 bulged nucleotide, or loop-out, in the guide strand does not allow cleavage and silencing of a target mRNA, whereas a bulge at the same position in the mRNA target does allow target cleavage (Wang et al., 2008). Even when targeting six-sites in the 3' UTR of a luciferase reporter in HeLa cells, there was no silencing of a reporter by an siRNA that had a loop-out in the guide strand at position 5 of the seed region yet was perfectly paired to target at every other position (unpublished data, Wee and Zamore). In *C. elegans*, a target site for *let-7* in the 3' untranslated region (UTR) of *lin-41* forms a bulge in the target at the nucleotide that is paired to the nucleotide at position 5 of *let-7*, and is able to silence a reporter target when one there is an additional target site nearby that does not contain a bulge at position 5, showing that the geometry of a target bulge in the seed region is tolerated by Argonaute proteins (Lee et al., 2003; Vella et al., 2004).

Apart from the seed region, the next most conserved target pairing region in miRNA families is to four contiguous nucleotides at positions 13-16 and is called 3' supplemental pairing (Figure 5) (Grimson et al., 2007). The seed-nucleation model for target recognition by a guide RNA proposed that a conformation shift in the Argonaute protein is required in order for the guide RNA to pair completely to target beyond the seed region, allowing the paired guide and target to form two helical turns (Bartel, 2009). The seed nucleation

model proposes that a thermodynamic shortcut to allow pairing beyond the seed, but avoid a conformational shift in the protein, could be to sacrifice central pairing to the target and restart contiguous pairing at positions 13-16 of the guide. The reduced target affinity caused by having mismatches in the central region of the guide can be energetically offset by not requiring the conformational shift in the Argonaute protein in order for the 3' end of the guide to pair with the target. Allowing 3' supplementary pairing to target, after the centrally unpaired region, avoids the energetic cost of release of the central region of the miRNA from the Argonaute protein during target pairing.

When *T. thermophilus* Argonaute was crystallized with a 19 nt target RNA, the target paired to the guide RNA from position 2 to position 16 of the guide strand (Wang et al., 2009). Mismatches at positions 10 and 11 of the guide RNA caused disorder in the structure between positions 12-19 so that the 3' end of the guide strand was retained in the PAZ domain. A guide strand that paired perfectly only up to position 12 or 15 with target was prevented from slicing the target, when the 3' end was retained in the PAZ domain. In contrast, a 3' truncated guide strand, pairing to only 9 nucleotides of target, was able to cleave the target. This data supports the mechanistic distinction between silencing by catalytic Ago2 and the non-catalytic Argonautes based on extent of pairing. It has been proposed that in order for Ago2 to cleave target, the 3' end of the guide RNA must release from the PAZ domain (Wang et al., 2009). More extensive pairing to the target would favor cleavage of target by Ago2, but cleavage would be inhibited until pairing propagated to the 3' end. However, multiple rounds of target cleavage by Ago2 are favored when the 3' end of the guide is mismatched or competitively blocked. Thus it appears that release of the target cleavage

product when paired to the 3' end of the guide RNA is the limiting step in target cleavage (Haley and Zamore, 2004; Ameres et al., 2007). Finally, 3' supplemental or compensatory pairing contributes little to target-binding specificity and affinity, and might be dispensable for silencing by non-catalytic Argonaute proteins, since most miRNAs do not cleave their targets (Doench and Sharp, 2004; Yekta et al., 2004; Brennecke et al., 2005; Davis et al., 2005; Lewis et al., 2005; Lim et al., 2005; Grimson et al., 2007; Baek et al., 2008; Selbach et al., 2008; Friedman et al., 2009; Shin et al., 2010).

Argonaute protein mechanisms for silencing

One of the unsolved mysteries of small RNA silencing is the mechanism through which miRNAs regulate the expression of targets. The first miRNA target interaction was discovered in *C. elegans* (Lee et al., 1993). The 3' UTR of the *lin-14* mRNA contained 7 sites with extensive, but not complete complementarity to the *lin-4* miRNA. Mutation of either *lin-4* or *lin-14* genes produced the same defect in developmental transitions of larvae, causing the mis-expression of proteins at incorrect stages of development resulting in improper execution of cell fates in the developing worm, called a heterochronic mutant phenotype (Ambros and Horvitz, 1984; Chalfie et al., 1981; Horvitz and Sulston, 1980; Sternberg and Horvitz, 1984; Sulston and Horvitz, 1981; Lee et al., 1993; Wightman et al., 1993). Interestingly, deletion of some of the complementary sequences caused a weaker phenotype than when all were deleted from the 3' UTR of *lin-14*. None of the mutants showed a change in abundance of *lin-14* mRNA, however, mutation of the sequence in the 5' end of *lin-4* caused persistent expression of the LIN-14 protein, suggesting that an RNA:RNA interaction, *lin-4* binding to the *lin-14* 3' UTR, inhibited translation (Lee et al.,

1993). The discovery of another small RNA, *let-7*, which paired to the 3' UTR of *lin-41* mRNA, showed that this mechanism for regulation was not exclusive to worms, because the *let-7* nucleotide sequence and temporal expression pattern was conserved in *Drosophila* embryos and across bilateral animals (Pasquinelli et al., 2000; Reinhart et al., 2000).

Similarity in the antisense nature of regulation of *lin-14* and *lin-41* by *lin-4* and *let-7* together with their hairpin-like precursor structure led to the confluence of miRNA mediated silencing and two similar gene silencing phenomena: co-suppression in plants and RNA interference discovered in worms (Fire et al., 1991; Guo and Kemphues, 1995; Baulcombe, 1996; Fire et al., 1998). Biochemical studies in plants, worms and the establishment of an in vitro system from extracts of syncytial blastoderm of *Drosophila* embryos quickly led to the discovery that the trigger for silencing was processed from long double stranded RNA (dsRNA) into a small RNA duplex which directed nuclease activity of a ribonucleoprotein, RISC containing Argonaute 2 (Tuschl et al., 1999; Hamilton and Baulcombe, 1999; Hammond et al., 2000; Parrish et al., 2000; Zamore et al., 2000; Hammond et al., 2001). A major breakthrough in the field was made by using synthetic small RNA duplexes, that bypassed the need for a long double stranded RNA precursor trigger, to silence endogenous mRNAs. This allowed transient transfection of siRNAs into cells and overcame the antiviral Protein Kinase RNA-activated (PKR) response triggered by long dsRNA in mammalian cells (Elbashir et al., 2001a).

Soon after, two important discoveries moved the field forward. First, an endogenously programmed miRNA in RISC when presented with a target mRNA to which it paired with perfect complementarity, caused cleavage and

destruction of multiple target mRNAs in vitro (Hutvagner and Zamore, 2002). Reciprocally, Doench and Sharp showed that an exogenously supplied siRNA that paired imperfectly, with central bulges, to a target did not direct target cleavage, but instead caused the mRNA target to be translationally repressed, similar to *lin-4: lin-14* in worms (Doench et al., 2003; Doench and Sharp, 2004). They could still detect mRNA from the luciferase reporter target on a Northern blot, but the luciferase protein activity was decreased. These two studies led to the model that the degree of complementarity between a small RNA and its target determined the mechanism of silencing. Extensive pairing with target mRNA could cause cleavage and an endogenously loaded miRNA was capable of directing cleavage of a target to which it paired with perfect complementarity, as was an exogenously supplied siRNA. Sharp and Doench showed that an siRNA could act like a miRNA: when the siRNA had central bulges or mismatches to multiple target sites in the 3' UTR of a reporter mRNA, the siRNA caused translational repression.

Translational repression or mRNA destabilization?

Several large-scale proteomic analyses support bioinformatically-based predictions that a single miRNA can repress hundreds of target mRNAs (Lewis et al., 2003; Rajewsky and Socci, 2004; Lewis et al., 2005; Grimson et al., 2007; Baek et al., 2008; Selbach et al., 2008; Friedman et al., 2009). After years of studies on the mechanism of miRNA target regulation, two opposing models remain for how miRNAs effect silencing of their mRNA targets. One model is that miRNAs reduce the translation efficiency of an mRNA, without decreasing mRNA abundance, and the other model proposes that the reduced protein level is a result of mRNA destabilization by miRNA-directed decapping or deadenylation.

Two groups examined the impact of inhibiting a miRNA or its over-expression on translational efficiency by quantitative mass spectroscopy of metabolically labeled proteins. One study concluded that the dominant effect for certain targets was decreased translational efficiency (Selbach et al., 2008), while another group concluded that there was a strong correlation between the decrease in mRNA and protein abundance effected by miRNAs (Baek et al., 2008). These studies may have conflicting results because measurement of the mRNA levels by microarray analysis from a single time point can be distorted by comparison to protein quantitation after metabolic labeling— a process occurring over a period of time. However, a more quantitative analysis was carried out that quantitatively measured ribosome density by deep sequencing analysis of ribosome protected mRNA fragments and compared them to the mRNA array or RNA-seq data (Ingolia et al., 2009; Guo et al., 2010). The ribosome protected fragment analysis measures levels of mRNAs in the cell and the effect of the miRNA on the mRNA level can be cross-correlated to the amount of ribosome protected fragments of mRNA, eliminating the time discrepancy between mRNA and protein measurements of the previous studies. This method also reports on the levels of thousands of mRNAs, in contrast to the proteomic analyses, which skews the data toward more highly expressed proteins. Using the more quantitative ribosome profiling approach, two groups showed that a majority of the reduced protein output corresponded to destabilization of the mRNA targets, however, for some remaining target mRNAs there was a decrease in ribosome density consistent with the model for inhibition of translation (Hendrickson et al., 2009; Guo et al., 2010).

Translational repression at initiation or elongation?

For those mRNA targets not destabilized by their miRNAs, there is controversy for the mechanism of repression. Translation can be inhibited at the initiation step or at the elongation step. Repression of a target mRNA depends on disruption of the interaction between the 5' cap and poly-A tail. miRNA binding to the 3' UTR could block translation initiation, by preventing binding of the 40S ribosome initiation complex through competition for cap binding proteins (Mathonnet et al., 2007; Thermann and Hentze, 2007). In *Drosophila*, Ago1 blocks a step after cap recognition, whereas Ago2 binds to eIF4E and specifically blocks eIF4E-eIF4G interaction that is required for translation initiation (Iwasaki et al., 2009). Interestingly, when *Drosophila* Ago2 is bound to target mRNA, the affinity of Ago2 for eIF4E is dramatically enhanced (Iwasaki et al., 2009). Data also suggest that a target mRNA can be repressed even if it is not adenylated at its 3' end, while messages containing an adenosine cap, instead of a guanosine cap, or a viral internal ribosome entry site are not subject to repression by miRNA binding to the 3' UTR (Humphreys et al., 2005; Pillai et al., 2005; Wang et al., 2006; Wu et al., 2006; Wakiyama et al., 2007; Eulalio et al., 2008; Eulalio et al., 2009; Iwasaki et al., 2009).

Alternatively, elongation could be inhibited if miRNA binding caused ribosomes to stop translocating. In mammalian cells, evidence for inhibition of translation initiation is supported by polysome gradient analysis that showed *let-7* binding or Ago2 protein tethered to the 3' UTR of a target mRNA shifted the density of the target because fewer ribosomes bound to it than to a control target not bound by *let-7* or tethered to Ago2 (Pillai et al., 2005). In contrast, in *C. elegans*, no loss of ribosome density occurred for the *lin-14* and *lin-28* mRNAs

when bound by *lin-4* miRNA, suggesting repression occurs post-initiation (Moss et al., 1997; Olsen and Ambros, 1999; Seggerson et al., 2002). Between the first and second larval stages there is a 10-fold change in LIN-14 protein, but by comparison of wild-type worms to the *lin-4* miRNA mutant, *lin-4* miRNA causes only a 4-fold change in LIN-14 protein, suggesting that the additional decrease in protein is caused by destabilization of *lin-14* mRNA (Wightman et al., 1993; Bagga et al., 2005). A unified model in support of inhibition of translational elongation and resolving discrepancies between ribosome density on a target will be more difficult and may prove to be target or cell type specific.

Adding to the uncertainty, insertion of a stem-loop structure in the 5' UTR that prevented translation initiation still allowed de-adenylation and decay of target mRNA to occur upon miRNA binding to the 3' UTR in mammalian cells, suggesting that miRNA binding can still cause target destabilization even in the absence of ongoing translation (Mishima et al., 2006; Wu et al., 2006; Eulalio et al., 2007; Wakiyama et al., 2007; Eulalio et al., 2009). Whether or not deadenylation is the direct result of miRNA binding to the 3' UTR or a consequence of bound miRISC preventing circularization between 5' cap and polyA tail is unclear because there is currently no system that allows one to understand the kinetics of miRNA-mediated translational repression. An explanation for the inconsistent results obtained from the various model systems is that the outcome of miRNA binding to a target may be specific to the cell type or that translation initiation of a target is initial response, followed by destabilization.

Localization of silenced targets

Repression of a target by an Argonaute protein may be influenced by

other proteins recruited to an mRNA after miRISC binding. GW182 protein binds to Ago proteins through an Ago-hook motif and tethering of GW182 to a 3' UTR of a target mRNA causes repression independent of a small RNA (Behm-Ansmant et al., 2006; Till et al., 2007). Humans have three GW182 paralogs: TNRC6A, TNRC6B and TNRC6C (Eulalio et al., 2007). GW182 causes posttranscriptional regulation of a subset of mRNAs by localizing them to cytoplasmic foci called processing-bodies, or P-bodies (Liu et al., 2005b; Meister et al., 2005). GW182 is a component of P-bodies which are thought to be the cytoplasmic destination of silenced target mRNAs where the target is decapped and degraded, 5' to 3' by the exonuclease Xrn1 (Lim et al., 2005; Liu et al., 2005a; Pillai et al., 2005; Valencia-Sanchez et al., 2006). Although localization of miRISC-bound targets to P-bodies was an attractive hypothesis, in cell-free extracts targets are still silenced, arguing against localization to P-bodies as a requirement for silencing (Chu and Rana, 2006). In *Drosophila*, Ago1 requires GW182 for translational repression and ATP-dependent deadenylation, whereas Ago2 represses translation independent of GW182 (Iwasaki et al., 2009).

Stress granules are cytoplasmic RNA granules that contain Ago2 as well as translation initiation factors. The presence of Ago2 in stress granules allows for the possibility that silenced targets are sequestered to stress granules and suggests that target mRNAs may be detained there while waiting to be reinitiated for translation (Parker and Sheth, 2007). Target mRNAs that are bound by Ago2 may be able to be reinitiated for translation, whereas targets bound by Ago1 may be destabilized through decapping and deadenylation in P-bodies. Far from the original hypothesis that the extent of pairing between target and miRNA determines the mechanism for silencing, it may be that individual

Ago proteins and their ability to recruit other proteins may dictate the mode of target silencing (Iwasaki et al., 2009). A model where the identity of the Argonaute bound to a target effects different mechanisms of silencing may also be the reason that some targets are translationally repressed, while the majority of targets are destabilized.

Cooperative function of miRNAs

Cooperativity is used to describe the complex interactions between molecules during ligand binding and multimolecular complex assembly. A classical example of cooperativity is oxygen binding to hemoglobin, where the fourth and last oxygen binds more tightly than the first. Essential to cooperativity is that interactions between molecules are linked through structures. Configurational cooperativity means that pre-organization of the first and second bound molecules increases the affinity for a third molecule (Whitty, 2008). In a target mRNA with multiple binding sites for miRNAs, the effect can be to increase the dependence of the output, expression of protein, on the concentration of the miRNA, causing a sharper dose-response threshold for silencing.

The potential for cooperative silencing by miRNAs was first considered when it was discovered that the 3' UTR of *lin-14* mRNA contained multiple target sites for *lin-4* and soon after target sites for *lin-4* and *let-7* were identified in the 3' UTR of *lin-14* and *lin-28* mRNAs (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000). The presence of multiple candidate sites in 3' UTR sequences is a useful predictor of an mRNA being regulated by miRNA (Lewis et al., 2003). Sharp and Doench observed that an exogenous siRNA that paired with a central bulge to a reporter target with four sites in its 3' UTR was able to cause

more repression than a reporter bearing two sites. In their system, there was an increase in repression that is typical of each target site contributing independently to repression, but the data suggested that there might be cooperativity in silencing by imperfectly pairing siRNAs. In their reporter system, an siRNA that paired perfectly to the target reporter mRNA showed no increase in repressive capacity when multiple sites existed in the target reporter, an expected result, because a perfectly pairing siRNA can cleave that target mRNA and need only cleave at one site to silence the reporter.

The concept of cooperativity was further pursued to determine how near to each other target sites needed to be in order to cause cooperative effects. By altering the spacing between the seed-matched target sites for endogenous *let-7* in the 3' UTR of a reporter mRNA, a minimum cooperative distance was determined to be between 13 and 35 nucleotides (Saetrom et al., 2007). Another analysis of cooperativity in silencing between two sites for miR-124 or two miRNAs, miR-1 and miR-133, showed repression was not greater than the multiplicative effect of independent silencing until the distance separating the two target sites was between 8 and 40 nucleotides (Grimson et al., 2007). In this case, the repression observed for the two cooperative sites was more than the amount of repression predicted by multiplying, or the product of, the repression observed for each site acting alone in separate reporters. Though none of these analyses showed biochemical cooperativity, the potential remains for multiple co-expressed miRNAs to cooperatively regulate target mRNAs. In flies, the *E(spl)* and *Bearded* mRNAs contain multiple target sites within a cooperative distance of each other for miR-2 (two sites) and miR-4 (three sites), respectively (Lai et al., 2005). In worms, *lin-14* (two *lin-4* sites), *lin-41* (two *let-7* sites), *lin-28* (a

Hill coefficient

If $n^H = 1$, then silencing exhibits no cooperativity.

If $n^H = n$, then silencing exhibits perfect cooperative behaviour.

If $1 < n^H < n$, then silencing exhibits a degree of positive cooperativity.
e.g., hemoglobin, n^H is about 3.

If $1 > n^H$, then silencing exhibits a degree of anti-cooperativity.
e.g., reduction in binding affinity due to repulsion between miRNAs.

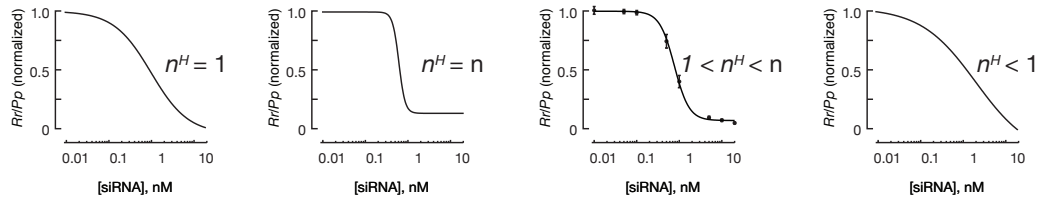


Figure 1.6: Hill coefficient measures the degree of cooperativity.

let-7 and a *lin-4* site), *hbl-1* / *lin-57* (four *let-7* sites), and *cog-1* (two *lsy-6* sites) mRNAs are targets for miRNAs and contain multiple sites within a cooperative configuration (Wightman et al., 1991; Moss et al., 1997; Reinhart et al., 2000; Abrahante et al., 2003; Johnston and Hobert, 2003). The mammalian *Hmga2* mRNA contains seven sites for *let-7* in its 3' UTR, two of which are within cooperative distance of each other (Mayr et al., 2007). Cooperative miRNA function to repress target mRNAs would ensure that targets are sensitive to small changes in the levels of their cognate co-expressed miRNAs.

Chapter II of this thesis provides an analysis of biochemical cooperativity in silencing of a target mRNA by four different siRNAs that pair to different

extents at multiple sites in the 3' UTR of a luciferase reporter mRNA. The role of Argonaute proteins was assessed by using mouse embryonic fibroblast cells derived from Ago knockout mice. Cooperativity in silencing was determined by measuring luciferase activity as a function of siRNA concentration and fitting the data to the Hill equation. The Hill coefficient is a measure of cooperativity in a biological system. When a system exhibits cooperativity, ligand bound to a site in a macromolecule enhances binding of subsequent ligands to the macromolecule and the Hill coefficient quantifies this effect. It measures the fraction of a macromolecule saturated by bound ligands as a function of the ligand concentration (Hill, 1910; Holt and Ackers, 2009). In the reporter system, the mRNA target is the macromolecule and the siRNA-RISC is the ligand. A Hill coefficient greater than 1 indicates positive cooperativity while a value of 1 indicates complete independence for binding (Figure 1.6).

Ago2 silenced a target mRNA without cooperativity when the siRNA paired with perfect complementarity to the mRNA target (Broderick et al., in press). Further, silencing by a perfect siRNA was non-cooperative even when Ago2 was unable to cleave the target: a catalytically inactive mutant Ago2 non-cooperatively silenced a target mRNA bearing multiple target sites. This contradicts the assumption that silencing caused by a perfect siRNA:mRNA target pairing was non-cooperative because Ago2 can cleave the target mRNA. This suggests that the geometry of the perfect pairing between the guide RNA and the target prevent Ago2 protein from adopting a conformation that allows cooperative binding or interaction with other proteins, causing both nearby and adjacent binding sites to act independently. In the absence of Ago2, cooperative interactions by non-catalytic Agos bound through a perfect siRNA:target pairing

at multiple sites facilitated silencing. This suggests that Ago2 is sensitive to the geometry of a small RNA: target pairing. In contrast, the non-catalytic Argonautes, Ago1, Ago3, and Ago4, are capable of cooperative silencing irrespective of the geometry of the siRNA:target pairing. For silencing in mammalian cells, the geometry of the small RNA: target pairing and the identity of the Argonaute protein in RISC both determine whether or not cooperativity facilitates silencing of a target mRNA.

The configuration of target sites contributes to the capacity for cooperativity. Argonaute proteins show a differential ability to facilitate cooperative silencing between target sites: target sites must be surprisingly near to each other to cause cooperative silencing by Ago1 (Broderick et al., in press). When three adjacent target sites were separated by 19 nt, cooperativity in silencing was lost, in the absence of Ago2. Ago1, Ago3 and Ago4 could not silence three sites separated by 19 nt with a perfect or a bulged pairing. Only Ago2 could silence the reporter with a perfect or a bulged pairing at non-adjacent sites. For non-adjacent sites, only Ago2 can silence at the intracellular RISC concentration achieved at the highest amount of siRNA transfected, likely because in the absence of cooperativity, the intracellular concentration of Ago1-, Ago3-, and Ago4-RISC is less than the dissociation constant for target binding for these Argonaute:siRNA complexes.

microRNA therapeutics

miRNAs regulate one-third of genes in the human genome (Bartel, 2009; Friedman et al., 2009). They have been implicated in pathways of disease like cancer, metabolic disease, viral infection, and immune disease. miRNAs provide the specificity determinants for Argonaute proteins to bind to and repress

expression of target mRNAs. Mutations occurring in miRNAs can lead to abnormal processing of a miRNA during biogenesis. Further, mutation of miRNA target sites within an mRNA can cause inappropriate regulation of a target mRNA. A specific instance of this situation is mutation in miR-96 of the nucleotide at position 5. Mutation at this position within the seed sequence decreases the level of expression of miR-96 and impairs target mRNA repression (Mencia et al., 2009). This single nucleotide mutation is associated with autosomal dominant, high-frequency, hearing loss in humans.

In addition to mutation of the sequence of miRNAs or their target sites, some miRNAs are deleted or their expression is reduced in some diseases. Chromosomal translocations that join two previously unlinked fragments of the genome together can cause inappropriate expression of a miRNA or target mRNA. In cancer, expression of most miRNAs is lower than normal due to

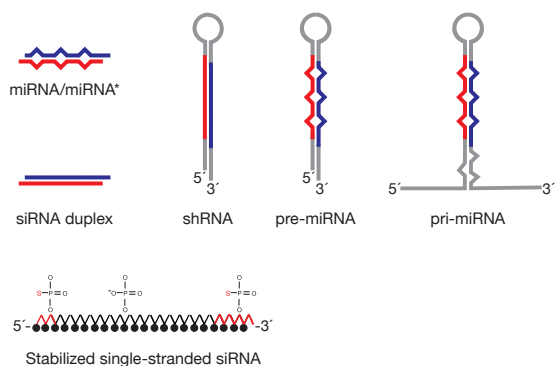


Figure 1.7: Types of exogenous small RNAs for expression in cells.

deletions at breakpoints and fragile sites. miR-15 and 16 are frequently deleted in B-cell lymphocytic cancer and their expression is reduced by 80% in

prostate cancer (Medina et al., 2008). Thus many miRNAs function similar to tumor suppressor genes. Replacement of miRNAs that have tumor suppressor function might augment traditional cancer chemotherapy. Expression of

exogenous small RNAs in cells is possible through transient or stable transfection or viral transduction of a pri-miRNA transgene, pre-miRNA, mature miRNA/miRNA*, small interfering RNA (siRNA) or short hairpin RNA (Figure 1.7). Using this strategy, intratumoral injection of exogenous *let-7* was found to block tumor development in a mouse model of non-small cell lung cancer (Johnson et al., 2007; Esquela-Kerscher et al., 2008; Kumar et al., 2008; Trang et al., 2010).

miRNAs can restrict the expression of traditional gene therapy approaches that replace defective protein-coding genes, underlying many genetic diseases. Limiting the expression of a therapeutic transgene to the correct tissue is a challenge, even when using a tissue specific promoter to express the transgene, because transgene expression occurs in non-target tissues due to leaky promoter control. Combining miRNA regulation with gene therapy is a strategy to 'de-target' expression of the transgene in non-target tissues.

Incorporation of miRNA target sites in the 3' UTR of transgenes provides a way to prevent the expression of the transgene in tissues that express the corresponding miRNA. For example, miR-122 is specifically expressed in hepatocytes and incorporation of miR-122 binding sites in the 3' UTR of a systemically delivered transgene will prevent its expression in liver, but not other tissues. This strategy was used to restrict the expression of a transgene in a lentiviral vector to astroglial cells (Colin et al., 2009). Starting with a lentivirus engineered to preferentially infect neurons and glia, miR-124 target sites were inserted in the 3' UTR to prevent transgene expression in neuronal cells, which express miR-124, and allow transgene expression in glial cells, which do not express miR-124. Injection of the vector into the hippocampus in mice produced

transgene expression in astrocytes and Bergmann glial cells, but not in pyramidal neurons or Purkinje cells. Since each site is only 21 nt long, binding sites for multiple, tissue-specific miRNAs can be incorporated in the 3' UTR, extinguishing transgene expression in many different tissues simultaneously.

Silencing an mRNA by tethering RISC

Many human diseases are caused by genes being expressed, when they should not be. Other diseases are produced by mutant genes that produce a defective protein product. For example, patients suffering from the devastating inherited neurodegenerative disorder, Huntington's disease, have both a normal copy of the huntingtin gene and a mutant, disease-causing copy. Turning off the disease gene, while retaining expression of the normal gene, would almost certainly cure the disease. Similarly, when viruses such as HIV infect human cells, they turn on their own genes to assist the spread of infection. Turning off invading viral genes—such as the HIV protease gene—would likely stop the viral infection. With few exceptions, current drugs are designed to block the action of protein products, not turn off the genes that direct production of the protein. Thus, HIV infection is treated with protease inhibitors, small molecule drugs that block HIV protease function, rather than blocking production of the HIV protease protein in the first place, because no strategy for turning off disease genes has been demonstrated to be clinically safe and effective. Toward that end, the goal is to develop novel nucleic acid reagents that turn off a gene in a specific tissue or cell type affected by disease or infection.

The RNAi pathway is the best-known example of a larger set of cellular pathways known as RNA silencing pathways (reviewed in Ghildiyal and

Zamore, 2009). The small RNAs that trigger RNAi are called small interfering RNAs. Since its discovery in 1998 (Fire et al., 1998), RNAi has emerged as the dominant method for blocking gene function in human cells. Although siRNAs hold great promise as human therapeutics and have rapidly become the standard method for target validation in cultured cells, siRNA technology is limited by our inability to deliver siRNAs only to a specific tissue or cell type and by the undesirable and unpredictable ability of siRNAs to turn off genes beyond those they were designed to target (Jackson et al., 2003).

miRNAs are a second class of small RNAs that turn genes off in animals and plants (Bartel, 2004). When these natural small RNAs bind to an mRNA, they block it from being translated into protein. The human genome contains more than 500 and perhaps as many as 1000 miRNAs, many of which are expressed only in specific cell types or tissues or during specific stages of vertebrate development (Landgraf et al., 2007). As much as one-third of all human genes have been proposed to be intrinsically regulated by miRNAs (Bartel, 2009; Friedman et al., 2009). Moreover, many viruses produce their own miRNAs; these are not normally made by the host cells, so they provide a new opportunity to identify infected cells (Cullen, 2009).

During my thesis research, I developed a novel strategy that recruits an endogenous miRNA to an mRNA not normally regulated by that miRNA, thereby blocking the mRNA from being translated into protein. Recruiting the miRNA to the mRNA effectively turns that gene off. The strategy uses oligonucleotide 'tethers' to recruit endogenous miRNAs to novel mRNA targets, turning off their expression (Figure 1.8). Each tether contains a region that binds the mRNA target and a region that binds the miRNA to be recruited, thereby

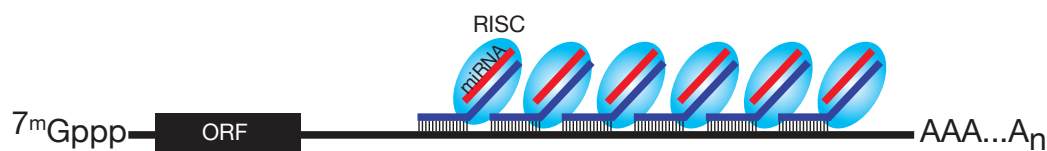


Figure 1.8: A modified RNA oligonucleotide (blue) binds an endogenous miRNA (red) and tethers RISC to the 3' UTR of an mRNA.

bringing the two together. This method is designed to harness the spatial and temporal specificity of miRNAs, so that the tethers trigger target mRNA repression only in cells where a specific miRNA species is expressed. This method can also be used to block viral gene expression in infected cells, which, unlike uninfected cells, will express viral miRNAs. Proof-of concept and preliminary experimental data are discussed on Chapter III.

Chapter II: Argonaute protein identity and pairing geometry determine cooperativity in mammalian RNA silencing.

Chapter II was a collaborative effort. Experimental plan and experiments were the author's own except for Supplemental Figure 2.S2, added in revision, which was a joint effort by the author and William E. Salomon.

Sean P. Ryder contributed intellectually to the mode of data analysis and interpretation and made this work far better than its original inception. His intellect was critical to the review process.

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Chapter II: Argonaute protein identity and pairing geometry determine cooperativity in mammalian RNA silencing.

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Running title: Requirements for cooperativity in RNA silencing

Abstract

Small RNAs loaded into Argonaute proteins direct silencing of complementary target mRNAs. It has been proposed that multiple, imperfectly complementary small interfering RNAs or microRNAs, when bound to the 3' untranslated region of a target mRNA, function cooperatively to silence target expression. We report that in cultured human HeLa cells and mouse embryonic fibroblasts, Argonaute1 (Ago1), Ago3 and Ago4 act cooperatively to silence both perfectly and partially complementary target RNAs bearing multiple small RNA-binding sites. Our data suggest that for Ago1, Ago3, and Ago4, multiple, adjacent small RNA-binding sites facilitate cooperative interactions that stabilize Argonaute binding. In contrast, small RNAs bound to Ago2 and pairing perfectly to an mRNA target act independently to silence expression. Non-cooperative silencing by Ago2 does not require the endoribonuclease activity of the protein: a mutant Ago2 that cannot cleave its mRNA target also silences non-cooperatively. We propose that Ago2 binds its targets by a mechanism fundamentally distinct from that used by the three other mammalian Argonaute proteins.

Keywords: Argonaute; cooperativity; microRNA; miRNA; RNAi; siRNA; RNA silencing

Introduction

In plants and animals, small silencing RNAs such as small interfering RNAs (siRNAs) and microRNAs (miRNAs) provide the specificity determinants for Argonaute proteins. A small RNA guide bound to an Argonaute protein is called the RNA-induced silencing complex (RISC) (Hammond et al., 2000; Hannon, 2002; Du and Zamore, 2007; Matranga and Zamore, 2007; Ghildiyal and Zamore, 2009); binding of RISC to the 3' untranslated region (UTR) of an mRNA silences its expression (Lee et al., 1993; Wightman et al., 1993; Olsen and Ambros, 1999; Lai, 2002; Doench et al., 2003; Grimson et al., 2007). Argonaute proteins are structural homologs of RNase H that typically cleave their target RNAs after the nucleotide paired to the tenth base of the small RNA guide (Elbashir et al., 2001a; Elbashir et al., 2001b; Tolia and Joshua-Tor, 2007). Cleavage requires three key amino acids—D, D, H—that form a magnesium-binding catalytic triad, which promotes nucleophilic attack by hydroxide on the phosphodiester bond (Kanaya et al., 1996; Haruki et al., 2000; Martinez and Tuschl, 2004; Schwarz et al., 2004; Song et al., 2004; Rivas et al., 2005).

The human genome encodes four Argonaute paralogs—Ago1, Ago2, Ago3, and Ago4, and most cultured mammalian cell lines express all four proteins, albeit in different proportions (Meister et al., 2004). Of the four mammalian Argonautes, only Ago2 retains the ability to catalyze site-specific, small RNA-directed endonucleolytic target cleavage (Liu et al., 2004; Meister et al., 2004). Like Ago2, Ago3 contains an apparent catalytic triad, but unlike Ago2, it lacks endoribonuclease activity. For Ago1 and Ago4 there is no catalytic triad,

explaining their lack of endoribonuclease activity (Meister et al., 2004; Rivas et al., 2005; Azuma-Mukai et al., 2008). Extensive, but not complete, complementarity between a small RNA guide and an mRNA is required for Argonaute-catalyzed target cleavage (Hutvagner and Zamore, 2002; Schwarz et al., 2002; Haley and Zamore, 2004; Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005). In contrast, small RNAs with only partial complementarity to their target mRNAs, especially those bearing mismatches near the cleavage site, cannot direct endonucleolytic cleavage of their target (Holen et al., 2002), but instead reduce the stability of the target mRNA (Guo et al.) and, in some conditions, cause translational repression (Doench et al., 2003; Doench and Sharp, 2004).

Experimental and computational analyses suggest that a single miRNA can regulate hundreds of genes, because a target mRNA need only pair with the seed sequence of a small RNA—comprising nucleotides 2 through 7 or 8—to recruit RISC and promote repression (Lewis et al., 2003; Doench and Sharp, 2004; Rajewsky and Socci, 2004; Brennecke et al., 2005; Krek et al., 2005; Lewis et al., 2005; Lim et al., 2005; Grimson et al., 2007; Baek et al., 2008; Selbach et al., 2008; Friedman et al., 2009). Multiple, partially complementary small RNAs, when bound to the 3' UTR of a luciferase reporter target mRNA, may function cooperatively to repress its translation (Doench et al., 2003; Bartel and Chen, 2004), and most mRNAs contain multiple potential miRNA-binding sites in their 3' UTRs (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000; Abrahante et al., 2003; Lin et al., 2003; Bartel, 2004; Grimson et al., 2007; Friedman et al., 2009). However, the molecular basis for cooperativity in small RNA silencing remains unknown.

Here, we show that both the nature of siRNA:mRNA target pairing and the identity of the Argonaute protein to which the small RNA is bound determine whether multiple target sites act cooperatively to recruit RISC. Small RNAs that pair perfectly to multiple target sites silenced non-cooperatively when the small RNA guide acts through Ago2, whereas silencing directed by either perfectly or imperfectly pairing small RNAs bound to Ago1, Ago3, or Ago4 acted cooperatively to silence mRNA bearing multiple small-RNA-binding sites. Cooperativity required adjacent sites. Surprisingly, non-cooperative silencing by perfectly pairing small RNAs bound to Ago2 did not require target cleavage, as catalytically inactive mutant Ago2 silenced essentially as well as wild-type. Finally, we find that computationally predicted modes of miRNA:target pairing required far more small RNA to achieve repression than more extensively but still incompletely paired small RNA guides. We propose that cooperative binding of RISC to multiple adjacent sites, combined with high intracellular concentrations of miRNAs, allows robust regulation of mRNA targets by Ago1, Ago3, and Ago4.

Results

Experimental paradigm

At least three distinct regulatory mechanisms could explain the enhanced silencing of reporter mRNAs containing multiple miRNA-binding sites (Fig. 2.1). A *cooperative binding* model posits that the binding of a miRNA:Argonaute protein complex to one site increases the affinity of a second miRNA:Argonaute complex for an adjacent site (Fig. 2.1A). In this model, the binding of the first

bulged siRNA would have a higher dissociation constant, K_D^A than subsequent binding events, K_D^B and K_D^C ; we predict that the amount of siRNA required to silence a reporter would decrease with an increasing number of target sites as cooperativity between bound Argonautes increases. Such cooperativity in small RNA-directed silencing might arise from direct interactions between adjacent Argonaute proteins. Alternatively, a pair of Argonaute proteins might be bridged by one or more additional proteins. In a *cooperative function* model, multiple miRNA:Argonaute complexes bind to the target mRNA independently, but the interaction of one miRNA:Argonaute complex could recruit binding proteins which block translation of the target mRNA or decrease the stability of the target (Fig. 2.1B). Historically, such protein targets of RISC have been envisioned to include components or regulators of the ribosome, but more likely correspond to factors that promote accumulation of the target RNA in a P-body, where it would be degraded (Guo et al.; Liu et al., 2005; Rehwinkel et al., 2005; Eulalio et al., 2007; Parker and Sheth, 2007). In the *cooperative function* model, we predict that the presence of three bulged siRNAs on the target would have a lower inhibitory constant, K_i^{ABC} than for the presence of two (K_i^{AB}) or one (K_i^A) bulged siRNA; the amount of siRNA required to silence a reporter would decrease with increasing number of target sites occupied by Argonautes and/or a protein factor X until the concentration of Argonaute or factor X becomes limiting. Finally, in a *multiple independent sites* model, each miRNA:Argonaute complex binds and acts independently, but the presence of multiple miRNA-binding sites in the target increases its effective miRNA occupancy: i.e., the probability that the target mRNA is bound by at least one miRNA is increased by the presence of multiple sites (Fig. 2.1C). Such statistical effects cause the

macroscopic binding constant, K , representative of all possible combinations of target mRNA with n sites where at least one site is occupied, would be determined by the statistical factors of identical microscopic binding constants, k , to give a fractional saturation of target: $[1/n]k$ (Cantor and Schimmel, 1980). If we assume that the IC_{50} is governed by binding and that the microscopic binding constant for a single site is essentially identical to the macroscopic binding constant for the one-site target, then we expect the IC_{50} for the three-site target to be $1/3(IC_{50}, \text{one-site target})$.

In contrast to the cooperativity ascribed to miRNA-directed changes in mRNA stability or translation, small RNA-guided target cleavage—that is, RNAi—is thought to be non-cooperative, with each RISC acting independently at each complementary site on the target mRNA. The presence of multiple, independent small RNA-binding sites in a target would increase its effective occupancy by RISC: the probability that the target mRNA is cleaved by at least one molecule of RISC is increased by the presence of multiple sites.

To evaluate the efficacy of silencing and the extent of cooperativity directed by a small silencing RNA bound at one or multiple sites on an mRNA, we established an experimental system comprising six *Renilla* luciferase reporter plasmids, each expressing an mRNA bearing one to six identical, adjacent target sites in its 3' UTR (Fig. 2.2, left). We tested four siRNAs whose guide strands pair to different extents with the target sites (Fig. 2.2, top). The four siRNAs enabled evaluation of four siRNA:mRNA target RNA binding modes— perfect pairing, bulged pairing (mismatched at positions 9 and 10 of the guide strand), seed pairing with supplemental 3' pairing (matching the target at positions 2–8 and 13–16 of the guide strand), and seed-only pairing (paired only at positions 2–8 of

the guide strand). Each siRNA duplex was designed to ensure preferential loading of its guide strand into RISC (Schwarz et al., 2003). Because all of our experiments comparing distinct modes of miRNA:target pairing employed a common target reporter mRNA, our strategy avoids differences in local target mRNA structure that might confound interpretation.

For each of the 24 reporter-siRNA combinations tested in HeLa cells, we calculated the concentration of siRNA required to achieve half-maximal silencing (IC_{50}) and the Hill coefficient (n^H), a measure of cooperativity, using dose-response data from at least 12 independent experiments, each evaluating silencing at ≥ 10 siRNA concentrations and spanning a 2,000-fold concentration range. For each siRNA, we confirmed that the siRNA was inherently active by validating its ability to silence a *Renilla* luciferase reporter containing a single, fully complementary siRNA-binding site (Fig. 2.3). For the four siRNAs, the mean IC_{50} values \pm standard deviation for the corresponding perfect, single-site reporter mRNA ranged from 0.27 ± 0.22 nM to 1.33 ± 0.78 nM, establishing that all four siRNAs were active.

Silencing by perfect pairing at multiple sites is not cooperative

Next, we targeted each reporter for silencing by a perfect siRNA to determine if increasing the number of target sites reduced the amount of siRNA needed to silence the reporter. Based on the *multiple independent sites* model, we anticipated that a reporter mRNA bearing more target sites would be more likely to recruit RISC and would therefore show a reduced IC_{50} for a fully complementary (“perfect”) siRNA. Instead, our data suggest that RISC neither binds nor functions appreciably better when the target contained multiple sites (Fig. 2.2). In fact, reporter mRNAs bearing three ($IC_{50} = 0.75 \pm 0.93$ nM), four ($IC_{50} = 0.25 \pm 0.09$

nM), five ($IC_{50} = 0.41 \pm 0.33$ nM) or six ($IC_{50} = 0.30 \pm 0.14$ nM) perfect sites had essentially indistinguishable IC_{50} values and were silenced only slightly better than a reporter bearing a single perfect site ($IC_{50} = 0.63 \pm 0.25$ nM). None of the six reporters displayed positive cooperativity with the perfectly matched siRNA, with the Hill coefficients ranging from $n^H = 0.8 \pm 0.2$ (six-site reporter) to 1.2 ± 0.2 (four-site reporter). None of the Hill coefficients were significantly different from $n^H = 1$ (p -value > 0.05 ; Supplemental Fig. 2.S1). Moreover, when the same perfect siRNA was used to silence a reporter bearing three sites separated by 19 nt, the IC_{50} and the Hill coefficient were similar to the mRNA reporter with a single site ($IC_{50} = 0.37 \pm 0.39$ nM, $n^H = 1.0 \pm 0.2$, Fig. 2.4).

In general, silencing by the perfect siRNA was well described by a sigmoidal curve with a Hill coefficient of one, irrespective of the number of sites (Fig. 2.2, *left*). We conclude that, when guided by a perfectly pairing siRNA, each RISC acts independently from other RISCs that bind to nearby target sites.

Multiple, bulged sites act cooperatively

Central internal loops—or “bulges”—typically block siRNA- or miRNA-directed target cleavage, the most potent post-transcriptional silencing mechanism (Holen et al., 2002; Du et al., 2005; Dahlgren et al., 2008; Huang et al., 2009). Consistent with that view, effective silencing by a small RNA that forms a central bulge when paired to its target site required a higher concentration of siRNA than did the corresponding perfect siRNA, even when comparing multiple bulged sites to a single perfect site (Table 2.1 and Fig. 2.2). In fact, we were unable to achieve half-maximal silencing of a *Renilla* luciferase reporter bearing one or two bulged sites even at 20 nM transfected siRNA. For a reporter bearing six bulged sites, the IC_{50} was nearly three times greater than that for perfect sites. Unlike silencing

mediated by perfect sites, silencing via bulged sites showed positive cooperativity, with a Hill coefficient of 2.5 ± 0.8 ($p = <0.0001$ for six sites; Table 2.1).

Seed matches and supportive pairing

Although bulged sites have been shown to effectively silence both reporter mRNAs and endogenous genes (Zeng et al., 2002; Doench et al., 2003; Doench et al., 2003), they rarely occur for natural miRNAs and their endogenous targets (Lewis et al., 2003; Vella et al., 2004; Yekta et al., 2004; Brennecke et al., 2005; Lewis et al., 2005; Grimson et al., 2007). Instead, miRNAs generally pair with the mRNAs they regulate at positions 2–8 of the guide strand, the seed sequence (Lewis et al., 2003; Brennecke et al., 2005; Krek et al., 2005; Lewis et al., 2005). Additional base pairs between the mRNA and miRNA positions 13–16 (Grimson et al., 2007) and target adenosines flanking the seed match sequence at position 1 (t1A) and 9 (t9A) (Lewis et al., 2005) enhance the likelihood that a miRNA will regulate a putative mRNA target.

We tested seed-matched (t1A) sites with supplemental 3' pairing and seed only (t1A) sites for their ability to regulate reporter mRNA bearing one to six siRNA-binding sites. A seed match plus supplemental 3' pairing required far more siRNA to achieve silencing equivalent to the bulged sites (Fig. 2.2). For example, the bulged siRNA regulated the three-site reporter with an $IC_{50} = 1.9 \pm 0.5$ nM, whereas half-maximal silencing for the same reporter with the siRNA pairing with both the seed and supplemental 3' nucleotides could not be achieved even using 20 nM siRNA. With six sites in the reporter, the siRNA with seed plus supplemental 3' pairing achieved an $IC_{50} = 3.7 \pm 1.4$ nM. The seed siRNA was even less potent, reaching half-maximal silencing at an siRNA

concentration of 10 ± 2.4 nM only for the reporter mRNA with six sites; the IC_{50} could not be reliably determined for mRNAs with fewer than six sites. Our data suggest that the intracellular concentration of functional miRISC exceeds the RISC concentration we achieved using transfected, synthetic siRNA duplexes.

Most studies of small RNA-directed silencing report the extent of repression (“fold-repression”) for a single concentration of small RNA. To permit comparison of our data to those in the published literature, we used our data to calculate the observed “fold repression” of the multiple-site reporters by the seed-only siRNA (Supplementary Table 2.S1). Like Grimson et al. (2007) before us, we observe a ≥ 1.4 -fold repression of targets bearing two or more small RNA-binding sites when using the seed-only siRNA. For three or more sites, the observed repression, which ranged from 1.8- to 4.8-fold, was significantly different from that predicted by a multiple, independent sites model ($0.002 \leq p \leq 0.04$; Supplementary Table 2.S1).

Cooperativity requires adjacent target sites

Silencing for bulged sites displayed positive cooperativity for all multiple-site reporter mRNAs for which we could measure the IC_{50} and Hill coefficient. To test if the sites need to be adjacent in order to observe positive cooperativity, we altered the sequence of every other target site in the six-site *Renilla* luciferase mRNA to create a three-site reporter in which 19 nucleotides separate each site targeted by the siRNAs (Fig. 2.4A). Silencing of this expanded three-site reporter mRNA by the bulged siRNA required >15 -fold more siRNA and showed no evidence of cooperativity ($IC_{50} \geq 20$ nM; $n^H = 0.8 \pm 0.1$; Fig. 2.4A) relative to the reporter mRNA in which the three sites were adjacent ($IC_{50} = 1.3 \pm 0.8$ nM; $n^H = 1.6 \pm 0.4$; Fig. 2.4B).

In theory, these data might reflect reduced target-site accessibility in the expanded three-site reporter (Brown et al., 2005; Ameres et al., 2007; Tafer et al., 2008). We view this as unlikely. First, both the adjacent and expanded three-site reporters were silenced equally well by a perfectly pairing siRNA ($IC_{50} = 0.37 \pm 0.39$ nM versus 0.22 ± 0.15 nM; Fig. 2.4C,D). Second, antisense oligonucleotide-directed RNase H cleavage at each of the target sites occurred with similar rates (6.1 , 6.0 and 6.6 nM min⁻¹ for sites 1, 2 and 3 respectively) (Supplemental Fig. 2.S2). Finally, the RNase H cleavage kinetics fit better to a model of independent action compared to a dependent, sequential model for cleavage at each site (Supplemental Fig. 2.S2).

Silencing by the perfectly paired siRNA was non-cooperative for both the expanded ($n^H = 1.0 \pm 0.2$) and original ($n^H = 1.1 \pm 0.1$) three-site reporter mRNAs (Fig. 2.4C,D). The observation that cooperative silencing by a small RNA requires that fewer than 19 nt separate the RISC-binding sites to promote efficient, cooperative silencing suggests that cooperativity springs from interactions between adjacent Argonaute-siRNA complexes, rather than cooperative recruitment of proteins involved in subsequent steps in repressing mRNA expression.

Ago2-RISC binding prevents cooperative silencing

A simple explanation for why Ago2 acts non-cooperatively to silence a multiple-site reporter with a perfect guide is that silencing reflects the endonucleolytic activity unique to mammalian Ago2. To test this idea, we evaluated silencing of the six-site reporter mRNA in three mouse embryonic fibroblast (MEF) cell lines derived from an Ago2 knockout mouse: Ago2^{-/-} MEFs, Ago2^{-/-} MEFs reconstituted with mouse Ago2, and Ago2^{-/-} MEFs reconstituted with a mutant

mouse Ago2 in which aspartic acid 669 was changed to alanine (D669A) (O'Carroll et al., 2007). The D669A mutant Ago2 cannot cleave an RNA target (Liu *et al.*, 2004). In Ago2^{-/-} MEF cells, the perfect siRNA and the bulged siRNA were both cooperative: $n_{perfect}^H = 1.6 \pm 0.4$, $p = 0.03$, and $n_{bulged}^H = 1.8 \pm 0.3$, $p = 0.006$ (Fig. 2.5A). These data suggest that Ago1, Ago3 and Ago4 bind cooperatively to a reporter mRNA bearing multiple small RNA-binding sites, irrespective of the nature of small RNA:target pairing.

As expected, repression mediated by a perfectly pairing siRNA was non-cooperative ($n_{perfect}^H = 1.0 \pm 0.1$) in Ago2^{-/-} MEFs reconstituted with over-expressed Ago2 (Fig. 2.5B, Fig. 2.6). In contrast, silencing directed by the bulged siRNA in the Ago2-reconstituted cells was cooperative ($n_{bulged}^H = 1.5 \pm 0.2$, $p = 0.02$; Fig. 2.5B). To test whether the apparent cooperativity observed in reconstituted Ago2 MEF cells was caused by Ago2 over-expression, we measured silencing in Ago1^{-/-} MEF cells, which express far less Ago2 mRNA and protein than reconstituted Ago2^{-/-} MEF cells (Fig. 5D, Fig. 6, Supplemental Table 2.S2). (All four Argonautes are expressed in the HeLa line we used [Supplemental Fig. 2.S3].) We detected no cooperativity for silencing by the perfect siRNA in the Ago1^{-/-} MEFs ($n_{perfect}^H = 1.1 \pm 0.1$). However, silencing by the bulged siRNA was cooperative ($n_{bulged}^H = 1.7 \pm 0.2$, $p = 0.003$), suggesting that Ago2 is capable of cooperative silencing (Fig. 2.5D).

To test whether Ago3 or Ago4 contributes to the cooperativity that we observed for a bulged siRNA in the Ago1^{-/-} MEFs, we used siRNAs to deplete Ago3 and Ago4 mRNAs before transfecting the reporter plasmids and bulged siRNA. Ago4 mRNA was reduced 50% compared to Ago1^{-/-} MEF cells transfected with a control siRNA. By qRT-PCR, we detected Ago3 mRNA two

threshold cycles after detection of Ago1 mRNA in the Ago1^{-/-} MEF cells, indicating that its expression is probably functionally inconsequential in our analysis (data not shown). The level of Ago3 protein in Ago1^{-/-} MEFs was very low and our attempts to reduce it further by RNAi were unsuccessful (Supplemental Fig. 2.S4A). Under these conditions, n^H for silencing by the bulged siRNA was not significantly different from the null hypothesis (non-cooperative binding), although the Hill coefficients for the bulged and perfect siRNAs were significantly different ($n_{bulged}^H = 1.8 \pm 0.5$ versus $n_{perfect}^H = 1.0 \pm 0.1$, $p = 0.03$; Supplemental Fig. 2.S4B).

As a final test of the idea that non-cooperative silencing reflects target cleavage, we analyzed silencing directed by a perfectly pairing siRNA in Ago2^{-/-} MEFs reconstituted with D669A mutant Ago2. In the cells reconstituted with catalytically inactive Ago2, the single-site reporter was not silenced by the perfect siRNA (Supplemental Fig. 2.S5). Surprisingly, cells reconstituted with catalytically inactive Ago2 exhibited non-cooperative silencing of the six-site reporter by a perfect siRNA ($n_{perfect}^H = 1.1 \pm 0.1$), while silencing by a bulged siRNA displayed positive cooperativity ($n_{bulged}^H = 1.5 \pm 0.3$, $p = 0.04$) (Fig. 2.5C). This unexpected result suggests that target cleavage *per se* is not required for non-cooperative silencing mediated by Ago2. Rather, both the identity of the Argonaute protein and the nature of pairing between the small RNA and its target determine if RISC bound to multiple sites in the 3' UTR of an mRNA can collaborate to generate cooperativity in silencing.

Only Ago2-RISC can repress a reporter with non-adjacent sites

In HeLa cells, the mRNA with the expanded target sites (Fig. 2.4A) was less efficiently silenced by a bulged siRNA than an mRNA in which the three sites

were adjacent (Fig. 2.4B). We propose that RISCs bound to adjacent sites collaborate to achieve efficient silencing. Is Ago2 required to silence an mRNA in which the small RNA binding sites cannot collaborate? We tested silencing of the expanded three-site reporter mRNA by the perfect and bulged siRNAs in the Ago2^{-/-} MEFs. Silencing of the expanded three-site reporter was completely dependent on Ago2: little or no silencing was observed in the Ago2^{-/-} MEFs for either type of small RNA:target pairing (Fig. 2.7C,D). In contrast, both perfect and bulged siRNAs cooperatively silenced the reporter bearing three adjacent small RNA-binding sites in the Ago2^{-/-} MEFs (Fig. 2.7A,B). Notably, in the absence of Ago2, silencing by the perfect siRNA of the reporter containing three adjacent sites was highly cooperative ($n_{perfect}^H = 2.1 \pm 0.3$, $p = 0.007$). We conclude that for widely spaced sites, only Ago2 can silence at the intracellular RISC concentration achieved at the highest amount of siRNA transfected, likely because in the absence of cooperativity, the intracellular concentration of Ago1-, Ago3-, and Ago4-RISC is less than the K_D for target binding for these Argonaute:siRNA complexes.

Reconstituting the Ago2^{-/-} MEFs with either wild-type or catalytically inactive mouse Ago2 rescued silencing of the expanded three-site reporter (Fig. 2.7E–J). Silencing showed no significant cooperativity for the perfect siRNA in MEFs reconstituted with wild-type ($n_{perfect}^H = 1.1 \pm 0.1$) or catalytically inactive Ago2 ($n_{perfect}^H = 1.2 \pm 0.2$). Silencing by the bulged siRNA was cooperative for both the wild-type Ago2-reconstituted ($n_{bulged}^H = 1.6 \pm 0.1$, $p = 0.02$; Fig. 2.7F), and the catalytically inactive Ago2 MEFs ($n_{bulged}^H = 1.3 \pm 0.2$, $p = 0.03$; Fig. 2.7H). Intriguingly, in the absence of Ago1, silencing of the expanded three-site reporter by the bulged siRNA was highly cooperative ($n_{bulged}^H = 2.5 \pm 0.2$, $p = 0.006$; Fig. 2.7J).

Discussion

In our assays, Ago2 non-cooperatively silenced mRNAs bearing multiple, perfectly complementary small RNA-binding sites, even when its endoribonuclease activity was inactivated by mutation. This finding is surprising, because we and others have assumed that endonucleolytic cleavage by Ago2 explained its lack of cooperativity in silencing when guided by a perfectly pairing siRNA. Clearly, a more complex explanation is warranted. We suggest that the Ago2 conformation associated with perfect small RNA:target pairing precludes protein:protein interactions, causing both nearby and adjacent binding sites to act independently. Alternatively, Ago2 protein, when guided by a small RNA that pairs extensively with its target mRNA might be bound by proteins that prevent its association with factors promoting cooperativity.

Silencing via multiple small RNA-binding sites is likely always cooperative for Ago1, Ago3 or Ago4, irrespective of the type of pairing between the small RNA and its target. We suggest that these non-catalytic Argonaute proteins adopt a single conformation when bound by different modes to their mRNA targets or that the conformations produced by both perfect and bulged small RNAs are compatible with protein:protein interactions between adjacent RISC molecules. Intrinsic differences between Ago2 and Ago1, Ago3, and Ago4 may dictate the combination of Ago proteins capable of cooperative silencing.

Cooperativity versus statistical effects for closely apposed target sites

We find that multiple, imperfect binding sites need to be surprisingly close in order to mediate cooperative silencing. Our data fundamentally agree with

previous reports, but differ quantitatively in the precise inter-site distance that supports cooperativity (Grimson et al., 2007; Bartel, 2009). We note that the precise inter-site distance that supports cooperative interactions may reflect the intracellular concentrations of Argonaute proteins and associated factors, as well as the local structure or sequence of the mRNA target. When testing silencing of a two-site reporter, Grimson et al. observed that a seed-matched siRNA transfected at 25 nM cooperatively silenced a reporter bearing two 3' UTR target sites spaced 8–40 nt apart (counting the number of nucleotides between the 3' end of the first site and the 5' end of the second site); expanding the distance to 56 nt disrupted cooperative silencing (Grimson et al., 2007). We note that these authors defined cooperativity as an excess of silencing when the observed repression for a two-site reporter was compared to the product of the repression observed for each site acting alone. Enhanced silencing measured in this way may correspond to true cooperativity or may simply reflect the statistical effects* of multiple independent sites. Our Hill analyses distinguish between these two possibilities and suggest that RISCs bound to adjacent sites cooperate to confer greater silencing than would be expected from statistical effects alone.

*Statistical effects result from the simultaneous occupancy of multiple, independent binding sites of similar affinities even in the absence of cooperativity (Cantor and Schimmel, 1980). This effect, with increasing site occupancy causes a steep threshold response that appears non-additive. In contrast, cooperativity results in the concerted loading of sites at a lower overall concentration of ligand (e.g., miRISC) and a sharp dose-response to a relatively small increase in ligand concentration.

Supplemental 3' pairing reduces the amount of small RNA required to repress an mRNA

The amount of siRNA required to repress an mRNA target is determined by the number of small RNA-binding sites, the spacing of the sites, and the extent of complementarity beyond the seed sequence at each site. Compared to an mRNA in which the siRNA seed sequence alone paired with the small RNA-binding sites, an mRNA in which seed-pairing was supplemented with additional 3' base pairs required slightly less siRNA to achieve comparable repression, particularly for multi-site target RNAs (Table 2.1, Fig. 2.2). These data reinforce the view that has emerged from previous computational analyses of miRNA target binding: 3' supplemental pairing provides a small but measurable increase in the affinity of a small RNA for its target (Grimson et al., 2007).

Nonetheless, it is striking how much more siRNA is needed to regulate a target containing small RNA-binding sites with a seed-match only or a seed-match plus supplementary pairing, compared to a target containing sites that fully pair to the small RNA but for a central bulge. While our current data do not permit direct estimation of the binding affinity of a small RNAs for a reporter mRNA within a cell, they suggest that one explanation for the remarkably high intracellular abundance of some miRNAs is that most miRNAs bind weakly to the mRNAs they regulate.

Materials and Methods

Plasmids

Renilla luciferase vector pRL-TK (Promega, Madison, WI, USA) containing target sites for CXCR4 and a modified linker sequence (Doench et al., 2003) was mutated from TAG to CTC (lower case letters in the oligonucleotides) at nucleotides 387–389 of the *Renilla* luciferase open reading frame to generate mismatches with seed positions 5, 6, and 7 of the siRNA guide strand by PCR-directed mutagenesis using DNA oligonucleotides: 5'-CTT GTT TGG CAT TTC ATT ACt ccT ATG AGC ATC AAG ATA AGA TC-3' (sense), 5'-GAT CTT ATC TTG ATG CTC ATA Gga gTA ATG AAA TGC CAA ACA AG-3' (antisense). Mutagenesis was confirmed by sequencing, and then 5' phosphorylated oligos containing the target sites and pairing to create appropriate ends were cloned into the *XbaI* and *ApeI* sites of the mutant pRL-TK. Supplemental Table S3 lists the sequences of the DNA oligonucleotides used to construct target sites. psiCheck2 (Promega) reporters were constructed by digesting psiCheck2 with *NheI* and *NotI* and inserting the 3' UTR target site-containing *NheI*–*NotI* fragment from the mutant pRL-TK vectors. Table 2 lists the oligonucleotide sequences used to generate plasmid reporters. Dual Reporter Luciferase assays were conducted using Dual Luciferase Assay Reagents (Promega) in a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA) according to the manufacturers' directions.

Cell Culture and Transfection

HeLa CCL2 cell cultures were maintained at 37°C and 5% CO₂ in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated FBS (Invitrogen) and 50 U/ml penicillin and streptomycin (Invitrogen). MEF cells were cultured in DMEM (Invitrogen) supplemented with 15% heat inactivated FBS (Invitrogen), 50 U/ml penicillin and streptomycin (Invitrogen), 0.1 mM NEAA (Invitrogen), 2 mM glutamine (Invitrogen). Cells were seeded at a density of 0.1×10^6 cells per well in 24 well plates in DMEM (Invitrogen) containing 10% heat inactivated FBS (Invitrogen). Twenty-four hours later, cells were washed 3 times in 500 µl PBS (Invitrogen), and then 400 µl DMEM with serum was added to each well. Renilla luciferase plasmid (0.025 mg), firefly luciferase plasmid pGL3 (0.025 mg), and 20 nM siRNA were mixed with 99 µl DMEM and 1 µl DharmaFECT Duo (Dharmacon, Lafayette, CO, USA) per well. A control siRNA (CXCR4) was used to equalize the total amount of siRNA in each transfection. Cells are incubated with 0.5 ml final volume of DMEM plus serum containing 100 µl of transfection reagent nucleic acid mixture for 24 h.

siRNA Annealing

Single-stranded guide and passenger siRNA strands (Supplemental Table S4; Dharmacon) were annealed by incubating 10 µM each strand in 500 µl annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C, followed by 1 h at 37°C.

Luciferase Assays

Cells were washed once in 500 µl PBS and lysed in 100 µl of Passive Lysis Buffer (Promega) at room temperature for 20 min in 24 well plates. For each well 10 µl

lysate was read in triplicate using dual luciferase reagents (Promega) in a Turner Biosystems luminometer controlled by Veritas software (Turner). *Renilla* luciferase activity for each concentration of transfected siRNA was normalized to the corresponding firefly luciferase activity.

Data Analysis

The individual biological replicates for normalized *Renilla* luciferase activity versus siRNA concentration was fit using Igor Pro 6.10 (Lake Oswego, Oregon, USA) to the Hill equation to determine IC_{50} and n^H . Fitting was weighted using the standard error of each mean value. Throughout this study, the standard deviation is reported for mean IC_{50} and n^H values. The Hill coefficients from each replicate were subjected to the Student's t-test to determine p -values at 95% confidence using GraphPad Prism (La Jolla, CA, USA).

Statistical Testing

To test if the individual Hill coefficients from the replicates of each experiment followed a Gaussian distribution, data were subjected to the Kolmogorov–Smirnov, D'Agostino & Pearson omnibus and Shapiro-Wilk normality tests. By all three tests, all Hill coefficient data was normally distributed. The p -values at 95% confidence were calculated using an unpaired, one sample, two-tailed Student's t-test (GraphPad Prism; La Jolla, CA, USA) to test whether n^H was significantly different from the null hypothesis that $n^H = 1$ (i.e., non-cooperative). An unpaired, two-tailed Student's t-test with Welch's correction at 95% confidence, which does not assume equal variances, was used to test the significance of differences in n^H a perfect and bulged siRNA. For non-normally

distributed fold-repression data, we used the non-parametric Wilcoxon Signed-Rank test at 95% confidence to determine *p*-values.

Western Blotting

Forty micrograms cell lysate in cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% v/v NP-40) containing Complete, mini, EDTA-free protease inhibitor (Roche, Indianapolis, IN, USA) were separated by 4–20% HEPES-SDS-PAGE and transferred at 4°C in Tris-bicine buffer to nitrocellulose membrane overnight at 30 V. Membranes were blocked in 5% w/v milk-TBST (100 mM Tris Cl pH 7.5, 150 mM NaCl, 0.1% TWEEN 20) for 1 h and incubated overnight at 4°C with primary antibody diluted in 3% milk-TBST. Rabbit anti-human and mouse Ago2 antibody (Cell Signaling Technologies, Danvers, MA, USA) (Li et al., 2010) or rabbit anti-human and mouse Ago1 antibody (MBL International, Woburn, MA, USA) was diluted 1:1,000 and rabbit anti-actin antibody (Bethyl Laboratories, Montgomery, TX, USA) was diluted 1:5,000. After three 5 min washes in TBST the membranes were incubated 1 h with secondary goat anti-rabbit HRP-conjugated antibody (GE Healthcare, Piscataway, NJ, USA) diluted 1:10,000. After five, 5 min washes in TBST, the membranes were incubated for 5 min in Super Signal West-Dura Extended Duration Substrate (Pierce, Rockford, IL, USA). Chemiluminescent signal was recorded using an LAS-4000 (Fuji, Tokyo, Japan).

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Figures

Broderick et al., Figure 1

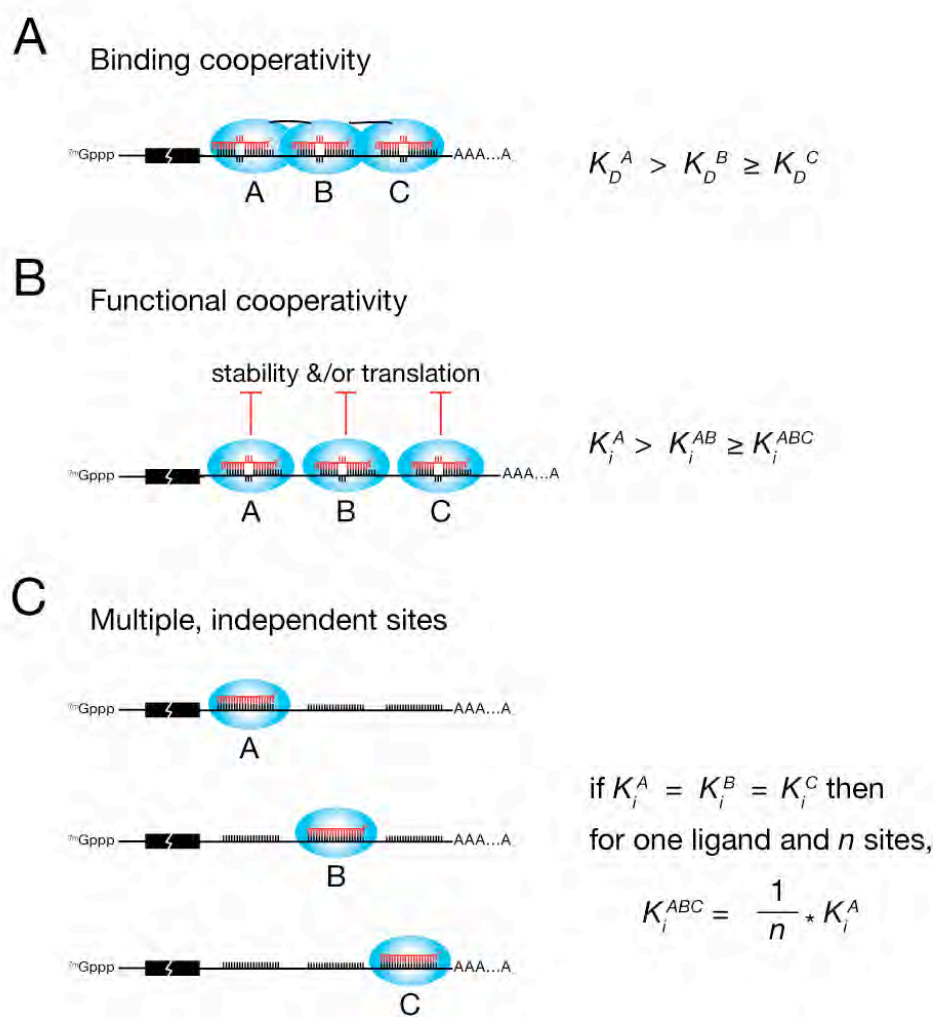


Figure 2.1. Models for cooperativity in silencing.

FIGURE 2.1. Potential sources of cooperativity in the repression of a target mRNA by the small RNA-directed Argonaute complex, RISC. (A) Cooperative binding. RISC binding at multiple target sites increases site occupancy by mutually stabilizing subsequent binding of RISCs. (B) Cooperative function. RISC binding at multiple sites may increase the likelihood that repressive factors, such as nucleases, are recruited to the mRNA. (C) Multiple independent sites. Each RISC functions independently, so the multiple sites increase the probability of repression, but do not influence each other.

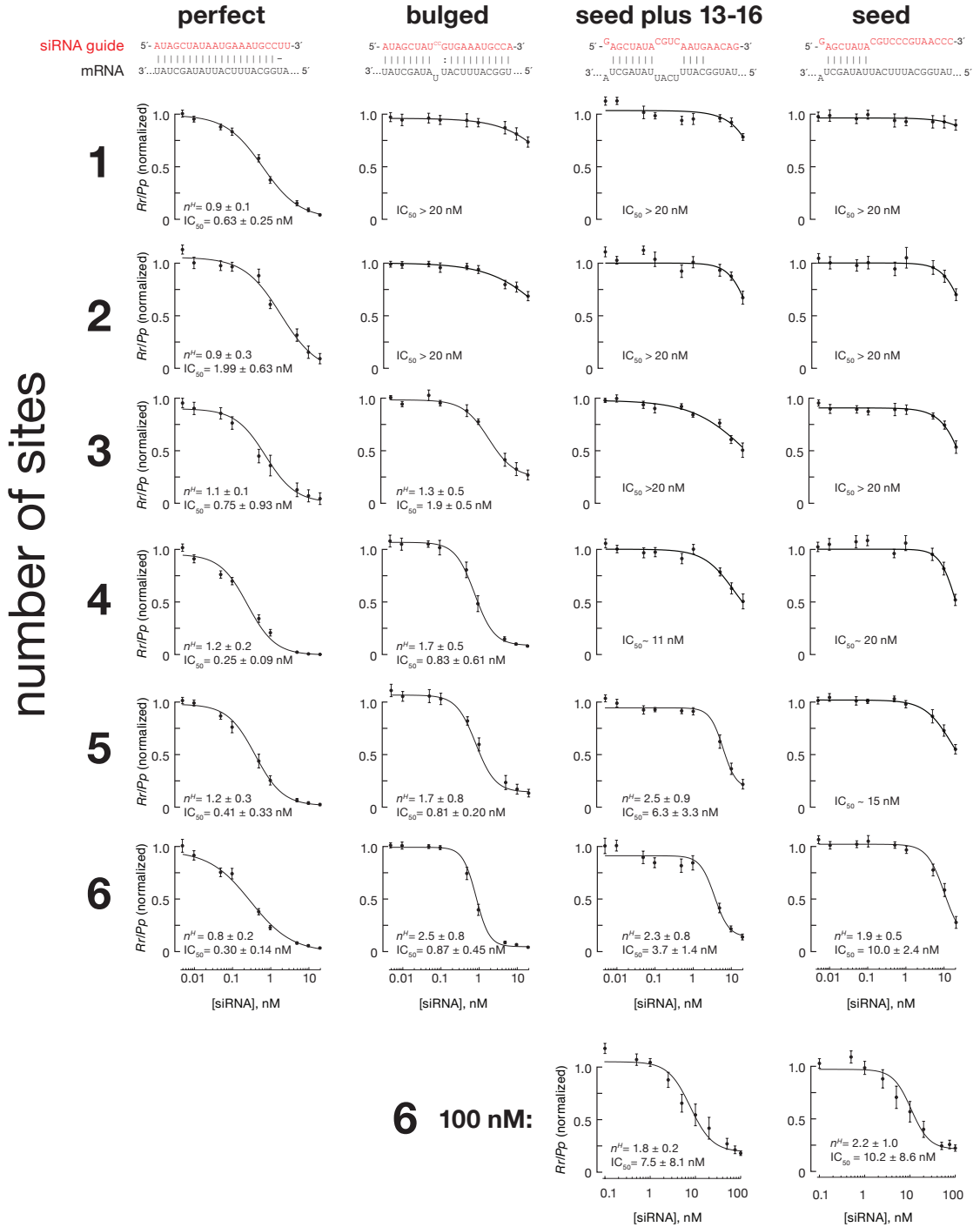
Figure 2.2: Silencing of *Rr* luciferase mRNA by siRNA pairing to different extents at multiple sites.

FIGURE 2.2. Extent of pairing and target site number determine both efficacy and cooperativity in small RNA-directed silencing in HeLa cells. Silencing of a *Renilla* luciferase reporter mRNA bearing 1–6 target sites in its 3′ UTR, relative to a firefly luciferase internal control, was determined at different siRNA concentrations. Pairing between the siRNA guide (red) to the 3′ UTR sites (black) is shown at top. IC_{50} and Hill coefficient (n^H) were calculated for each dose-response curve. Throughout this study, values are reported as mean \pm standard deviation for IC_{50} values and n^H ; error bars indicate standard error for ≥ 12 biological replicates. The curves correspond to the concentration-dependence of silencing expected for the mean IC_{50} and n^H values.

Broderick et al., Figure 3

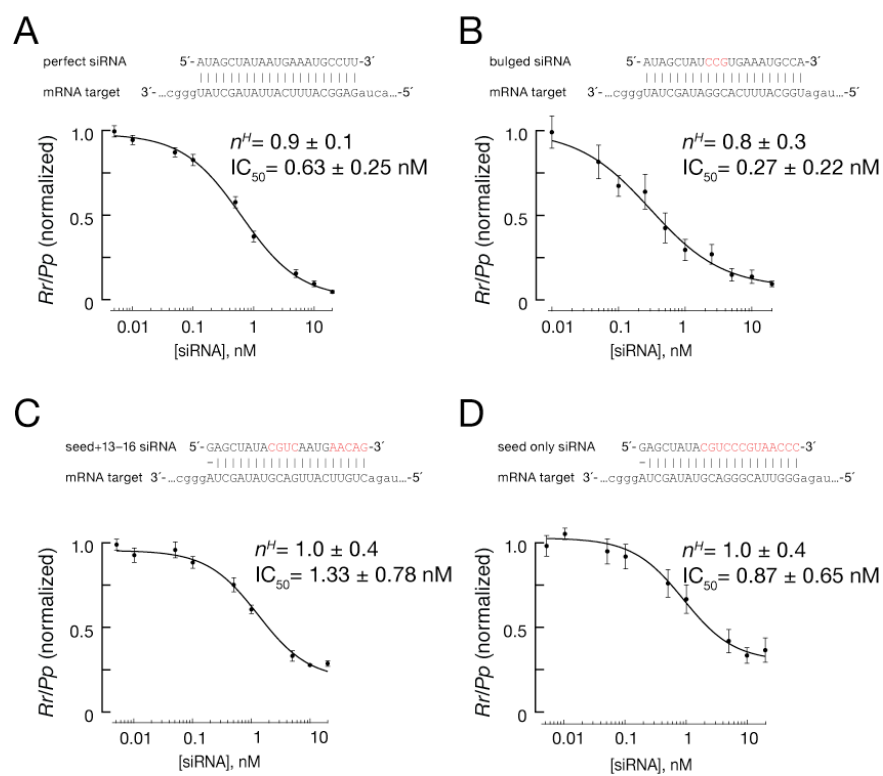


Figure 2.3: Validation of siRNAs.

FIGURE 2.3. siRNA validation in HeLa cells. Each siRNA was functional in silencing a reporter containing a single perfect target site. (A) Perfect siRNA. (B) Bulged siRNA. (C) siRNA with seed plus supplementary 3' pairing (nts 13–16). (D) siRNA with only seed pairing. The curves correspond to the concentration-dependence of silencing expected for the mean IC_{50} and n^H values (\pm standard deviation) calculated from 3 independent trials.

Broderick et al., Figure 4

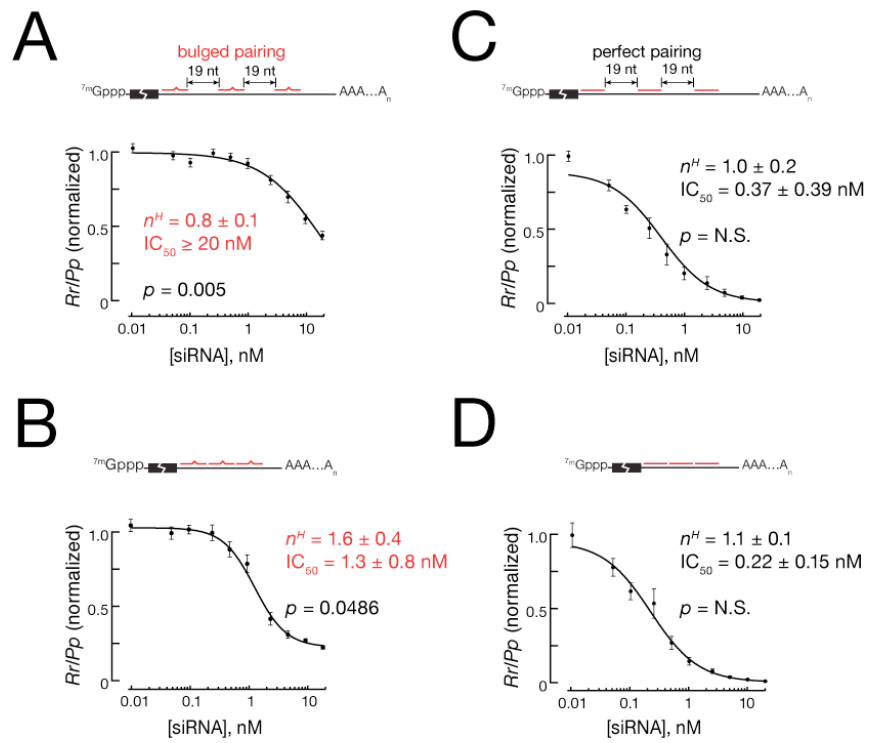


Figure 2.4: Silencing of *Rr* luciferase mRNA by siRNA pairing to three adjacent or non-adjacent sites.

FIGURE 2.4. Cooperative binding of RISC requires adjacent target sites in HeLa cells. Three sites spaced 19 nt apart (*A*) require more siRNA to achieve half-maximal silencing, compared to three adjacent sites (*B*), and act non-cooperatively. In contrast, a perfectly matched siRNA silences a three-site reporter with sites separated by 19 nt (*C*) or a reporter with three adjacent sites (*D*) with equal efficacy and without detectable cooperativity. The three adjacent-site experiments in this figure were performed independently from those in Fig. 2. A one sample, two-tailed Student's t-test was used to calculate the *p*-values at 95% confidence for the Hill coefficients to determine if n^H was significantly different from the null hypothesis: $n^H = 1$ (i.e., non-cooperative).

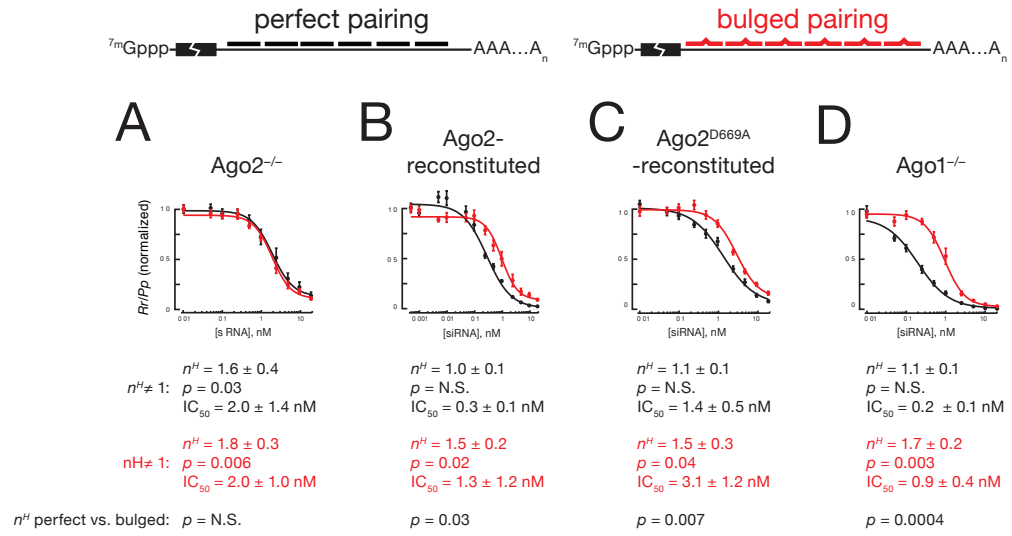


Figure 2.5: Silencing of *Rr* luciferase by a perfect or bulged pairing to six sites in MEFs.

FIGURE 2.5. Silencing in Ago2^{-/-} MEFs or Ago2^{-/-} MEFs reconstituted with mouse Ago2 or catalytically inactive, mutant Ago2^{D669A} or Ago1^{-/-} MEFs. (A) In the absence of Ago2, silencing by a perfect site ($n^H = 1.6 \pm 0.4$, $p = 0.03$) is equally cooperative as a bulged site ($n^H = 1.8 \pm 0.3$, $p = 0.006$). (B) Mouse Ago2 expression restored non-cooperative silencing by the perfect siRNA (black; $n^H = 1.0 \pm 0.1$); silencing directed by a bulged siRNA became less cooperative (red; $n^H = 1.5 \pm 0.2$, $p = 0.02$) than in the absence of Ago2 (red in (A); $n^H = 1.8 \pm 0.3$). (C) Catalytically inactive mouse Ago2^{D669A} likewise restored non-cooperative silencing by a perfect siRNA (black; $n^H = 1.1 \pm 0.1$), but silencing by the bulged siRNA (red; $n^H = 1.5 \pm 0.3$, $p = 0.04$), was cooperative. (D) In the absence of Ago1, silencing by the perfect siRNA was not cooperative (black; $n^H = 1.1 \pm 0.1$) but silencing by the bulged siRNA was cooperative (red; $n^H = 1.7 \pm 0.2$, $p = 0.003$). A one sample, two-tailed Student's t-test was used to calculate the p -values at 95% confidence for the Hill coefficients to determine if n^H was significantly different from the null hypothesis: $n^H = 1$ (i.e., non-cooperative).

Broderick et al., Figure 6

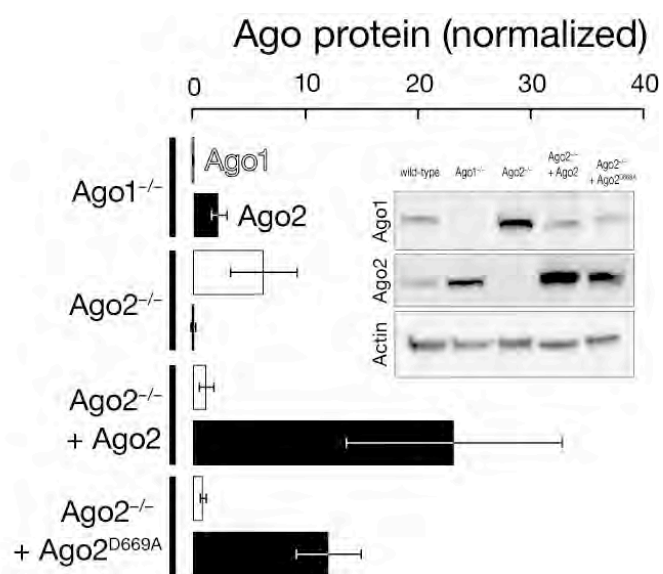


Figure 2.6: Argonaute protein levels in MEFs cells.

FIGURE 2.6. Ago1 and Ago2 protein levels in MEF cells. Ago2 was detected by Western blotting using a rabbit anti-Ago1 antibody that recognizes both mouse and human Ago1 and a rabbit anti-Ago2 antibody that recognizes both mouse and human Ago2. Ago protein levels were normalized to actin, and the level of Ago protein in wild-type MEFs was set to 1. Data are mean \pm standard deviation for three trials. Inset shows representative data from a single experiment.

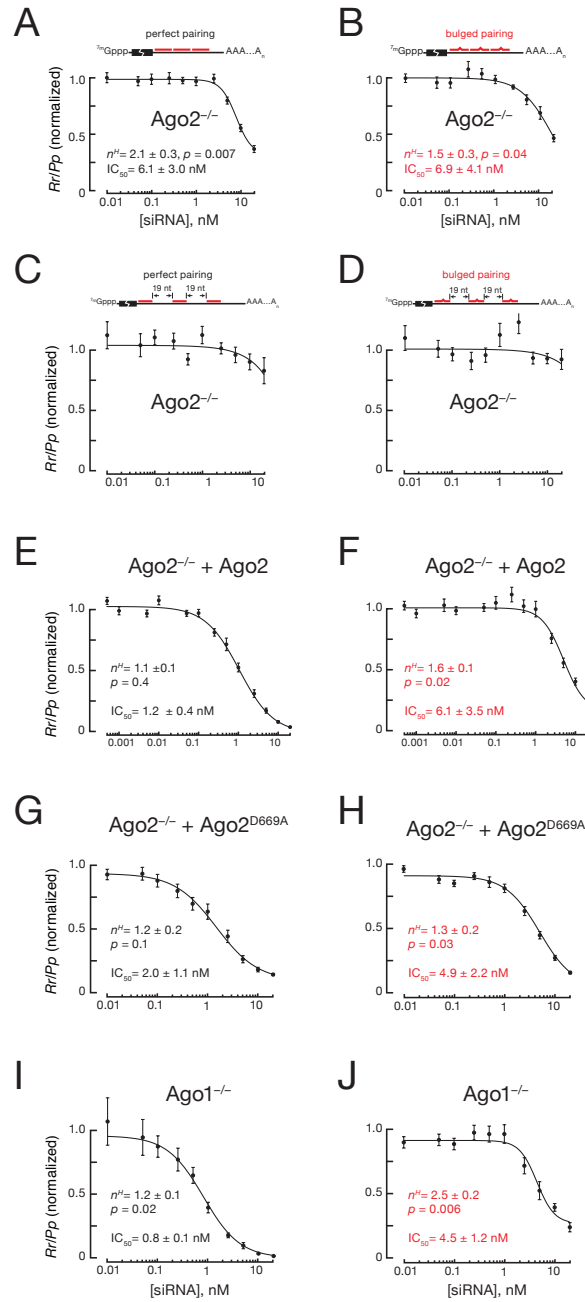


Figure 2.7: Silencing of *Rr* luciferase mRNA at three non-adjacent sites requires Ago2.

FIGURE 2.7. In the absence of Ago2, effective silencing requires adjacent sites. (A,B) Both perfect ($n^H = 2.1 \pm 0.3$, $p = 0.007$) and bulged ($n^H = 1.5 \pm 0.3$, $p = 0.04$) adjacent sites were silenced cooperatively in the absence of Ago2. (C,D) In Ago2^{-/-} MEFs, three target sites spaced 19 nt apart did not silence the reporter. (E,F) Expressing mouse Ago2 in the Ago2^{-/-} MEFs allowed three distant sites to silence the reporter. (G,H) Expressing catalytically inactive, mutant Ago2^{D669A} also allowed three distant sites to silence the reporter. (I,J) In the Ago1^{-/-} MEFs the three distant sites silenced the reporter. A one sample, two-tailed Student's t-test was used to calculate the p -values at 95% confidence for the Hill coefficients to determine if n^H was significantly different from the null hypothesis: $n^H = 1$ (i.e., non-cooperative).

Table 2.1. Concentration-dependence and cooperativity for distinct siRNA:target pairing modes using reporters bearing one to six siRNA-binding sites.

N.D., not determined. IC₅₀ values (nM) and Hill coefficients (nH) of the fitted curves are reported as mean values \pm standard deviation for the IC₅₀ and Hill coefficients for at least 12 trials.

Number of sites	Perfect		Bulged		Seed plus 13–16		Seed only	
	IC ₅₀	<i>n</i> ^H	IC ₅₀	<i>n</i> ^H	IC ₅₀	<i>n</i> ^H	IC ₅₀	<i>n</i> ^H
1	0.63 \pm 0.25	0.9 \pm 0.1	>20	N.D.	>20	N.D.	>20	N.D.
2	1.99 \pm 0.63	0.9 \pm 0.2	>20	N.D.	>20	N.D.	>20	N.D.
3	0.75 \pm 0.93	1.1 \pm 0.1	1.9 \pm 0.5	1.0 \pm 0.5	>20	N.D.	>20	N.D.
4	0.25 \pm 0.09	1.2 \pm 0.2	0.83 \pm 0.61	1.7 \pm 0.5	~11	N.D.	~20	N.D.
5	0.41 \pm 0.33	1.2 \pm 0.3	0.81 \pm 0.20	1.7 \pm 0.8	6.3 \pm 3.3	2.5 \pm 0.9	~15	N.D.
6	0.30 \pm 0.14	0.8 \pm 0.2	0.87 \pm 0.45	2.5 \pm 0.8	3.7 \pm 1.4	2.3 \pm 0.8	10.0 \pm 2.4	1.9 \pm 0.5

Argonaute protein identity and pairing geometry determine cooperativity in mammalian RNA silencing

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Supplemental Methods

Quantitative RT-PCR

Cells were harvested and total RNA purified using Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol, then 10 μ g RNA was treated with 20 units of Turbo DNase (Ambion, Austin, TX, USA) in 100 μ l at 37°C for 20 min. RNA was extracted with 100 μ l acid phenol (EMD Chemicals, Gibbstown, NJ, USA) and precipitated using 0.1 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes absolute ethanol. Precipitated RNA was washed with 900 μ l 70% ethanol and then dissolved in 50 μ l water. RNA purity and concentration was determined by absorbance. RNA (0.1 μ g) was reversed transcribed using Superscript III Reverse Transcriptase (Invitrogen) with oligo(dT) primers (Invitrogen) according to manufacturer's protocol. A parallel reaction containing water instead of reverse transcriptase provided a negative control. After reverse transcription, quantitative PCR was performed using 1 μ l of the cDNA reaction. PCR reactions (25 μ l) used SYBR green PCR mix (Bio-Rad Laboratories, Hercules, CA, USA) and specific primers (Supplemental Table S5). PCR conditions were: 94°C, 4 min, then 40 cycles of 94°C, 15 sec; 60°C, 15 sec; 72°C, 30 sec; 72°C, 7 min using an Opticon 2 instrument (Bio-Rad). PCR data was analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) and DART PCR (Pfaffl, 2001; Peirson et al.,

2003). Cycle number data was analyzed by normalizing the cycle threshold for Argonaute mRNA to that of GAPDH. For the analysis of mRNA in MEF cells, the levels of Argonaute mRNA were normalized first to the cycle threshold for GAPDH, then to the cycle threshold for Argonaute mRNA from wild-type MEF cells.

RNAi Transfections

Cells were seeded at a density of 3×10^6 cells in 100 mm plates in DMEM (Invitrogen) containing 15% heat inactivated fetal bovine serum (FBS; Invitrogen). Twenty-four hours later, cells were washed 3 times in 5 ml PBS (Invitrogen), and then 8.8 ml DMEM containing 10% v/v FBS was added to each well. Each siRNA (20 nM) was transfected in 1.2 ml DMEM and 20 μ l DharmaFECT 1 transfection reagent (Dharmacon; Table S3). Cells were incubated with 10 ml DMEM containing 10% FBS and 1.2 ml transfection reagent/nucleic acid mixture for 16 h. Next, the cells were washed 3 times with PBS, then grown in DMEM supplemented with 15% FBS, 50 U/ml penicillin and streptomycin (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 2 mM glutamine (Invitrogen). Twenty-four hours later, the cells were seeded into 24-well plates at 0.1×10^6 cells per well.

Western Blotting

Western blotting was as described in the main text except 40 μ g each protein lysate was resolved by 5% SDS-PAGE and transferred to nitrocellulose membrane in Tris-glycine buffer overnight at 4°C at 30V. Mouse anti-human/anti-mouse Ago3 antibody (Active Motif, Carlsbad, CA, USA) (Azuma-Mukai *et al.*, 2008), diluted 1:1000, was used to detect Ago3.

RNase H Cleavage

Target accessibility was measured by the ability of an antisense oligonucleotide (5'-mGmCmU mAdTdA dAdTdG dAdAdA dTdGdC dCmCmG mCmG-3' [m, 2'-O-methyl]) to direct RNase H cleavage at the target sites of the synthetic RNA target containing the expanded three target sites (generated by in vitro transcription and gel purified). The purified RNA was capped with guanylyl transferase, S-adenosylmethionine, and [α - 32 P] guanosine triphosphate and then gel purified. Cleavage reactions were assembled on ice in 60 mM Tris-HCl, pH 7.8, 60 mM KCl, 2.5 mM MgCl₂ and contained 700 nM synthetic RNA target, 7 μ M antisense oligonucleotide, and 50 U/ml f.c. RNase H (New England Biolabs, Ipswich, MA, USA). The reaction was incubated at 37°C; cleavage was stopped by adding 8M urea, 25 mM EDTA, pH 8.0, 0.025% w/v xylene cyanol FF, 0.025% w/v bromophenol blue. RNA was resolved by urea-denaturing, polyacrylamide gel electrophoresis and analyzed using a FLA-9000 phosphorimager (Fuji) at 50 μ m resolution.

Supplemental References

- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. *Methods* **25**: 402-408.
- Peirson SN, Butler JN, Foster RG. 2003. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res* **31**: e73.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45.

Supplemental Table 2.S1. Observed fold-repression of 1–6 site reporters for seed only and seed plus 3' supplemental pairing siRNAs transfected at 20 nM.

Expected fold repression was calculated as the repression for one site, to the power of the number of sites in the reporter (n), 1.13n for seed and 1.32n for seed plus 3' supplemental. Wilcoxon Signed-Rank test was used to determine the p-value for significance of the observed fold-repression compared to that expected as calculated from the reporter containing a single target site.

		Fold-repression			
		Number of sites	Observed (median; minimum–maximum)	Expected	<i>p</i> -value (Wilcoxon rank test)
Seed only siRNA		1	1.1	1.1	
		2	1.4; 1.0–2.8	1.2	N.S.
		3	1.8; 1.3–5.0	1.5	0.03
		4	2.2; 1.3–7.7	1.6	0.003
		5	4.8; 1.1–14	1.9	0.04
		6	4.1; 1.4–18	2.1	0.002
Seed plus 3' supplemental pairing		1	1.3	1.3	
		2	1.5; 0.7–3.0	1.7	N.S.
		3	2.5; 1.1–5.7	2.3	N.S.
		4	2.0; 1.1–5.6	3.0	N.S.
		5	4.8; 1.7–21	4.0	N.S.
		6	8.4; 3.5–27	5.3	N.S.

Supplemental Table 2.S2. Change in Argonaute mRNA abundance, compared to wild-type MEF cells; standard deviations are for three technical replicates.

MEF	Argonaute	mean \pm standard
Genotype	mRNA	deviation
<i>Ago1</i> ^{-/-}	Ago1	0.31 \pm 0.04
	Ago2	0.73 \pm 0.06
	Ago3	0.61 \pm 0.05
	Ago4	0.63 \pm 0.11
<i>Ago2</i> ^{-/-}	Ago1	0.55 \pm 0.07
	Ago2	0.024 \pm 0.00
	Ago3	0.20 \pm 0.02
	Ago4	0.24 \pm 0.09
<i>Ago2</i> ^{-/-} + <i>Ago2</i>	Ago1	0.63 \pm 0.05
	Ago2	36 \pm 3
	Ago3	0.26 \pm 0.05
	Ago4	0.09 \pm 0.03
<i>Ago2</i> ^{-/-} + <i>Ago2</i> ^{D669A}	Ago1	2.2 \pm 0.3
	Ago2	17 \pm 1
	Ago3	0.61 \pm 0.05
	Ago4	0.33 \pm 0.05

Supplemental Table 2.S3. Oligonucleotides used to produce reporter plasmids.

Target sites	Sequences (sense, antisense)
1	5'-CTA GAG GCA TTT CAT TAT AGC TAT GGC CC-3', 5'- CAT AGC TAT AAT GAA ATG CCT-3'
2	5'-CTA GAG GCA TTT CAT TAT AGC TAT GGC ATT TCA TTA TAG CTA TGG GCC-3', 5'- CAT AGC TAT AAT GAA ATG CCA TAG CTA TAA TGA AAT GCC T-3'
3	5'-CTA GAG GCA TTT CAT TAT AGC TAT GGC ATT TCA TTA TAG CTA TGG CAT TTC ATT ATA GCT ATG GGC C-3', 5'- CAT AGC TAT AAT GAA ATG CCA TAG CTA TAA TGA AAT GCC ATA GCT ATA ATG AAA TGC CT-3'
4	5'-CTA GAG GCA TTT CAT TAT AGC TAT GGC ATT TCA TTA TAG CTA TGG CAT TTC ATT ATA GCT ATG GCA TTT CAT TAT AGC TAT GGC CC-3', 5'- CAT AGC TAT AAT GAA ATG CCA TAG CTA TAA TGA AAT GCC ATA GCT ATA ATG AAA TGC CAT AGC TAT AAT GAA ATG CCT-3'
5	5'-CTA GAG GCA TTT CAT TAT AGC TAT GGC ATT TCA TTA TAG CTA TGG CAT TTC ATT ATA GCT ATG GCA TTT CAT TAT AGC TAT GGC ATT TCA TTA TAG CTA TGG GCC-3', 5'- ATA GCT ATA ATG AAA TGC CAT AGC TAT AAT GAA ATG CCA TAG CTA TAA TGA AAT GCC ATA GCT ATA ATG AAA TGC CAT AGC TAT AAT GAA ATG CCT-3'
6	5'-CTA GAG GCA TTT CAT TAT AGC TAT GGC ATT TCA TTA TAG CTA TGG CAT TTC ATT ATA GCT ATG GCA TTT CAT TAT AGC TAT GGC ATT TCA TTA TAG CTA TGG CAT TTC ATT ATA GCT ATG GGC C-3', 5'- CAT AGC TAT AAT GAA ATG CCA TAG CTA TAA TGA AAT GCC ATA GCT ATA ATG AAA TGC CAT AGC TAT AAT GAA ATG CCA TAG CTA TAA TGA AAT GCC ATA GCT ATA ATG AAA TGC CT-3'
3-site expanded	5'-CTA GAG GCA TTT CAT TAT AGC TAT AAA CGG GAC GGC GCA CGC GGG CAT TTC ATT ATA GCT ATA AAC GGG ACG GCG CAC GCG GGC ATT TCA TTA TAG CTA TGG GCC-3', 5'-CAT AGC TAT AAT GAA ATG CCC GCG TGC GCC GTC CCG TTT ATA GCT ATA ATG AAA TGC CCG CGT GCG CCG TCC CGT TTA TAG CTA TAA TGA AAT GCC T-3

Perfect target for bulged siRNA	5' -CTAGATGGCATTTCACGGATAGCTATGGGCC-3' , 5' -CATAGCTATCCGTAAGATGCCAT-3'
Perfect target for seed plus 13-16 siRNA	5' -CTAGACTGTTCATTGACGTATAGCTAGGGCC-3' , 5' -CTAGCTATACGTCAATGAACAGT-3'
Perfect target for seed siRNA	5' -CTAGAGGGTTACGGGACGTATAGCTAGGGCC-3' , 5' -CTAGCTATACGTCCCGTAACCCT-3'

Supplemental Table 2.S4. Synthetic siRNAs used in this study.

siRNA	Guide strand	Passenger strand
perfect	5' - AUAGCUAUA AUGAAUGCCUU - 3'	5' - GGCAUUUCAUU AUAGCUAGUU - 3'
bulged (10-11)	5' - AUAGCUAUccGUGAA AUGCCA - 3'	5' - GCAUUUCACGGAUAGCUAGUU - 3'
seed plus 13-16	5' - GAGCUAUACGUCAAUGAACAG - 3'	5' - GUUCAUUGACGU AUAGCUAUU - 3'
seed	5' - GAGCUAUACGUCCCGUAACCC - 3'	5' - GUUACGGGACGU AUAGCUAUU - 3'
CXCR4	5' - GUGUUAGCUUUGUGAAAACUU - 3'	5' - GUUUUCACAAAGCUAACAACG - 3'

Supplemental Table 2.S5. Synthetic siRNAs used to deplete Ago3 and Ago4; PCR primers used in this study.

siRNA	Guide strand	Passenger strand
Ago3	5' -UUUGCAAAGAUAGUUGUGCUU-3'	5' -GCACAACUAUCUUUGCAACUU-3'
Ago4	5' -UAAGGAAGCAUCCUGGUUCUU-3'	5' -GAACCAGGAUGCUUCCUUCUU-3'
control	5' -GUGUUAGCUUUGUGAAAACUU-3'	5' -GUUUUCACAAAGCUAACAACG-3'

PCR Oligos	Forward primer	Reverse primer
Ago1	5' -GTACGGCGGCCGGAACCGGCCATTG-3'	5' -CAAGGTTGACCTTGATAGGCATC-3'
Ago2	5' -GGAGGTCTGTACATTGTGGCAGGAC-3'	5' -GCCAGTCACGTCTGTCATCTCATCT-3'
Ago3	5' -GGAATTAGACAAGCCAATCAGCA-3'	5' -AGGGTGGTCATATCCTTCTGGA-3'
Ago4	5' -CTAACAGACTCCCAGCGTGTCA-3'	5' -GACTGGCTGGCCGTCTAGTCA-3'
GAPDH	5' -CGCTCTCTGCTCCTCCTGTT-3'	5' -CCATGGTGTCTGAGCGATGT-3'
Mouse Ago1	5' -CCCAGAAACAGTGTGAGAAG-3'	5' -TCCCTGCATCCTTGGAATC-3'
Mouse Ago2	5' -ATTCAAGTTCTACAAGTCCACCC-3'	5' -CTGATAGTCCTTCTCCAGCTTG-3'
Mouse Ago3	5' -ATACAGCCAATCCACTTCCTG-3'	5' -ATCTTTTCCACCTTCCCCAG-3'
Mouse Ago4	5' -AAAGGTTGGAAGGGTCTGG-3'	5' -TGGTGATTTTGCCTGGGAAG-3'
Mouse GAPDH	5' -CTTTGTCAAGCTCATTTCTGG-3'	5' -TCTTGCTCAGTGTCTTGC-3'

Supplemental Figures

Broderick et al., Supplemental Figure S1

number of sites		perfect	bulged	seed plus 13-16	seed
		siRNA guide 5'-AUAGCUAUAAUGAAUCCU-3' mRNA 3'...UAUCGAUAUACUUUACGGUA...5'	5'-AUAGCUAU ¹⁰ UGAAUCCCA-3' 3'...UAUCGAUA ₁₀ UACUUUACGGU...5'	5'- ⁵ AGCUAUA ^{CGUC} AUAGCAAC-3' 3'... ^A UCGAUA ₁₀ UACU ^U UACGGUAU...5'	5'- ⁵ AGCUAUA ^{CGUCCCGUA} ACCC-3' 3'... ^A UCGAUAUACUUUACGGUAU...5'
	1	not significantly different from $n^H = 1$	N.D. ($IC_{50} > 20$ nM)	N.D. ($IC_{50} > 20$ nM)	N.D. ($IC_{50} > 20$ nM)
	2	not significantly different from $n^H = 1$	N.D. ($IC_{50} > 20$ nM)	N.D. ($IC_{50} > 20$ nM)	N.D. ($IC_{50} > 20$ nM)
	3	not significantly different from $n^H = 1$	not significantly different from $n^H = 1$	N.D. ($IC_{50} > 20$ nM)	N.D. ($IC_{50} > 20$ nM)
	4	not significantly different from $n^H = 1$	$p = 0.002$	N.D. ($IC_{50} \sim 11$ nM)	N.D. ($IC_{50} > 20$ nM)
	5	not significantly different from $n^H = 1$	$p = 0.02$	$p = 0.02$	N.D. ($IC_{50} \sim 15$ nM)
	6	not significantly different from $n^H = 1$	$p = < 0.0001$	$p = 0.002$	not significantly different from $n^H = 1$
			6 100 nM:	$p = 0.002$	not significantly different from $n^H = 1$

Figure 2.S1. p -values for Hill coefficients.

FIGURE 2.S1. Analysis of the statistical significance of Hill coefficients presented in Fig. 2. Individual values for the Hill coefficient of each replicate experiment ($n = 12$), were analyzed using Student's t-test to determine if the experimentally determined Hill coefficient was significantly different at a 95% confidence level from a theoretical Hill coefficient equal to 1.

Broderick et al., Supplemental Figure S2

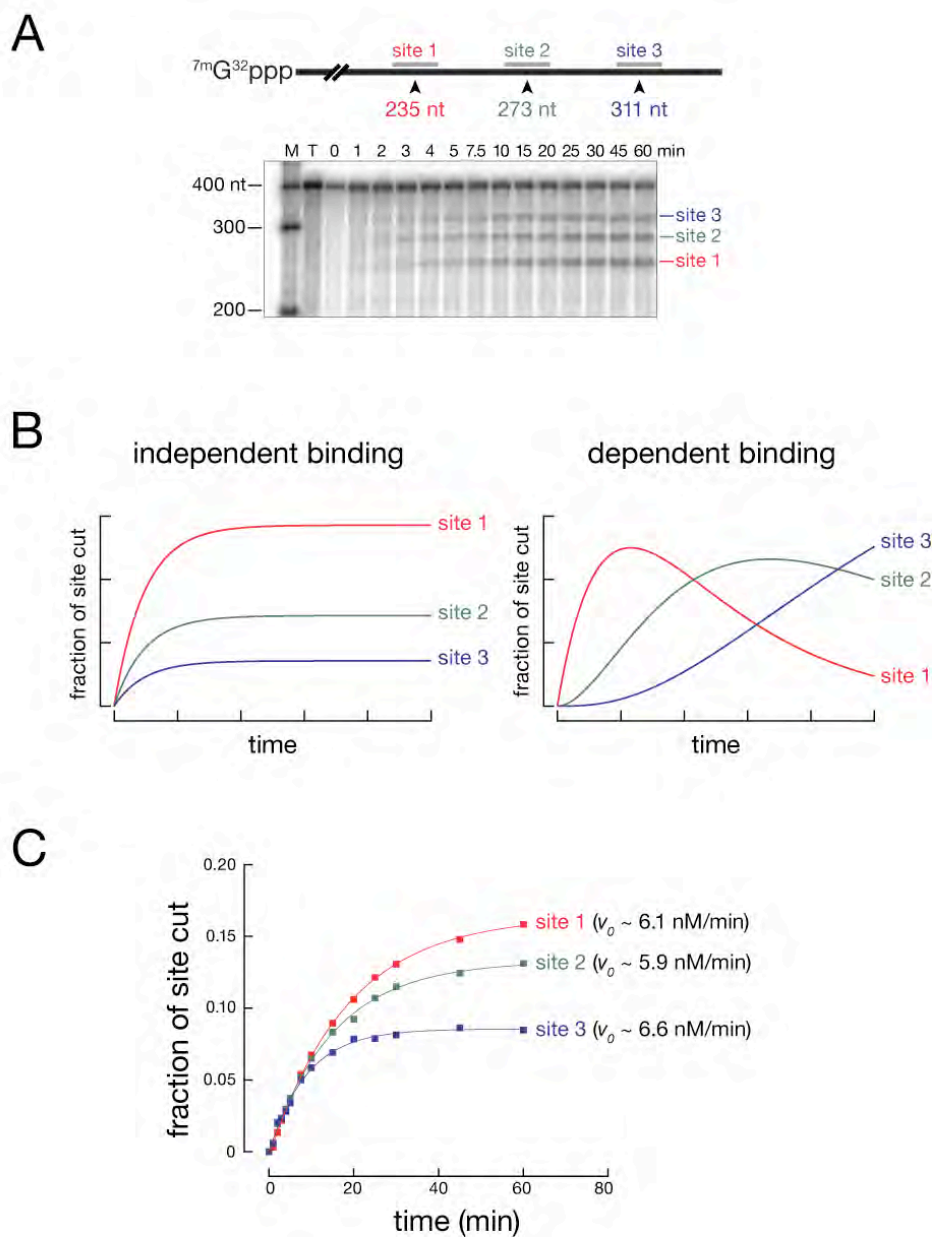


Figure 2.S2. Independence of cleavage at three non-adjacent targets sites by RNase in vitro.

FIGURE 2.S2. Each site in the expanded three-site reporter is equally accessible. We examined the accessibility of each site by measuring its sensitivity to oligonucleotide-directed RNase H cleavage. Cleavage at each site occurred with similar initial rates ($v_0 \sim 6.1, 5.9$ and 6.6 nM min^{-1}) and with kinetics that were consistent with a model assuming independent and unordered (i.e., random) rather than dependent and sequential cleavage of each site. (A) Oligonucleotide-directed RNase H cleavage of the expanded three-site reporter mRNA. (B) Expected results (from computer modeling) for independent and dependent binding models. (C) Quantification of the data in (A).

Broderick et al., Supplemental Figure S3

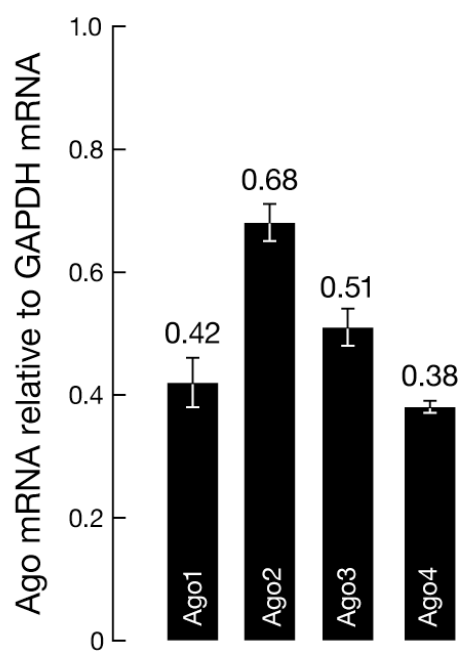


Figure 2.S3. Argonaute mRNA levels in HeLa cells relative to GAPDH.

FIGURE 2.S3. The relative abundance of Argonaute mRNAs, normalized to GAPDH, was determined by qRT-PCR for the HeLa cell line used in Figures 2–4. mRNA for Argonaute 2 was most abundant; relative levels were Ago2 > Ago3 > Ago1 and Ago4. Data is reported as mean \pm standard deviation for three technical replicates.

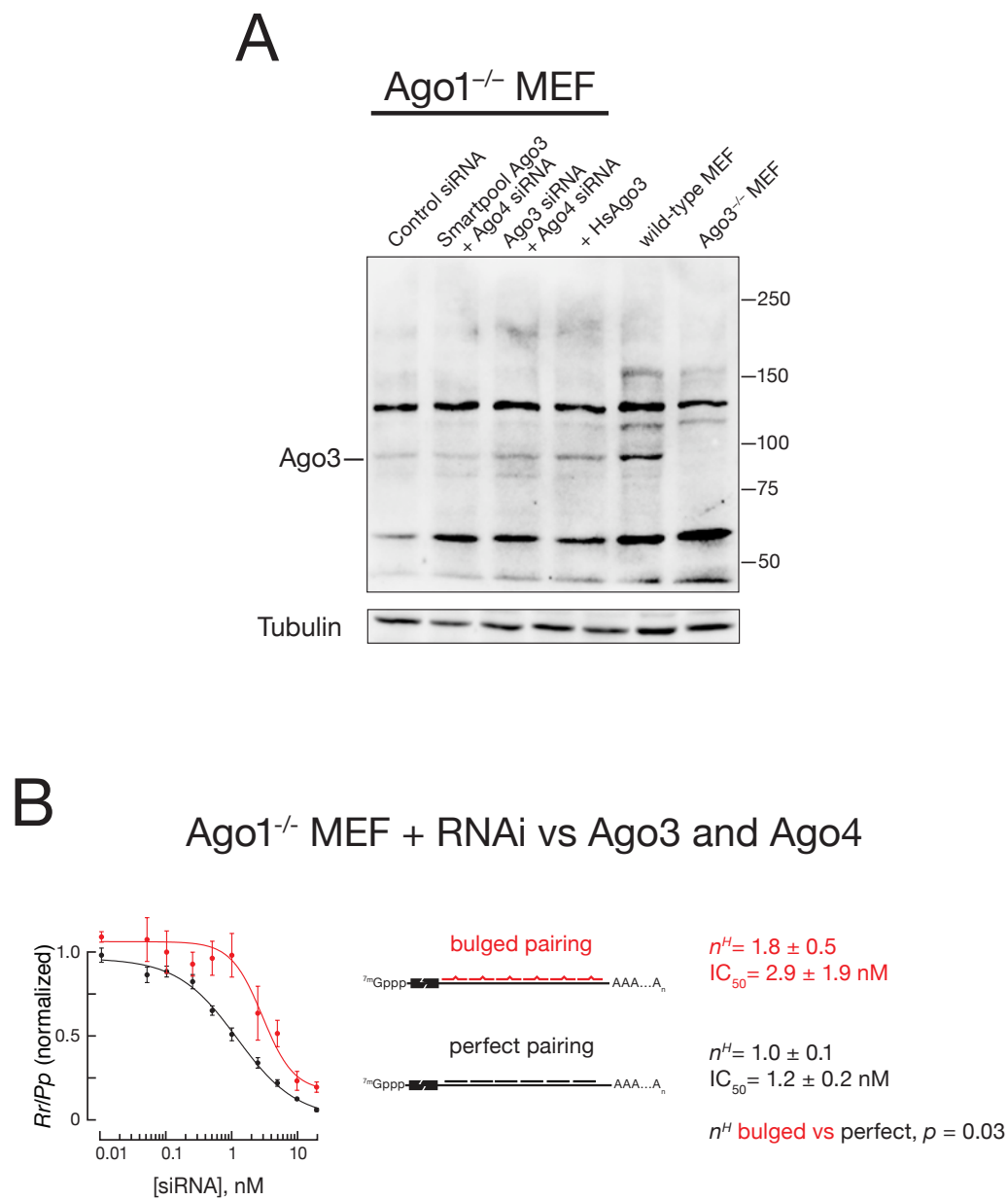


Figure 2.S4. Argonaute 3 protein level in Ago1^{-/-} MEFs (A) and silencing of *Rr* luciferase by perfect or bulged siRNA after RNAi for Argonaute 3 and Argonaute 4 (B).

FIGURE 2.S4. Endogenous Ago2 silences bulged sites cooperatively. (A) Ago3 protein abundance, relative to Tubulin, was measured by western blot. (RNAi against Ago3 and Ago4 in the Ago1^{-/-} MEFs halved the amount of Ago4 mRNA [data not shown].) (B) A bulged siRNA silenced the six-site reporter cooperatively ($n_{bulged}^H = 1.8 \pm 0.5$), compared to silencing by the perfect siRNA ($n_{perfect}^H = 1.0 \pm 0.01$) in Ago1^{-/-} MEF cells in which Ago3 and Ago4 had been depleted by RNAi.

Broderick et al., Supplemental Figure S5

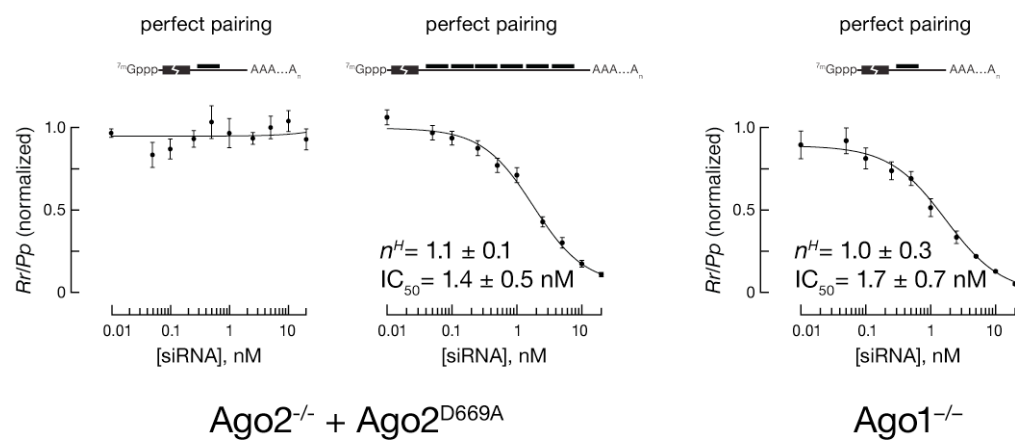


Figure 2.S5. Silencing by catalytically inactive Ago2 requires binding at multiple sites by a perfect pairing.

FIGURE 2.S5. A single target site reporter is not silenced in Ago2^{-/-} MEFs reconstituted with catalytically inactive Ago2^{D669A} (Ago2^{-/-} + Ago2^{D669A}; *left*). As controls, silencing of the one-site reporter in Ago1^{-/-} MEF cells (*right*) and silencing of the six-site reporter in Ago2^{-/-} + Ago2^{D669A} (*middle*) is shown. Silencing of the six-site reporter by catalytically inactive Ago2^{D669A} occurs without cooperativity ($n_{perfect}^H = 1.1 \pm 0.1$).

Chapter III: Oligonucleotide tethers recruit RISC to an mRNA and silence its expression.

This chapter explores ideas that are pending patent by the author, Phillip D. Zamore and Neil Aronin.

USPTO App. No. 20060293267, “Dual functional oligonucleotides for use as anti-viral agents” Broderick JA and Zamore PD.

USPTO App. No. 20050256072, “Dual functional oligonucleotides for use in repressing mutant gene expression” Broderick JA, Aronin N, Zamore PD

All data presented in this chapter is the author’s own. This manuscript is unpublished.

Summary

Harnessing an endogenous miRNA to induce gene silencing may restrict silencing of a co-expressed gene to a specific cell type. A molecule that—in principle—could combine antisense technology and RNAi is a 2′-O-methyl oligonucleotide “tether” that contains a 5′ region complementary to an endogenous miRNA in RISC and a 3′ region complementary to the target mRNA. 2′-O-methyl oligonucleotides incorporate in each nucleotide a methoxyl group in place of the ribose 2′ hydroxyl, thereby conferring endonuclease resistance. 2′-O-methyl oligonucleotides bind tightly to complementary sequences in siRNAs and miRNAs in the RISC and are irreversible inhibitors of small RNA function in *Drosophila* embryo lysates, HeLa cell lysates, and *in vivo* in HeLa cells and *C. elegans* larvae. The 2′-O-methyl oligonucleotide used to inhibit

a miRNA in RISC can be extended by adding sequence that is complementary to a target mRNA. This “tether” could recruit RISC to a target mRNA. A 2′-O-methyl oligonucleotide tether would be expected to function only when two conditions are met. First, the tether must be complementary to a region of the endogenous miRNA that recruits the RISC. Second the tether must bind the target mRNA through complementary base pairing. If the required miRNA or target mRNA is not present in the cell then the tether should not function. More than one target site in the 3′ untranslated region (UTR) of a target mRNA is necessary to induce silencing of the mRNA when there is imperfect pairing between the miRNA and the target. Therefore binding of a single tether to a non-targeted mRNA should not suffice to cause silencing. Only when multiple tether molecules bind the target is robust silencing anticipated. Thus, oligonucleotide tethers should be significantly more specific than either traditional antisense or siRNA methods, where partial pairing of the nucleic acid to an mRNA unrelated in sequence to the intended target can elicit an ‘off-target’ response. In addition, during viral infection tethers may be able to recruit viral miRNAs in RISC to silence viral or host genes that promote or are required for viral propagation. Preliminary data confirm the prediction: an oligonucleotide tether that recruits the *let-7* miRNA to silence an exogenous reporter gene, in HeLa cells and mouse embryonic fibroblast cells, directs robust silencing using only nanomolar concentrations of oligonucleotide. This chapter contains data from the proof of concept experiments and the initial characterization of the mechanism of gene silencing by oligonucleotide tethers.

Introduction

Small noncoding RNAs (~21-23 nucleotides long), called microRNAs (miRNAs), guide the endogenous pathway of mRNA silencing (Figure 3.1). Most miRNAs reside in their own genes, distinct from the mRNAs whose stability or translation they regulate (Bartel, 2004; Cai et al., 2004). miRNA expression is often tissue specific and developmentally important (Pasquinelli and Ruvkun, 2002; Olsen and Ambros, 1999). Many animal miRNAs are phylogenetically conserved and some show organ-specific expression (Sempere et al., 2004; Landgraf et al., 2007). Endogenous miRNAs establish right and left neuronal symmetry in *C. elegans* (Johnston and Hobert, 2003; Ambros et al., 2003). miRNAs have been proposed to temporally regulate development of the mammalian central nervous system (Krichevsky et al., 2003; Kim et al., 2004). miRNAs are transcribed by RNA polymerase II as primary microRNAs (pri-miRNAs) (Lee et al., 2004; Bracht et al., 2004). RNase III endonuclease Drosha (Lee et al., 2003), together with the small dsRNA-binding protein DGCR8 (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004) convert pri-miRNAs to pre-miRNAs, ~70 nt stem-loop structures. Drosha establishes the 5' or 3' end of the miRNA. Pre-miRNAs are moved to the cytoplasm by Exportin-5 (Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004; Zeng and Cullen, 2004). In the cytoplasm, the RNase III enzyme Dicer converts pre-miRNAs into mature double stranded miRNAs ~25 nt long called miR/miR* (or miR-5p/miR-3p). The miRNA duplex is assembled into an Argonaute protein, forming the RNA-induced silencing complex (RISC), and the miR* strand is destroyed. The miRNA guides RISC to bind a target mRNA. When incorporated into RISC, miRNAs direct the post-transcriptional silencing of their

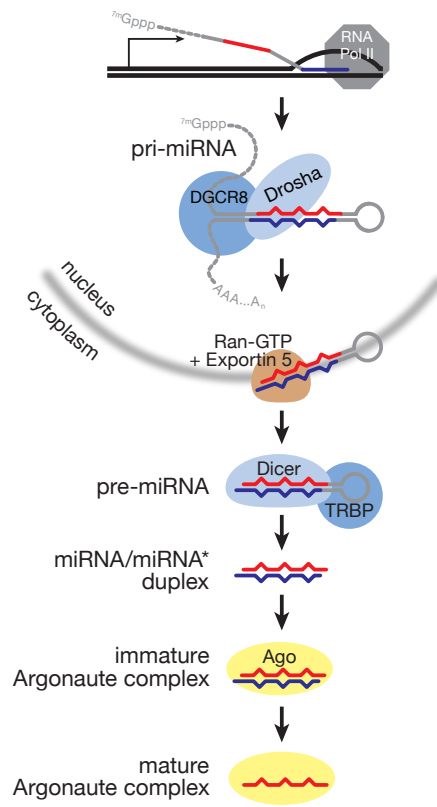


Figure 3.1. MicroRNA biogenesis pathway.

mRNA targets. Like siRNAs, plant and animal miRNAs can direct cleavage of their mRNA targets when the two are extensively complementary, but cause destabilization or repress mRNA translation when they are not (Hutvagner and Zamore, 2002; Doench et al., 2003; Doench and Sharp, 2004).

Because the specificity of RISC is determined by base pairing between the miRNA and its target mRNA, RISC specificity can, in theory, be modified by oligonucleotides. Current methods for regulating gene expression using antisense oligonucleotides or RNAi allow exogenous nucleic acids to repress expression of cellular genes. However, each of these methods has specific limitations. A concern for antisense technology is that unmethylated CpG (Cytosine-phosphate-Guanine) dinucleotides activate mammalian B cells and natural killer cells in culture (Krieg et al., 1995). DNA and RNA antisense oligonucleotides have a short half-life *in vivo*, so chemical modification of the oligonucleotides is required to stabilize them against nucleolytic degradation and to improve their biodistribution and pharmacokinetics. Some modified antisense oligonucleotide chemistries that improve antisense performance *in vitro* show toxicity and lethal side effects in animal studies (Crooke, 2004). But most importantly, antisense technology has shown poor efficacy *in vivo*, perhaps because it does not exploit a robust biological pathway. In contrast, RNAi technology uses a powerful cellular pathway for repressing gene expression. However, chemically unmodified siRNAs may prove unstable *in vivo*, and—unlike antisense technology—siRNA delivery methods are in their infancy. Expression of siRNAs by viral vectors *in vivo*, as an alternative strategy to the use of synthetic siRNAs, is fraught with the same difficulties plaguing all gene-therapy approaches: the difficulty of developing long lasting viral vectors,

producing therapy-grade, high titer virus stocks, and by the inherent immunostimulatory side effects associated with all current viral vectors. Clearly, a technology is needed that combines the delivery and stability of antisense technology with the robust target-specific silencing elicited by siRNAs. Furthermore, the silencing effects of neither antisense nor RNAi technology can be restricted to a particular cell type.

Harnessing an endogenous miRNA to induce gene silencing may restrict silencing to a specific cell type. A molecule that—in principle—could combine antisense technology and RNAi is a 2′-O-methyl oligonucleotide “tether” that contains a 5′ region complementary to an endogenous miRNA in RISC (miRNA-programmed RISC (miRISC)) and a 3′ region complementary to the target mRNA (Figure 2). 2′-O-methyl oligonucleotides incorporate in each nucleotide a methoxyl group in place of the ribose 2′ hydroxyl of RNA, thereby conferring endonuclease resistance (Sproat et al., 1989; Iribarren et al., 1990). 2′-O-methyl oligonucleotides bind tightly to complementary sequences in siRNAs and miRNAs in RISC and are irreversible inhibitors of small RNA function in *Drosophila* embryo lysates, HeLa cell lysates, and in vivo in HeLa cells and *C. elegans* larvae (Hutvagner et al., 2004; Meister et al., 2004). The 2′-O-methyl oligonucleotide can be extended by adding sequence that is complementary to a target mRNA, creating a tether that could recruit miRISC to a target mRNA. A 2′-O-methyl oligonucleotide tether would be expected to function only when two conditions are met. First, the tether must be complementary to a region of the endogenous miRNA in RISC. Second, the tether must bind the target mRNA through complementary base pairing (Figure 3.2). If either the required miRNA or target mRNA is not present in the cell, then the tether should not function. The

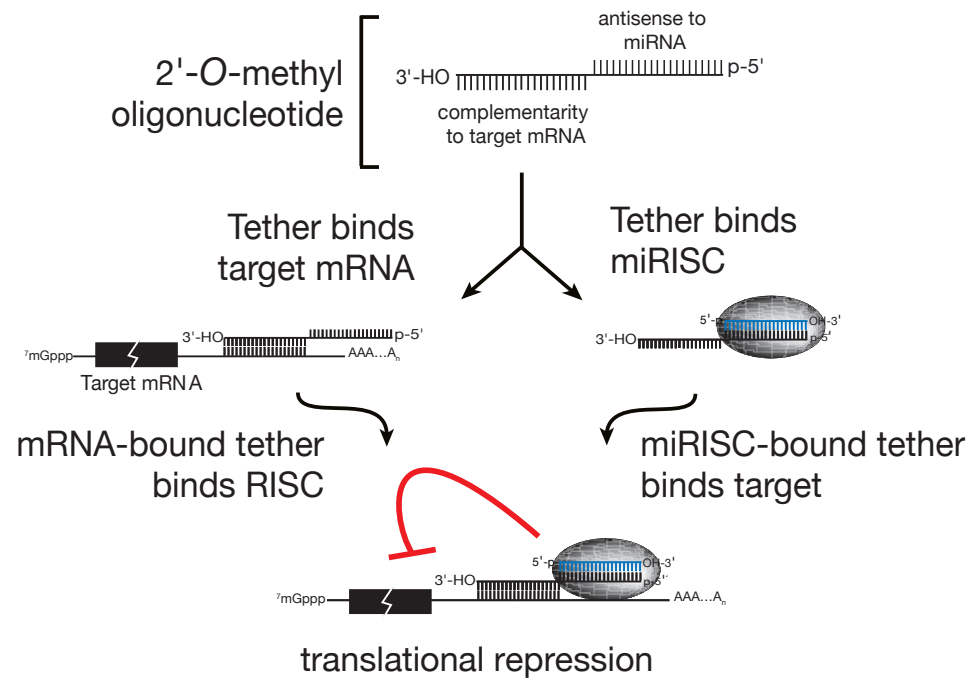


Figure 3.2. Oligonucleotide tether binds a miRNA in RISC and the 3' UTR of mRNA.

work described in Chapter II of this thesis shows that more than one target site in the 3' untranslated region (UTR) of a target mRNA is necessary to induce silencing of the mRNA; increasing the number of target sites within an mRNA increases the level of silencing. Therefore binding of a single tether to an unintended mRNA should not suffice to cause silencing. Only when multiple tether molecules bind the target is robust silencing anticipated (Figure 3.3). Thus, oligonucleotide tethers should be significantly more specific than either traditional antisense or siRNA methods, where partial pairing of the nucleic acid to an mRNA other than the intended target can elicit an 'off-target' response (Jackson et al., 2003).

Results

The oligonucleotide tether is designed to be bifunctional. One end of the tether recruits, through nucleotide base pairing, a miRNA that has been incorporated into RISC (Figure 3.3). The other end of the tether binds the target mRNA through base pairing. The expected result is that the tethering of RISC will inhibit expression of protein from the mRNA. Preliminary data confirm the prediction: an oligonucleotide tether that recruits endogenous *let-7* miRNA to silence an exogenous reporter gene in HeLa cells and mouse embryonic fibroblast cells (MEFs) directs robust silencing using only nanomolar concentrations of oligonucleotide. The proof of principle experiment showed that the tether silenced a reporter luciferase mRNA by recruiting RISC to six sites in the 3' UTR. To test this, we designed a tether that had 24 nucleotides of complementarity to multiple sites in the 3' UTR of a luciferase reporter and 21

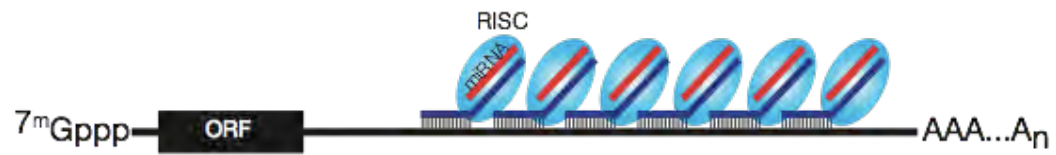


Figure 3.3. Oligonucleotide tether (Blue) binds to miRNA (Red) and recruits RISC to multiple sites in the 3' UTR.

nucleotides of complementarity to an exogenous guide siRNA in RISC. To examine oligonucleotide tether function in the absence of the recruited miRNA, we tested a tether that functions by recruiting a plant miRNA, miR-166. Since miR-166 is not expressed in HeLa cells, the tether silences only when miR-166 is transfected as an asymmetric siRNA into the cells (Schwarz et al., 2003). This system allows assessment of any antisense effects of the tether alone. When 24 nucleotides of the oligonucleotide tether are complementary to the target sites in the 3' UTR of a luciferase reporter and miR166 is transfected in the culture, we expected that RISC would be recruited to the mRNA through the exogenous miR-166. Using an in vivo reporter assay to test the ability of a tether to recruit RISC to luciferase mRNA expressed in HeLa cells, luciferase activity is reduced by 80-90%. Notably, the tether has little or no effect on reporter expression when the recruited miRNA is not present in the cells (Figure 3.4, "tether plus GFP siRNA"). Thus, the tethers function in a miRNA-dependent, rather than an antisense, mode.

To test the ability of the tether to recruit an endogenously expressed miRNA in RISC, we used a tether that has complementarity to *let-7* miRNA. The control to which all experimental samples were compared was a culture that received an oligonucleotide tether that cannot bind the target sites in the 3' UTR of the luciferase mRNA because it contains the sense sequence instead of the complement of the target sequence. Recruiting an endogenous miRNA, *let-7*, in RISC to the 3' UTR of the luciferase mRNA silenced the reporter by more than 60% (Figure 3.4). Mouse embryonic fibroblast cells derived from Ago1 or Ago2 null mice were used to determine which Argonaute protein in RISC, when tethered to the luciferase reporter mRNA, mediates silencing. The IC₅₀ for

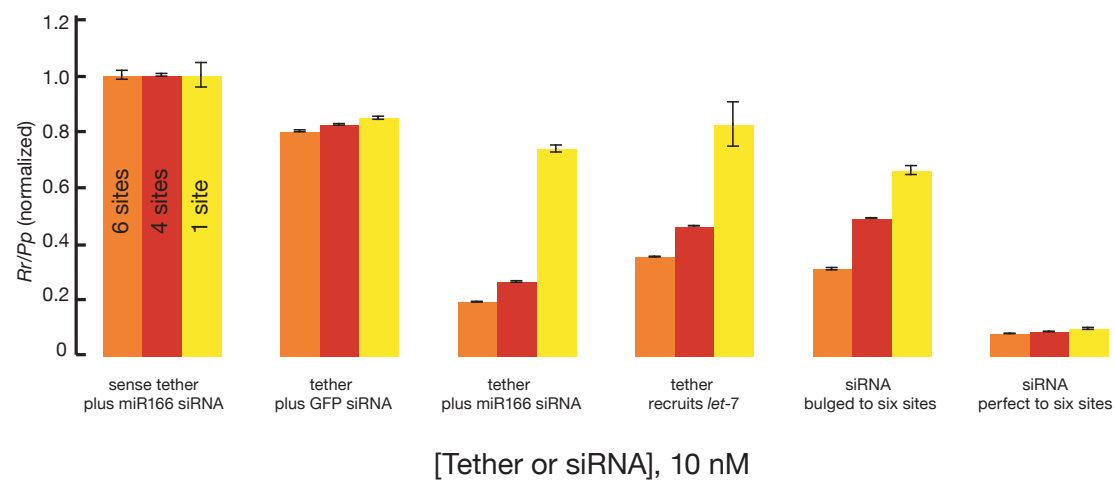


Figure 3.4. Silencing of *Rr* luciferase mRNA by tethering RISC to target sites in the 3' UTR.

silencing by the tether that targeted six sites in the 3' UTR of the luciferase reporter mRNA and paired to *let-7* in RISC was determined in wild-type MEF cells, and MEF cells derived from Ago1^{-/-} or Ago2^{-/-} mice. In wild-type MEF cells, which express all four Argonaute proteins, the IC₅₀ for silencing by the tether that paired perfectly to *let-7* was 0.08 ± 0.01 nM (Figure 3.5A).

Interestingly, in the absence of Ago1, the IC₅₀ for silencing by the perfectly pairing tether was 0.11 ± 0.02 nM— similar to the IC₅₀ for silencing in wild-type MEF cells (Figure 3.5A,B). The level of Ago2 protein in Ago1^{-/-} MEF cells is 2 fold higher than in wild-type MEF cells. This suggest that silencing by a tether is not limited by the concentration of Ago2, so it was surprising that the IC₅₀ for silencing did not decrease in the Ago1^{-/-}. This can be confirmed in the Ago2^{-/-} MEF cells that are cells reconstituted with wild-type Ago2 which expresses 30 fold more Ago2 protein than wild-type MEFs to see if the IC₅₀ for silencing decreases with more Ago2. In the absence of Ago2, the tether silenced with an IC₅₀ of 0.17 ± 0.11 nM (Figure 3.5C). The maximum amount of silencing in the Ago2^{-/-} MEF cells was 2 fold less than in the wild-type MEF cells. This suggests that Ago1, Ago3 and Ago4 proteins are less efficient at mediating silencing when tethered to a target mRNA, probably because their expression in MEF cells is lower than the K_D required to achieve silencing.

One consideration we had for the tether strategy was that providing a perfect target for a miRNA could cause destabilization of the miRNA (Ameres et al., 2010). To see if the extent of pairing to the miRNA changed the ability of the tether to silence the reporter containing six sites, we designed tethers pairing

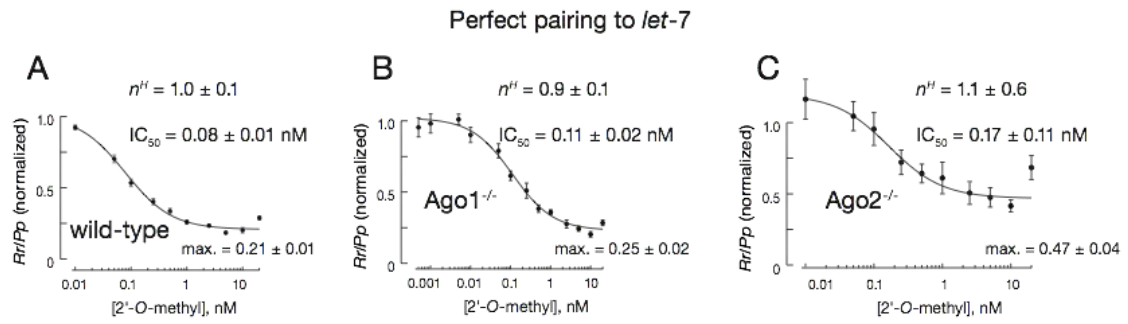


Figure 3.5. Silencing by a tether that binds *let-7* in wild-type mouse embryonic fibroblast cells (A) or cells lacking Ago1 (B) or Ago2 (C).

partially to *let-7*: a bulge at nucleotides 9-10, seed plus 13-16 and a seed only pairing (Figure 3.6).

In wild-type MEF cells, the tethers pairing to the seed or the seed plus positions 13-16 of *let-7* did not silence the reporter when targeting six sites in the 3' UTR (Figure 3.7A,B). In contrast, more extensive pairing to *let-7* caused silencing: a bulged pairing to *let-7* silenced the reporter with an IC_{50} of 0.46 ± 0.02 nM (Figure 3.7C). Maximum silencing by the bulged pairing to *let-7* was similar to that achieved by the perfect pairing (Figure 3.7C,D). At 10 nM, the perfect and bulged pairing tethers achieved 80% of the silencing caused by the siRNA that bound directly to the mRNA and paired perfectly to six target sites (Figure 3.7C,D,E). These results suggest that Ago proteins can mediate silencing without being bound directly to the target mRNA and that a tether can exploit endogenous miRNAs to recruit RISC to a target mRNA that is not normally regulated by miRNAs.

To figure out the minimal sequence of complementarity required to specifically bind the target mRNA and the minimal sequence required to recruit RISC through the oligonucleotide tether to the target mRNA, I did experiments with a tether that binds to exogenously transfected miR-166 in HeLa cells. To further analyze the function of the tether to specifically bind the target mRNA, truncations of the tether were made to determine the minimal sequence needed to silence the six-site reporter. The 3' end of the tether was reduced from 24 to 21,18,15,12 and 9 nucleotides in length while retaining all 21 nucleotides of

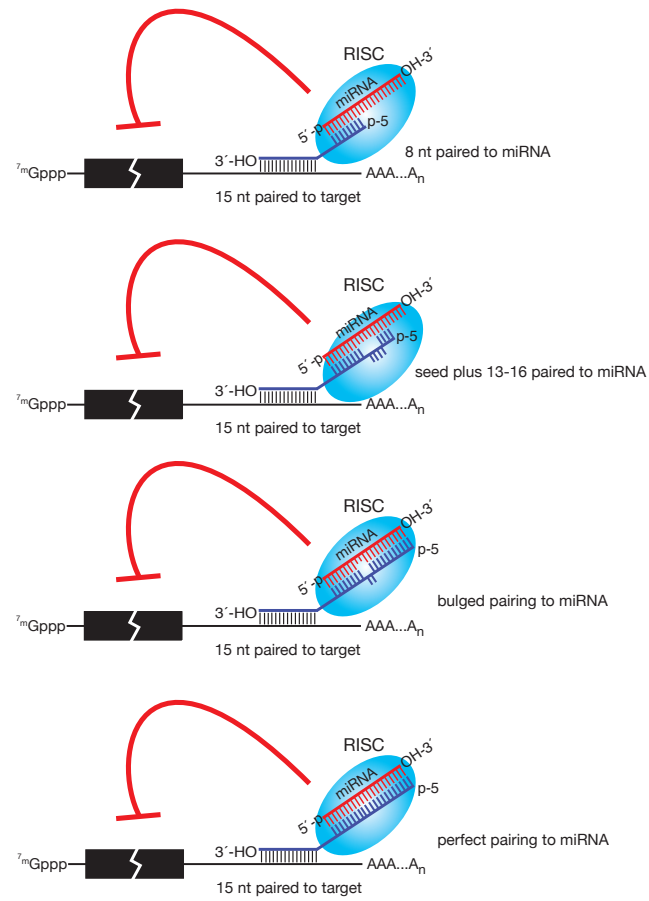


Figure 3.6. Tethers with different pairing to *let-7*.

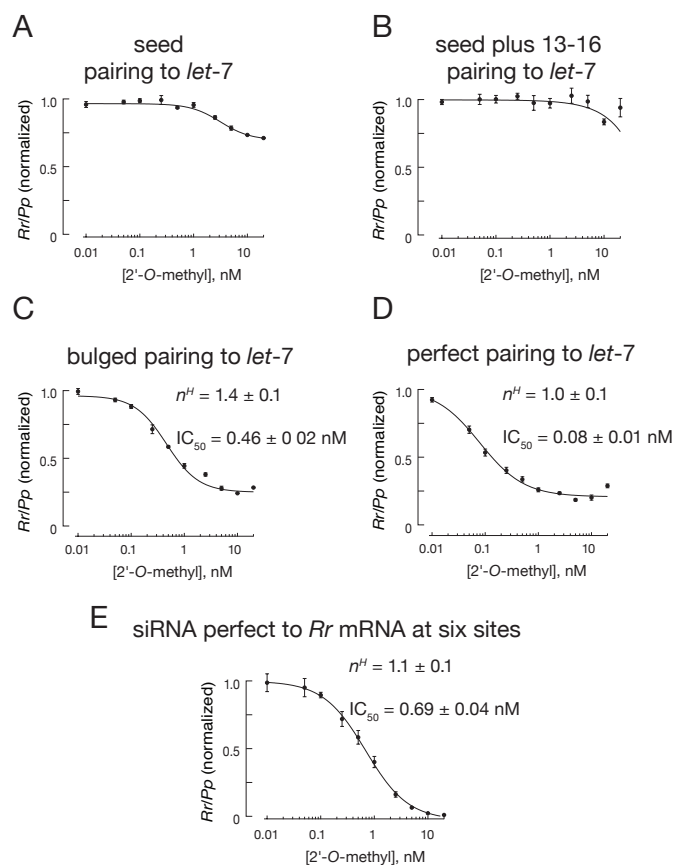


Figure 3.7. Silencing of a six-site reporter mRNA by tethers that pair to different extents with *let-7* or an siRNA that pairs perfectly to target sites.

pairing to the miR-166. The 5' end of the tether that is required to recruit RISC was also truncated. Truncations of the original 21 nucleotide sequence complementary to a miRNA within the oligonucleotide tether were made in two nucleotide increments to 19, 17, 15, 13, 11, and nine nucleotides. Luciferase expression from the reporter was silenced by 80% with 10 nM of tether that paired to the target with 15 or more nucleotides at six sites (Figure 3.8A). When the tether was truncated at its 5' end, 13 nucleotides was the shortest extent of complementarity to miR-166 that silenced the luciferase reporter (Figure 3.8B). Based on these results we have shortened the sequence of the tethers from 45 nt to 28 nt (Figure 3.9). To silence a reporter mRNA, an oligonucleotide tether need only contain ~28 nucleotides, 15 of which are specificity determinants for the target mRNA and 13 of which recruit the miRNA-programmed RISC.

To increase the specificity of the tether for pairing to the target sequences, locked nucleic acids (LNAs) were incorporated into the tether. LNAs contain 2', 4' methylene bridges that increase the specificity of oligonucleotides by constraining the ribose sugar moiety into the 3'-endo conformation, preorganizing the bases for pairing. LNAs increase the RNA:RNA melting temperature of the oligonucleotide by 2.4°C per base (Nielsen et al., 1999; Braasch and Corey, 2001). We compared tethers that contained either the LNA or 2'-O-methyl modified RNA. One tether recruited an exogenously transfected miR-166 (Figure 3.10A) and one recruited endogenous *let-7* (Figure 3.10B). The 2'-O-methyl modified tether that recruited miR-166 silenced the reporter by 80% when miR-166 was co-transfected, but not when GFP siRNA was co-transfected (Figure

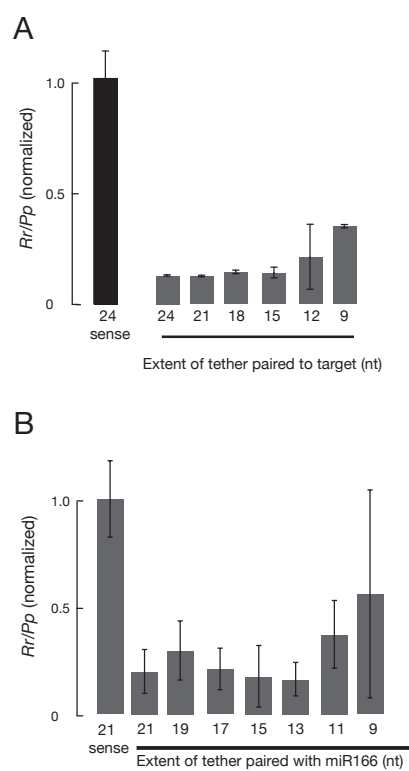


Figure 3.8. Silencing of six-site reporter by tethers with different extents of pairing to target mRNA (A) or miRNA (B).

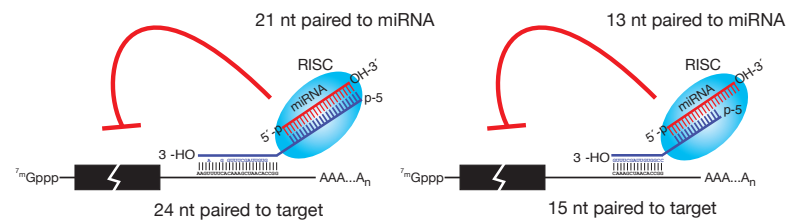


Figure 3.9. Tethers 28 nt in length silenced the luciferase reporter (*right*). Sequence of the tether that binds the miRNA was reduced to 13 nt from 21 nt and the sequence that paired to target was reduced to 15 nt from 24 nt of target pairing (*left*).

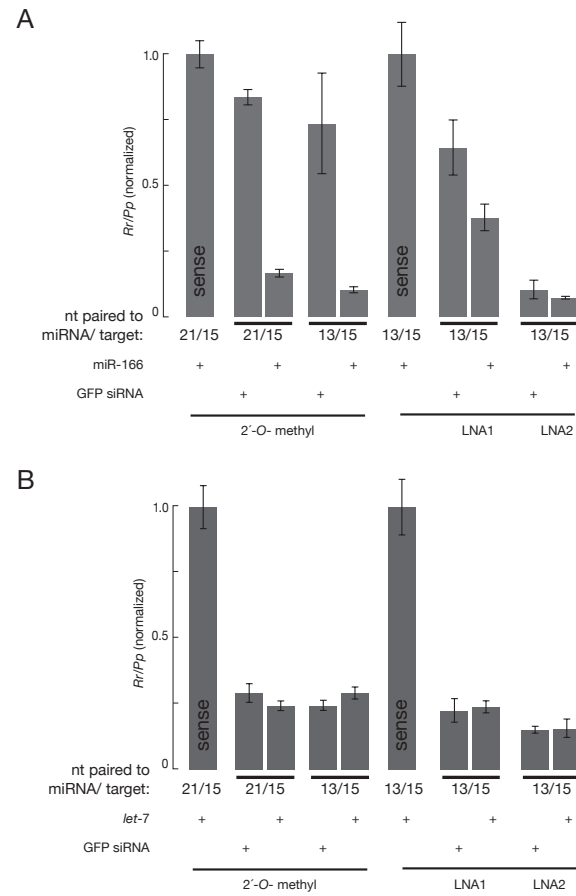


Figure 3.10. Silencing of a six-site reporter by 10 nM of tether with 2'-O-methyl or LNA modifications, pairing to miR-166 (A) or endogenous *let-7* (B).

3.10A). We tested two separate LNA modified tethers that differed in the placement of the LNAs within the sequence of the tether. LNA1 was not as effective as the 2'-O-methyl modified tether to silence the reporter in the presence of miR-166 (Figure 3.10A). LNA2 caused silencing even in the absence of miR-166, possibly because the LNA modified tethers cause RNase H digestion of the target mRNA. We also tested tethers that contained either the LNA or 2'-O-methyl modified RNA and recruited *let-7* to the reporter (Figure 3.10B). These tethers should silence the reporter using endogenous *let-7* and not require transfection of *let-7*. As expected, the tethers containing either type of RNA modifications and that recruited *let-7* were able to silence the reporter by > 50% (Figure 3.10B). The *let-7* tethers silenced the reporter to lesser degree than tethers that used the exogenous miR-166, a result that might be due to competition for endogenous targets of *let-7* in the cell. Shorter tethers, substituted with LNAs, were not more effective than the 2'-O-methyl tethers that recruit miR-166. A tether that contains 15 nt of sequence pairing to the target mRNA and 13 nucleotides of complementarity to a siRNA guide stand in RISC is necessary and sufficient to recruit RISC and induce silencing of the target reporter mRNA. By incorporating LNA substitutions into the tether sequence, the number of nucleotides required to silence the target mRNA might be reduced from 28 nt to less than 20 nt. Tethers that recruit other miRNAs may have increased effectiveness due to sequence specific effects.

Post-transcriptional gene silencing can occur by two mechanisms. Translational inhibition prevents the target mRNA from being translated by the

ribosome into protein by preventing translation initiation or blocking translational elongation of the target mRNA. Destabilization of target mRNA is thought to be the dominant form of silencing in animal cells. To determine if the reporter target mRNA is destabilized when tethered to RISC, we measured mRNA levels in HeLa cells by quantitative real-time RT-PCR. The experiments tested silencing of the six-site luciferase reporter by 2'-O-methyl tethers that either recruit exogenous miR-166 or endogenous *let-7* and compared silencing by a perfect or bulged pairing siRNA directly on the target mRNA (Figure 3.11). When comparing the target mRNA level to the luciferase activity, the tethers caused translational repression (Figure 3.12). Tethering RISC to the 3' UTR at six sites induced repression similar to a bulged siRNA that bound directly to the target mRNA. Further analysis of the effect of tether silencing on target mRNA levels in wild-type and Ago MEF cells is required to understand whether or not the target mRNA is destabilized or translationally repressed when RISC is tethered to the target through different endogenous miRNAs.

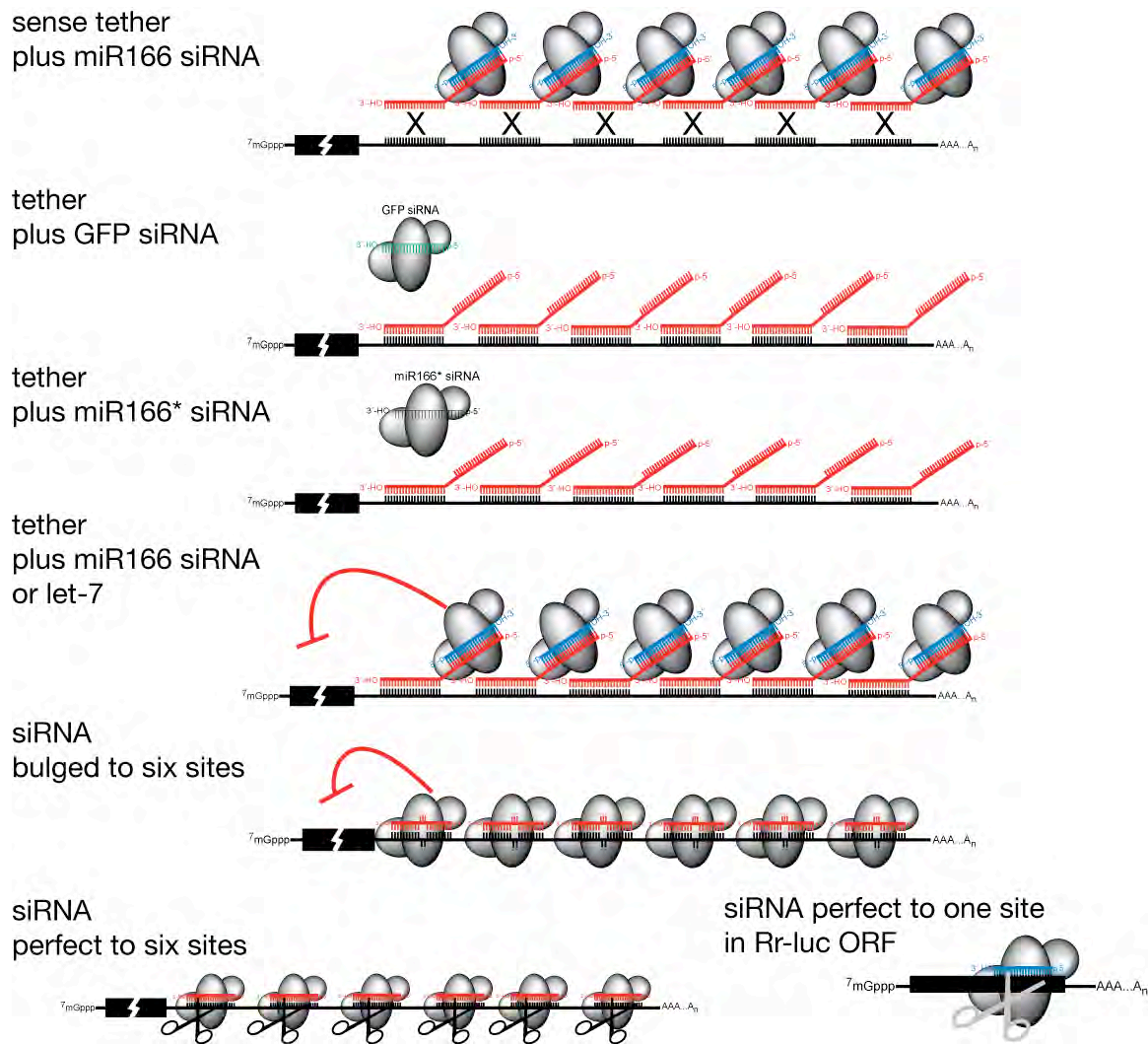


Figure 3.11. Tether pairing schemes used in experiments to measure mRNA levels by qRT-PCR and luciferase activity.

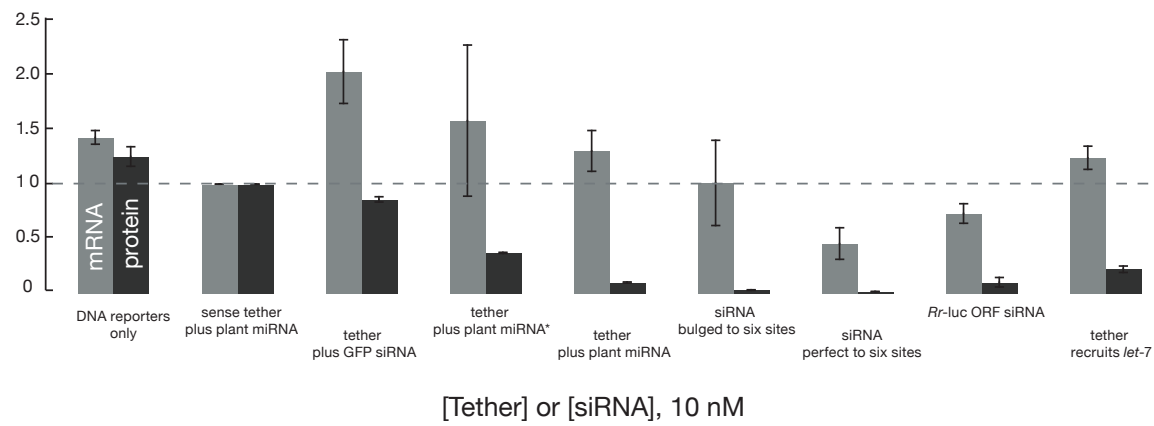


Figure 3.12. Tethers that recruit RISC to six sites in the 3' UTR of a reporter mRNA silence luciferase protein expression without destabilizing the mRNA.

Discussion

Oligonucleotide tethers recruit RISC to a target mRNA

The proof of principle experiments showed that it is possible to silence a target mRNA by tethering RISC to multiple sites in the 3' UTR. These results suggest that Ago proteins can mediate silencing without being bound directly to the target mRNA and that a tether can exploit endogenous miRNAs to recruit RISC to a target mRNA that is not normally regulated by miRNAs. Tethering RISC to a target mRNA through an oligonucleotide tether causes translational repression similar to a more sophisticated RISC tethering scheme (Pillai et al., 2004). In those experiments, human Ago2 was fused to the small, basic RNA-binding N-peptide from bacteriophage. Next, they placed multiple N-peptide-binding sites in the 3' UTR of a reporter mRNA and introduced both the Ago2 fusion protein and the reporter into human cells. The Ago2-N fusion protein bound the 3' UTR sites through the N-peptide rather than via a small RNA guide and silenced reporter protein production without reducing reporter mRNA levels. Translational repression by the Ago2-N fusion protein required more than three binding sites to achieve robust repression, similar to that of bulged siRNA:target pairings.

Preliminary experiments showed that the minimal length of an oligonucleotide required to tether RISC is 28 nucleotides, only ~7 nt longer than siRNAs or miRNAs. Since the tether is a single molecule, compared to exogenous duplex siRNA used for RNAi, it may be easier to deliver to target tissues than siRNA and prove to be an alternative strategy for silencing genes of interest. Our

data from HeLa cells showed that using a miRNA, the tether silences a target containing 4 target sites in the 3' UTR by >50% and silences > 60% when targeting six sites. Notably, because it does not silence when the reporter contains only a single site, it is unlikely that a tether will have 'off-target' effects on other non-target mRNAs. This needs to be explored further by determining the IC_{50} for silencing an mRNA containing a single target sequence to bind a tether.

Applications of tether silencing strategy

One application for the tether strategy is to target a sequence in the 3' UTR of huntingtin (*htt*) mRNA that contains a single nucleotide polymorphism which associates with the mutant allele of *htt* (Schwarz et al., 2006; DiFiglia et al., 2007; Liu et al., 2008; Pfister et al., 2009). This is dependent on the tether being able to discriminate target recognition to a single nucleotide difference that we may be able to achieve by using LNA modified tethers. If a tether can cause silencing of the mutant allele, then the progression of the disease may be slowed (Pfister and Zamore, 2009).

A further application is that a tether can recruit RISC through pairing to a virally expressed miRNA in a cell infected by a virus that expresses miRNAs. Mammalian viruses produce at least 66 distinct miRNAs (Cullen, 2009; Cullen, 2010). Viruses are able to manipulate the endogenous miRNA pathway in order to propagate. Some viral miRNAs collaborate with cellular miRNAs to silence mRNAs and thwart the immune response to infection. One known function of viral miRNAs is to thwart the immune response during infection by regulating the expression of the major histocompatibility complex class I chain-related molecule B (MICB), a natural killer cell ligand (Stern-Ginossar et al., 2007). A

second function for viral miRNAs may be to repress host mRNAs so as to maintain viral latency (Nachmani et al., 2010; Murphy et al., 2008). Exploiting viral miRNAs by using a tether to redirect viral miRNAs to silence viral mRNAs may some day be used to coax latent viruses into the more therapeutically tractable replicating state, allowing the elimination of reservoirs that enable viral reemergence after anti-viral therapy is completed.

Table 3.1. Synthetic siRNAs used in this study.

siRNA	Guide strand	Passenger strand
CXCR4 perfect	5' -GUGUUAGCUUUGUGAAAACUU-3'	5' -GUUUUCACAAAGCUAACAACG-3'
CXCR4 bulged	5' -UGUUAGCUGGAGUGAAAACUU-3'	5' -GUUUUCACUCCAGCUAACCCA-3'
<i>Rr</i> ORF siRNA	5' -AUAGCUAUAUGAAAUGCCUU-3'	5' -GGCAUUUCAUUUAUAGCUAGUU-3'
miR166*	5' -GGAUCAGGCUUCAUCCACUU-3'	5' -UUGGGAUGAAGCCUGAUCCGG-3'
miR166	5' -CCGGAUCAGGCUUCAUCCCAA-3'	5' -GGGAUGAAGCCUGAUCCGUAU-3'
GFP	5' -GAACUUCAGGGUCAGCUUGCCG-3'	5' -GCAAGCUGACCCUGAAGUUAU-3'

Table 3.2. Synthetic tether oligonucleotides used in this study.

Tether name	Sequence 5'-3'
T.13miR166/ 15 CXCR4/LNA1	aaGccTgaTccGgcmCggTgtTagmCttTg
T.13miR166/ 15 CXCR4/LNA2	AagmCctGatmCcgGccGgtGttAgcTttG
T.13 miR166/15 sense CXCR4/LNA1	aaGccTgaTccGggGccAcaAtcGaaAc
T.13 miR166/15 sense CXCR4/LNA2	AagmCctGatmCcgGggmCcamCaaTcgAaamC
T13 let7/ 15 CXCR4/LNA1	accTacTacmCtcAccGgtGttAgcTttg
T13 let7/ 15 CXCR4/LNA2	AccTacTacmCtcAccGgtGttAgcTttG
T.13 let7/15 sense CXCR4/LNA1	accTacTacmCtcAggmCcamCaaTcgAaac
T.13 let7/15 sense CXCR4/LNA2	AccTacTacmCtcAggmCcamCaaTcgAaamC
T.21miR166/24 CXCR4	mU . mU . mG . mG . mG . mA . mU . mG . mA . mA . mG . mC . mC . mU . mG . mA . mU . mC . mC . mG . mG . mC . mC . mG . mG . mU . mG . m U . mU . mA . mG . mC . mU . mU . mU . mG . mU . mG . mA . mA . mA . mA . mC . mU . mU
T.21miR166/21CXCR4	mU . mU . mG . mG . mG . mA . mU . mG . mA . mA . mG . mC . mC . mU . mG . mA . mU . mC . mC . mG . mG . mC . mC . mG . mG . mU . mG . m U . mU . mA . mG . mC . mU . mU . mU . mG . mU . mG . mA . mA . mA . mA

Tether name	Sequence 5'-3'
T.21miR166/18CXCR4	mU . mU . mG . mG . mG . mA . mU . mG . mA . mA . mG . mC . mC . mU . mG . mA . mU . mC . mC . mG . mG . mC . mC . mG . mG . mU . mG . mU . mU . mA . mG . mC . mU . mU . mU . mG . mU . mG . mA
T.21miR166/15CXCR4	mU . mU . mG . mG . mG . mA . mU . mG . mA . mA . mG . mC . mC . mU . mG . mA . mU . mC . mC . mG . mG . mC . mC . mG . mG . mU . mG . mU . mU . mA . mG . mC . mU . mU . mU . mG
T.21miR166/12CXCR4	mU . mU . mG . mG . mG . mA . mU . mG . mA . mA . mG . mC . mC . mU . mG . mA . mU . mC . mC . mG . mG . mC . mC . mG . mG . mU . mG . mU . mU . mA . mG . mC . mU
T.21miR166/10.CXCR4	mU . mU . mG . mG . mG . mA . mU . mG . mA . mA . mG . mC . mC . mU . mG . mA . mU . mC . mC . mG . mG . mC . mC . mG . mG . mU . mG . mU . mU . mA . mG
T.21miR166/9.CXCR4	mU . mU . mG . mG . mG . mA . mU . mG . mA . mA . mG . mC . mC . mU . mG . mA . mU . mC . mC . mG . mG . mC . mC . mG . mG . mU . mG . mU . mU . mA
T.21miR166/24senseCXCR4	mU . mU . mG . mG . mG . mA . mU . mG . mA . mA . mG . mC . mC . mU . mG . mA . mU . mC . mC . mG . mG . mG . mG . mC . mC . mA . mC . mA . mA . mU . mC . mG . mA . mA . mA . mC . mA . mC . mU . mU . mU . mU . mG . mA . mA
T.21miR166/15senseCXCR4	mU . mU . mG . mG . mG . mA . mU . mG . mA . mA . mG . mC . mC . mU . mG . mA . mU . mC . mC . mG . mG . mG . mG . mC . mC . mA . mC . mA . mA . mU . mC . mG . mA . mA . mA . mC
T13 let7/15CXCR4	mA . mC . mC . mU . mA . mC . mU . mA . mC . mC . mU . mC . mA . mC . mC . mG . mG . mU . mG . mU . mU . mA . mG . mC . mU . mU . mU . mG
T.21let7/15CXCR4	mA . mC . mU . mA . mU . mA . mC . mA . mA . mC . mC . mU . mA . mC . mU . mA . mC . mC . mU . mC . mA . mC . mC . mG . mG . mU . mG . mU . mU . mA . mG . mC . mU . mU . mU . mG
T.21let7/ 15 sense CXCR4	mA . mC . mU . mA . mU . mA . mC . mA . mA . mC . mC . mU . mA . mC . mU . mA . mC . mC . mU . mC . mA . mG . mG . mC . mC . mA . mC . mA . mA . mU . mC . mG . mA . mA . mA . mC
T.21let7/24 sense CXCR4	mA . mC . mU . mA . mU . mA . mC . mA . mA . mC . mC . mU . mA . mC . mU . mA . mC . mC . mU . mC . mA . mG . mG . mC . mC . mA . mC . mA . mA . mU . mC . mG . mA . mA . mA . mC . mA . mC . mU . mU . mU . mU . mG . mA . mA

Tether name	Sequence 5'-3'
T.let-7perfect/15 <i>Rr</i>	5' – mAmCmUmAmUmAmCmAmAmCmCmUmAmCmUmAmCmCm UmCmAmCmUmAmUmAmAmUmGmAmAmAmUmGmCmC – 3'
T.let-7bulged/15 <i>Rr</i>	5' – mAmCmUmAmUmAmCmAmAmCmUmCmUmAmCmC mUmCmAmCmUmAmUmAmAmUmGmAmAmAmUmGmCmC – 3'
T.let-7seed13-16/15 <i>Rr</i>	5' – mAmCmAmAmAmAmGmCmCmUmAmCmCmUmCmA mCmUmAmUmAmAmUmGmAmAmAmUmGmCmC – 3'
T.let-7seed/15 <i>Rr</i>	5' – mCmUmAmCmCmUmCmA mCmUmAmUmAmAmUmGmAmAmAmUmGmCmC – 3'

Table 3.3. PCR primers used in this study.

PCR Oligos	Forward primer	Reverse primer
Reverse transcription		5' -CACTGCATTCTAGTTGTGGTTTGTCC-3'
<i>Rr luc</i>	5' -GTGAGGCACTGGGCAGGTGTCCAC-3'	5' -CATTCATTTGTTTACATCTGGCCC-3'
<i>Pp luc</i>	5' -AGAGATACGCCCTGGTTCCT-3'	5' -CCAACACCGGCATAAAGAAT-3'
GAPDH	5' -CGCTCTCTGCTCCTCCTGTT-3'	5' -CCATGGTGTCTGAGCGATGT-3'

Materials and Methods

Plasmids

Renilla luciferase vector pRL-TK (Promega, Madison, WI, USA) containing target sites 6, 4 or one site for CXCR4 and a modified linker sequence (Doench et al., 2003) were used to test all tethers with complementarity to the CXCR4 target site. For tethers to test the different types of pairing to *let-7*, a separate six-site reporter in psiCheck2 was constructed as follows. *Renilla* luciferase vector pRL-TK (Promega, Madison, WI, USA) containing target sites for CXCR4 and a modified linker sequence (Doench et al., 2003) was mutated from TAG to CTC (lower case letters in the oligonucleotides) at nucleotides 387–389 of the *Renilla* luciferase open reading frame to generate mismatches with seed positions 5, 6, and 7 of the siRNA guide strand by PCR-directed mutagenesis using DNA oligonucleotides: 5'-CTT GTT TGG CAT TTC ATT ACt ccT ATG AGC ATC AAG ATA AGA TC-3' (sense), 5'-GAT CTT ATC TTG ATG CTC ATA Gga gTA ATG AAA TGC CAA ACA AG-3' (antisense). Mutagenesis was confirmed by sequencing, and then 5' phosphorylated oligos containing the target sites and pairing to create appropriate ends were cloned into the *XbaI* and *ApeI* sites of the mutant pRL-TK. The sequences of the DNA oligonucleotides used to construct the six target sites were sense :5'-CTA GAG GCA TTT CAT TAT AGC TAT GGC ATT TCA TTA TAG CTA TGG CAT TTC ATT ATA GCT ATG GCA TTT CAT TAT AGC TAT GGC ATT TCA TTA TAG CTA TGG CAT TTC ATT ATA GCT ATG GGC C-3', antisense: 5'- CAT AGC TAT AAT GAA ATG CCA TAG CTA TAA TGA AAT GCC ATA GCT ATA ATG AAA TGC CAT AGC TAT AAT GAA ATG CCA

TAG CTA TAA TGA AAT GCC ATA GCT ATA ATG AAA TGC CT-3'.

psiCheck2 (Promega) reporters were constructed by digesting psiCheck2 with *NheI* and *NotI* and inserting the 3' UTR target site-containing *NheI*–*NotI* fragment from the mutant pRL-TK vectors.

Cell Culture and Transfection

HeLa CCL2 cell cultures were maintained at 37°C and 5% CO₂ in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated FBS (Invitrogen) and 50 U/ml penicillin and streptomycin (Invitrogen). MEF cells were cultured in DMEM (Invitrogen) supplemented with 15% heat inactivated FBS (Invitrogen), 50 U/ml penicillin and streptomycin (Invitrogen), 0.1 mM NEAA (Invitrogen), 2 mM glutamine (Invitrogen). Cells were seeded at a density of 0.1×10^6 cells per well in 24 well plates in DMEM (Invitrogen) containing 10% heat inactivated FBS (Invitrogen). Twenty-four hours later, cells were washed 3 times in 500 μ l PBS (Invitrogen), and then 400 μ l DMEM with serum was added to each well. Renilla luciferase plasmid pRL-TK (0.025 mg), firefly luciferase plasmid pGL3 (0.025 mg), or psiCheck2 (0.025 mg) and 20 nM siRNA were mixed with 99 μ l DMEM and 1 μ l DharmaFECT Duo (Dharmacon, Lafayette, CO, USA) per well. A control siRNA (CXCR4) was used to equalize the total amount of siRNA in each transfection. Cells are incubated with 0.5 ml final volume of DMEM plus serum containing 100 μ l of transfection reagent nucleic acid mixture for 24 h.

siRNA Annealing

Single-stranded guide and passenger siRNA strands (Table 1; Dharmacon) were annealed by incubating 10 μ M each strand in 500 μ l annealing buffer (100 mM

potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C, followed by 1 h at 37°C.

Luciferase Assays

Cells were washed once in 500 μ l PBS and lysed in 100 μ l of Passive Lysis Buffer (Promega) at room temperature for 20 min in 24 well plates. For each well 10 μ l lysate was read in triplicate using dual luciferase reagents (Promega) in a Turner Biosystems luminometer controlled by Veritas software (Turner). *Renilla* luciferase activity for each concentration of transfected siRNA was normalized to the corresponding firefly luciferase activity.

Data Analysis

The individual biological replicates for normalized *Renilla* luciferase activity versus siRNA concentration was fit using Igor Pro 6.10 (Lake Oswego, Oregon, USA) to the Hill equation to determine IC_{50} and n^H . Fitting was weighted using the standard error of each mean value.

Quantitative RT-PCR

Cells were harvested and total RNA purified using Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol, then 10 μ g RNA was treated with 20 units of Turbo DNase (Ambion, Austin, TX, USA) in 100 μ l at 37°C for 20 min. RNA was extracted with 100 μ l acid phenol (EMD Chemicals, Gibbstown, NJ, USA) and precipitated using 0.1 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes absolute ethanol. Precipitated RNA was washed with 900 μ l 70% ethanol and then dissolved in 50 μ l water. RNA purity and concentration was determined by absorbance. RNA (0.1 μ g) was reversed transcribed using Superscript III Reverse Transcriptase (Invitrogen) with oligo(dT) primers

(Invitrogen) according to manufacturer's protocol. A parallel reaction containing water instead of reverse transcriptase provided a negative control. After reverse transcription, quantitative PCR was performed using 1 μ l of the cDNA reaction. PCR reactions (25 μ l) used SYBR green PCR mix (Bio-Rad Laboratories, Hercules, CA, USA) and specific primers (Table 3). PCR conditions were: 94°C, 4 min, then 40 cycles of 94°C, 15 sec; 60°C, 15 sec; 72°C, 30 sec; 72°C, 7 min using an Opticon 2 instrument (Bio-Rad). PCR data was analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) and DART PCR (Pfaffl, 2001; Peirson et al., 2003). Cycle number data was analyzed by normalizing the cycle threshold for reporter mRNA to that of GAPDH; then for *Rr* luciferase mRNA, the cycle threshold was normalized to the cycle threshold for *Pp* luciferase mRNA.

Quantitative RT-PCR References

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Chapter IV: General Discussion

Unique properties of Argonautes

A priority for the future of RNA silencing for both basic research and therapeutic application is determining whether or not each mammalian Argonaute protein has a specialized function. Defining the properties of each Argonaute protein may lead to more efficient target silencing strategies and uncover roles for individual Argonaute function in normal cells, developmental pathways and disease. Why does Ago2 cleave and repress translation of targets? Why are non-catalytic Argonautes necessary when Ago2 is bifunctional and there is no sorting of small RNAs in mammals?

One aspect of silencing to be considered is whether the expression level of Ago2 is modulated by the cell cycle or during stress response. Perhaps some target sites are not regulated until the concentration of Ago2 reaches a threshold. Interestingly, the IC_{50} for silencing by a perfect pairing to six sites is 10 fold lower for Ago2 than for Ago1 (Figure 2.5A,D). This could be because either Ago1 is much less abundant than Ago2 or that Ago1 uses only the seed of the guide to bind to target which reduces the on rate for Ago1 or the amount of time it remains bound to target. If the conformation of Ago1 protein allows pairing only with the seed, then this would explain why there is no difference in IC_{50} or Hill coefficient for silencing by the perfect or bulged pairing by Ago1 (Figure 2.5A). If Ago1 uses only the seed to pair to target, then that would mean that productive pairing to target beyond the seed region is only beneficial for silencing mediated by Ago2. A simple test to determine if pairing beyond the seed contributes to silencing by Ago1 would be to compare silencing by a seed only pairing to six

sites in the absence of Ago2 to that of the bulged and perfect pairings in the absence of Ago2.

Another interesting idea that my data support is that silencing by a perfect pairing was not limited by the amount of Ago2 protein in the cell. When Ago2 was over expressed in MEF cells, the IC_{50} for a perfect pairing to six sites is the same as in the Ago1^{-/-} MEF cells, that express 10 fold less Ago2 (Figure 2.5B,D). This indicates that there might be a limiting factor required for 'active' RISC, whether for loading or function, that is less abundant than the over expressed Ago2. We suspected this could be heat shock protein HSP70 or HSP90, which is required for RISC loading, but western blot analysis showed it to be equally as abundant as Ago2 (W. Salomon, pers. comm.). It could also mean that excess Ago2 is sequestered or not available to engage targets in the cytoplasm.

As more species of small RNAs are identified through cloning and deep sequencing of RNA from diverse tissues, perhaps new roles for Argonaute proteins will be revealed. A mystery that merits resolution is that Ago3 contains the catalytic triad, yet Ago3 has never been shown to be capable of cleaving a target mRNA. Perhaps Ago3 prefers an unidentified species of small RNA, containing modifications that have allowed them to evade capture by current small RNA cloning methods. Alternatively, Ago3 may require activation, through post-translational modification in order to cleave a target mRNA, an activity that may be restricted to certain cell types or occur during certain periods of development.

Generation of cooperativity

My research revealed one functional distinction between Argonaute proteins is their capacity to generate cooperativity in silencing between multiple

target sites. Using a quantitative approach of measuring IC_{50} and Hill coefficients and subjecting them to statistical testing, I detected true biochemical cooperativity in RNA silencing. The Hill equation was originally deduced to explain the equilibrium relationship of oxygen binding to hemoglobin and its sigmoidal binding curve (A.V Hill, 1910). This equation makes no assumptions about the molecular mechanisms that generate cooperativity. It does not describe kinetic rates and is insensitive to the microscopic binding constants of the component processes therein. The Hill equation is a phenomenological model that provides a convenient function for fitting experimental data. When considering the simultaneous effect of ligand binding to multiple sites, no model like the Hill equation has been derived to assess the effect of an applied perturbation on a cooperative system.

For Ago1, cooperativity between multiple miRNAs bound to adjacent sites in a target causes silencing and occurs regardless of the extent of target pairing. In contrast, Ago2 is sensitive to the extent of pairing between a small RNA guide and target, and silences non-cooperatively when perfectly paired to target and cooperatively when paired imperfectly to target. For Ago2, perfect pairing to a target may cause a conformational shift that precludes its association with other Argonautes or proteins that generate cooperativity. Yet to be discovered is whether cooperativity emanates solely from binding to target or from Ago:Ago interaction at nearby sites or Ago interaction with or recruitment of other proteins such as GW182.

Multiple partially complementary miRNAs bound to target may have a cooperative influence on the function of recruited proteins that cause destabilization or translational repression. My data showed that Ago1 was

unable to silence a reporter when 3 target sites were separated by 19 nucleotides, but when the three sites were adjacent Ago1 cooperatively silenced the reporter. Cooperativity generated by a protein-protein interaction between Argonautes or other proteins would not be disrupted by a distance of 19 nucleotides between target sites. The model reporter system used in my research cannot isolate the cooperative *binding* model from a cooperative *function* model (Figure 2.1). Though my data support a model for cooperative *binding* of bulged miRNAs (Figure 2.1A), it cannot not exclude a cooperative *function* model that multiple bound RISCs on a target mRNA generate cooperative interactions with proteins that increase repression (Figure 2.1B).

To illustrate the difference, consider cooperativity in transcriptional activation in eukaryotes and bacteriophage. Yeast GAL4 transcriptional activation protein and mammalian transcriptional activator ATF bind to DNA cooperatively. They also cooperatively increase transcription even when their target binding sites are saturated, indicating that they directly contact the transcriptional machinery (Carey et al., 1990; Lin et al., 1990). In contrast, λ repressor protein from λ bacteriophage binds multiple DNA sites cooperatively as a dimer at low concentrations of repressor protein, however, only one of the λ repressor dimers contacts the RNA polymerase to increase transcription non-cooperatively (Hochschild et al., 1983). My data could represent cooperative function of Argonautes bound at multiple sites, similar to that of GAL4 and ATF, or it could represent cooperative binding of Argonautes to target, where only one Ago protein is required to recruit a protein that causes silencing by either destabilization or translational repression. To isolate the exact nature of the cooperativity in silencing requires further investigation with a different

experimental approach. Perhaps in the future single molecule experiments to observe Argonaute binding to multiple target sites combined with in vitro translation will reveal the source of cooperativity.

Cooperativity between miRNAs that pair partially to target through Ago1 or Ago2 provides a mechanism where the expression of a target can be finely tuned, depending on the levels of co-expressed miRNAs. One striking result from my experiments was the capacity for Ago2 to cooperatively silence the reporter when paired to three bulged sites separated by 19 nucleotides whereas Ago1 was unable to silence the same reporter with a perfect of bulged pairing. In my model reporter system, Ago1 can only generate cooperativity when sites are adjacent to each other and only Ago2 can generate cooperativity in silencing when bound at multiple distant sites. If cooperativity by Ago1 is limited to adjacent sites, then how many adjacent target sites occur in protein coding genes? Until we know all the criteria that define a target site, this is impossible to know. How often do target sites fall within 19 nucleotides of each other in real protein coding genes? Bioinformatics analysis of Ago1 PAR-CLIP deep sequencing data from flies (Y. Tomari, unpublished) showed that 4346 protein coding genes (of ~14,000 protein coding genes in flies) have peaks for Ago1. The presence of two peaks, or hits, in the same gene within 19 nucleotides of each other occurs in 288 of the genes that bound Ago1 (J. Xu, pers. comm.). I predict that the same analysis on the Ago2-CLIP deep sequencing data will reveal that fewer target sites fall within 19 nt of each other because Ago2 is able to cooperatively silence more distant or non-adjacent sites.

Viral exploitation of cooperative regulation

Potentially, a perfect pairing through Ago2 could disrupt regulation of the

target by miRNAs bound through Ago1, providing a functional reason for why few miRNAs pair to targets with perfect complementarity. One instance where disrupting cooperativity could be exploited is during viral infection. Mammalian viruses produce at least 66 distinct miRNAs (Cullen, 2009; Cullen, 2010). One known function of miRNAs is to thwart the immune response during infection by regulating the expression of the major histocompatibility complex class I chain regulated molecule B (MICB), a natural killer cell ligand (Stern-Ginossar et al., 2007). Epstein-Barr virus, Kaposi's sarcoma-associated herpes virus and human cytomegalovirus (HCMV) express miRNAs that target separate sites in the MICB mRNA to prevent its expression. HCMV expresses a miRNA, miR-UL112, that targets the 3' UTR of MICB at a site overlapping the binding site for the cellular miRNA, miR-373. Interestingly, the MICB mRNA contains nearby sites for two other cellular miRNAs, miR-376a and miR-433. After 72 h of HCMV infection, the viral miRNA, miR-UL112, and the cellular miRNA, miR-376a, collaborate to silence the expression of MICB (Nachmani et al., 2010). Although it remains to be determined into which Argonaute protein viral miRNAs are loaded, a potential strategy used by viral miRNAs could be to load in Ago2 and pair extensively with target mRNAs in order to disrupt weaker regulation by cellular miRNAs of target mRNAs that impede successful infection. Perhaps viruses have evolved to exploit the unique properties of Argonaute proteins, thereby providing an advantage for the virus to propagate.

Non-redundant Argonaute functions

It will be important to look carefully at protein expression profiles of the three non-catalytic Argonautes to understand why mammals have retained them. Perhaps there is a developmental stage where expression of a specific non-

catalytic Argonautes is required. Are non-catalytic Argonautes redundant and why can loss of miRNAs in conditional Dicer null ES cells be tolerated (Kanellopoulou et al., 2005)? How can Ago2 substitute for non-catalytic Argonautes? And how does a mouse that is a triple knockout for Ago1,3 and 4 survive (G. Hannon, pers. comm.)? What if using miRNAs as guides for regulating targets is only a part-time function of Argonautes? Does the latest data, showing Argonaute cross-linked to mRNAs in the absence of a miRNA, hint at a novel mechanism of Argonautes associating with RNA binding proteins (Leung et al., 2011)? In our lab, we have begun experiments to look at Argonaute protein purified from MEF cells binding to target sites at the single molecule level. This project should detect and help resolve whether or not there are differential requirements for individual Argonaute proteins for binding based on extent of pairing between miRNAs and target mRNA.

Quantifying Argonaute expression profiles

We still do not know how Argonaute protein expression and function are regulated. Current methods to quantitate Argonaute levels are limited to measuring mRNA by qRT-PCR and the last published report to quantitate Argonaute mRNA levels in various tissues was by Sasaki et al., in 2004. Experiments to define tissue specific levels of Argonaute proteins and their distinct functional properties have been limited by the difficult task of identifying unique antigenic peptides from each Argonaute protein that will not cross-detect multiple Argonaute proteins. In my work, I have tested antibodies to mouse Ago1, Ago2 and Ago3 that are specific when blotted against MEF cells that are knockout for the individual Argonautes, but without MEF cells that lack Ago4, there is still no way to determine specificity for anti-Ago4 antibody. Once

we have the full complement of specific mammalian Argonaute antibodies, new experiments will be possible to look at regulation of Argonaute protein expression in all mammalian cell types.

Like the post-transcriptional regulation Argonautes effect upon other mRNAs, regulation of Argonaute mRNAs by miRNAs is a possible mode to control Argonaute protein expression, especially for Ago1 that has nearly 5 kilobases in its 3' UTR and for Ago2 that contains almost 2 kilobases in its 3' UTR. In Ago2^{-/-} MEF cells the level of Ago1 protein is much higher than in wild type MEF cells suggesting that Ago2 regulates expression of Ago1 protein. I have identified several candidate miRNAs that are computationally predicted to target the 3' UTRs of human Argonaute mRNAs. Additionally the stability of Argonaute proteins can be controlled by post-translational modifications such as hydroxylation, phosphorylation and ubiquitylation (Qi et al., 2008; Rybak et al., 2009; Zeng et al., 2008). Argonaute proteins themselves regulate many processes and so determining how Argonaute protein expression and function are regulated requires carefully designed experimental approaches. Continuation of basic research to define the roles that Argonaute proteins command from the molecular level via small RNAs may provide solutions to many unresolved aspects of mammalian RNA silencing.

Advancing therapeutic application of RNA silencing

Exploitation of robust RNA silencing mechanisms for successful application in humans depends on delivery of modified RNA to target tissues. Expression of modified RNA is not possible through viral packaging. Further, unmodified RNA expression in cells would be subject to degradation and have limited activity, even when expressed at high levels. Currently, effective delivery

of therapeutic RNAs to the intended tissue apart from direct injection, is difficult to achieve, but progress in the design of lipid nanoparticles seems promising (Whitehead et al., 2009).

In addition to delivery methods discussed in my review of microRNA therapeutics (see Appendix), innovative combinatorial formulation strategies are in progress. Before a therapeutic RNA can find its target mRNA or miRNA, it must make it through four barriers: 1) remain in the bloodstream (avoid filtering by kidneys), 2) extracellular matrix, 3) cellular uptake, 4) escape from endosomes (before lysosomal degradation). Some lipid-based formulations are able to pass through the cell membrane, but cannot escape the endosomes. Some formulations are great at releasing their cargo as the pH decreases in the lysosomal pathway, but cannot get through the cell membrane. Lipidoids are lipids with an amine-containing polar head group and nonpolar alkyl tails (Akinc et al., 2008). By combining two formulations that on their own were not able to silence a target in vitro, a library of 'binary' lipidoid formulations (combining two lipidoid formulations) was created and tested (Akinc et al., 2008; Siegwart et al., 2011; Whitehead et al., 2011). After screening the library by testing them in vitro in HeLa cells with luciferase reporters, an effective binary formulation, 86N₁₅-98O₁₃ was further tested in mice. 48 hours after tail vein injection of 5 mg/kg of siRNA, the formulation allowed silencing of factor VII protein expression in hepatocytes by 85% (Whitehead et al., 2011). Neither component of the formulation on its own was effective for silencing factor VII and ended up in the kidneys, similar to injection of naked (not formulated for delivery) siRNA. However, mixing the two lipidoids into binary nanoparticles allowed the siRNA to enter hepatocytes and silence the target with an IC₅₀ of 1.5

mg/kg (Whitehead et al., 2011). Further analysis of the binary formulation revealed that one component was responsible for getting through the cell membrane and the other component was critical for escaping the endosomal compartment. Pushing ahead with such innovative solutions to systemic delivery of therapeutic RNA ensures that human health will benefit from our efforts to understand RNA silencing.

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Appendix I: microRNA Therapeutics

microRNA Therapeutics

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Summary

MicroRNAs (miRNAs) provide new therapeutic targets for many diseases, while their myriad roles in development and cellular processes make them fascinating to study. We still do not fully understand the molecular mechanisms by which miRNAs regulate gene expression nor do we know the complete repertoire of mRNAs each miRNA regulates. However, recent progress in the development of effective strategies to block miRNAs suggests that anti-miRNA drugs may soon be used in the clinic.

Introduction

MicroRNAs (miRNAs) are 21–23 nt long RNAs that direct Argonaute proteins to bind to and repress complementary mRNA targets. The human genome contains more than 500 miRNAs, and each miRNA can repress hundreds of genes, regulating almost every cellular process^{1,2}. Individual miRNAs are often produced only in specific cell types or developmental stages.

Inappropriate miRNA expression has been linked to a variety of diseases^{3,4}. For example, the *let-7* miRNA prevents proliferation of cancer stem cells. miRNAs have roles in metabolic diseases such as obesity and diabetes; differentiation of adipocytes is promoted by miR-143 and insulin secretion is regulated by miR-375 in pancreatic-islet cells. Mutation of just a single nucleotide in the sequence of a miRNA or its mRNA target can eliminate target regulation. Mutation of the fifth nucleotide of miR-96 is associated with autosomal dominant, progressive, high-frequency hearing loss in humans; the mutation decreases the levels of miR-96 and impairs target mRNA repression⁵. A different mutation in miR-96 was discovered in a mouse mutant with hair cell defects and progressive hearing loss⁶. In contrast to mutation of miRNAs, normal miR-122 participates in the development of liver disease: hepatitis C virus (HCV) hijacks this miRNA, making miR-122 required for HCV to replicate in the liver⁷. Some viruses express their own miRNAs, presumably to repress cellular mRNAs that would otherwise interfere with viral infection⁸. Tissue-specific miRNAs may also be involved in the pathogenesis of cardiovascular, muscular and neurodegenerative diseases. Thus, molecules that alter the function or abundance of specific miRNAs represent a new strategy for treating human

disease.

miRNAs are transcribed by RNA polymerase II and matured by RNase III enzymes in two steps

miRNAs are transcribed from their own genes by RNA polymerase II. Consequently, miRNA primary transcripts (**pri-miRNAs**) begin with 5' 7-methylguanosine caps and end with 3' poly(A) tails. The **pre-miRNA**, a ~65 nt stem-loop structure that contains the miRNA and its corresponding miRNA* within its stem, resides within the pri-miRNA (Figure A.1). Cleavage of the pri-miRNA by the ribonuclease III (RNase III) enzyme, Drosha, releases the pre-miRNA stem-loop, which bears the 2 nt 3' overhanging ends characteristic of RNase III enzymes. The pre-miRNA is then exported to the cytoplasm, where its loop is removed by a second, RNase III enzyme, Dicer, that specifically recognizes the pre-miRNA structure, including its 2 nt 3' overhanging end. The resulting miRNA/miRNA* duplex is then loaded into a member of the Argonaute family of proteins. Subsequently, the miRNA* strand departs from the Argonaute protein, producing a mature, active miRNA:protein complex.

miRNAs provide the specificity determinants for Argonaute proteins

Binding of a miRNA:Argonaute protein complex to the 3' untranslated region (UTR) of an mRNA silences its expression¹. The human genome encodes four closely related Argonaute proteins, Ago1, Ago2, Ago3, and Ago4, and most tissues and cultured mammalian cell lines express all four, albeit in different proportions. Argonaute proteins are structural homologs of the DNA-guided ribonuclease, RNase H. Ago2 can cleave its target RNAs (after the nucleotide paired to the tenth base of the small RNA guide), but the other three human

Argonaute proteins have lost the capacity for such site-specific, small RNA-directed, endonucleolytic target cleavage.

Argonaute-catalyzed target cleavage requires extensive, but not complete, complementarity between the miRNA guide and an mRNA. However, human miRNAs generally base pair only partially with their target mRNAs. In fact, as few as six base pairs between a special region of the miRNA, the “seed sequence” (miRNA nucleotides 2 through 7 or 8), and an mRNA, may suffice to recruit Argonaute to repress the mRNA (Figure A.2). Consequently, most miRNAs do not direct Argonaute to slice the target mRNA, but instead the miRNA:Argonaute complex triggers general degradation of the target mRNA⁹. In some cases, the miRNA may block the translation of the mRNA into protein¹⁰. Because a miRNA need only pair only partially with its mRNA target, a single miRNA can repress hundreds of genes¹⁰⁻¹⁶. Most mRNAs contain multiple potential miRNA-binding sites in their 3' UTRs^{1,12}. Current computational estimates suggest that more than half of all human genes are regulated by miRNAs at some time or place in human development.

Viral miRNAs target cellular and viral mRNAs

Mammalian viruses produce at least 66 distinct miRNAs. Most miRNA-producing viruses are double-stranded DNA (dsDNA) viruses from the herpes virus family; no miRNAs have been detected from RNA viruses such as retroviruses or flaviviruses, or the papillomavirus, a dsDNA virus^{17,18}. One known function of viral miRNAs is to thwart the immune response during infection by regulating the expression of the major histocompatibility complex class I chain-related molecule B (MICB), a natural killer cell ligand¹⁹. Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV) and human

cytomegalovirus (HCMV) express miRNAs that target separate sites in the MICB mRNA to prevent its expression. Blocking these miRNAs might permit a more robust immune response to herpesvirus infection.

HCMV expresses a miRNA, miR-UL112, that targets the 3' UTR of MICB at a site overlapping the binding site for the cellular miRNA, miR-373. Interestingly, the MICB mRNA also contains nearby sites for two other cellular miRNAs, miR-376a and miR-433. After 72 hours of HCMV infection, the viral miR-UL112 and the cellular miR-376a collaborate to silence expression of MICB²⁰. KSHV express three miRNAs, each with the same seed sequence as a cellular miRNA. One of the viral miRNAs, miR-K12-11, represses the same set of mRNAs as its cellular counterpart, miR-155.

A second function for viral miRNAs may be to repress host mRNAs so as to maintain viral latency^{21,22}. KSHV miR-9* binds the 3' UTR of the major lytic-switch mRNA, preventing expression of the protein that controls viral reactivation from latency²³. Expression of two cellular miRNAs, miR-200b and miR-429, correlates with EBV lytic gene expression. These two microRNAs cause EBV to enter the lytic phase by repressing ZEB1 and ZEB2 protein expression²⁴. miRNA inhibitors may some day be used to coax latent viruses into the more therapeutically tractable replicating state, allowing the elimination of reservoirs that enable viral reemergence after anti-viral therapy is completed.

Artificially introducing or inhibiting miRNAs provides clues to their function

To understand the role of miRNAs in normal cellular processes and in human disease, we need tools to increase and decrease miRNA function or abundance. Expression of exogenous small RNAs in cells is possible through transient or stable transfection or viral transduction of a pri-miRNA transgene, pre-miRNA,

mature miRNA / miRNA*, small interfering RNA (siRNA) or short hairpin RNA (Figure 3). Using this strategy, intratumoral injection of exogenous *let-7* was found to block tumor development in a mouse model of non-small cell lung cancer²⁵⁻²⁸. Similarly, reintroduction of miR-26a in a mouse model of liver cancer caused regression of tumors²⁹. The opposite strategy—targeting miRNAs for inhibition—has yielded interesting results in vivo. For example, inhibition of miRNA-132 prevented angiogenesis in an orthotopic mouse model of ovarian and breast carcinoma and inhibition of miRNA-21, a miRNA that promotes oncogenesis, led to regression of malignant pre-B lymphoid tumors³⁰⁻³².

Antisense oligonucleotides (ASOs) are short, single-stranded RNA or DNA molecules that bind other nucleic acids by Watson-Crick base pairing. Traditional ASOs target a specific mRNA in order to block its translation into protein (e.g., morpholino ASOs) or to trigger its destruction by recruiting RNase H to hydrolyze the RNA strand of an RNA:DNA duplex (“gapmer” ASOs). ASOs are used in vitro and in vivo to discover gene function, and some ASOs are being tested in clinical trials. ASOs were first shown to inhibit specific miRNAs in cultured cells and in invertebrates in 2004. Subsequent studies have examined various chemical modifications of ASOs to improve their in vitro and in vivo stability and to improve their in vivo delivery. Moreover, some ASO chemistries can trigger the destruction of a miRNA through a mechanism recently discovered by using miRNA inhibitors. The stability of a miRNA is determined by the Argonaute protein with which it associates and the degree of sequence complementarity between the miRNA and its target mRNA³³. When a miRNA encounters a target to which it can pair extensively—including miRNA inhibitor oligonucleotides, it is tailed with adenosines or uridines and subsequently

degraded. This type of regulatory mechanism, dependent on the presence of a target and the extent of pairing to a complementary RNA, is critical to strategies that aim to inhibit or replace miRNAs to discover their roles in cellular processes or pathogenic mechanisms.

Modified antisense oligonucleotides can help define the molecular function of an individual miRNA

The molecular function of an individual miRNA can be discovered by inhibiting it and measuring the resulting changes in the levels of each mRNA or protein in the cell or by evaluating other phenotypic changes, such as developmental defects, cell proliferation, organ function, lipid metabolism, or behavior. ASOs engineered to withstand degradation by extra- and intracellular nucleases can effectively inhibit miRNAs in whole animals^{34,35}. The first ASOs used to inhibit miRNAs were composed of 2'-O-methyl ribose-modified RNA. Such 2'-O-methyl oligonucleotides proved to be effective miRNA inhibitors when introduced by lipid-mediated transfection into cultured human cells or by injection in whole nematodes (*Caenorhabditis elegans*). Dextran-conjugated ASOs can be injected into the *C. elegans* germ line and block the function of a specific miRNA function in the progeny³⁶.

“Antagomirs” were the first miRNA inhibitors demonstrated to work in mammals (Figure A.3). Because the amount of pre-miRNA was unchanged by the antagomir, ASOs likely target the mature miRNA^{37,38}. These synthetic ASOs contain 2'-O-methyl modified ribose sugars, terminal phosphorothioates, and at the 3' end a cholesterol group, which helps deliver the antagomir to cells. Cholesterol conjugation causes cellular uptake of the modified nucleic acid oligonucleotide by promoting its association with high-and low-density

lipoproteins that can bind cell surface membrane receptors: the Scavenger receptor BI for HDL and the LDL receptor for LDL. Intravenous injection of 80 mg of antagomir per kg mouse body weight on each of three successive days inhibited the corresponding miRNA in mouse liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenal glands. Nonetheless, antagomirs are unlikely to be used clinically, as they require higher doses to achieve the same efficacy as other ASO strategies.

Alternative RNA chemistries (Figure A.4) such as 2'-O-methoxyethyl (2'-MOE), 2'-fluoro, and 2',4' methylene ("locked nucleic acids" or LNAs) have greater affinity to bind and inhibit miRNA function in vivo than 2'-O-methyl RNA oligonucleotides³⁹⁻⁴¹. Alternative chemistries are also more resistant to degradation. In a test of stability of modified RNAs in 10% fetal bovine serum, 2'-fluoro RNA with LNA ends was less degraded after 24 hours than a 2'-O-methyl RNA with LNA ends or a DNA/LNA oligonucleotide, which was degraded within 2 hours³⁵. Phosphorothioates substitute a non-bridging oxygen atom on the phosphate group with a sulfur. Phosphorothioate bonds promote serum protein binding, thereby increasing the in vivo distribution and bioavailability of the ASO. A direct comparison of anti-miRNA oligonucleotide chemistries in vitro revealed that combining 2'-O-methyl and LNA with phosphorothioate ends was ~10 times more effective than the 2'-O-methyl or phosphorothioate modifications alone and twice as effective as the 2'-O-methyl with LNA modifications³⁵.

The 2',4' methylene bridge in LNAs constrains the ribose to the C3' endo conformation present in RNA:RNA and DNA:RNA helices. (DNA:DNA helices are C2' endo.) LNAs cannot interconvert between the C3' endo conformation,

which favors pairing with an RNA, and the C2' endo conformation, which does not. Consequently, an LNA modification increases RNA:RNA melting temperature by 2.4°C per modification and confers high specificity for their target sequences. Moreover, locked nucleic acids are resistant to many endonucleases. ASOs containing LNAs are effective probes for accurate detection of miRNAs by Northern blotting, in situ hybridization and, most importantly, are potent miRNA inhibitors in vivo.

The unique target mRNA-binding properties of a miRNA bound to an Argonaute protein—nearly all the binding specificity comes from the seed sequence—allow antisense miRNA inhibitors to be shorter than the miRNA itself. A 16 nt LNA-modified oligonucleotide complementary to miRNA-122 injected intravenously each day for five successive days at 10 mg/kg, lowered plasma cholesterol levels for more than 20 days in African green monkeys⁴². This pioneering non-human primate study established that LNA-modified anti-miRNA oligonucleotides are specific, stable, and non-toxic when administered intravenously. A subsequent study showed that Hepatitis C virus (HCV) replication could be inhibited in chimpanzees by a 15 nt LNA oligonucleotide targeting miRNA-122⁴³. Two chimpanzees were injected intravenously with 5 mg per kg of LNA inhibitor each week for 12 weeks. Two weeks after treatment ended, viral titer was 400- and 200-times lower in serum and liver, respectively. Free anti-miR-122 LNA was detected in liver for 8 weeks after treatment ceased, until week 25, at which point the drug had declined significantly and the level of miR-122 had increased. No liver toxicity was detected, and treatment was associated with improved liver histology, presumably due to prolonged suppression of viremia and normalization of the interferon pathway. No viral

escape was detected by sequence analysis of the HCV RNA target sites for miR-122 at the 16th week, in contrast to treatment with an antiviral non-nucleoside polymerase inhibitor with which resistance mutations occur after 2 days of treatment. miRNA-122 inhibition by LNA-modified oligonucleotides is now being tested in humans. A successful phase 1 trial has paved the way for a phase 2 study that will assess the safety and tolerability of weekly or bi-weekly subcutaneous injections of the anti-miR-122 LNA in 55 patients with chronic HCV genotype 1 infection.

Advancements in delivery formulations reduce the effective dose of ASOs

Delivering a therapeutic RNA to its target tissue starts with the challenge of its exiting the circulatory system into a target tissue, transiting the cell membrane, and, finally, escaping from endosomal vesicles into the cytoplasm. The size of an unconjugated therapeutic RNA is 7–20 kDa. Molecules smaller than 50 kDa are filtered by the kidneys and excreted. Transfer of therapeutic RNA from the blood to the target tissue is a challenge because anything longer than 5 nm diameter, including therapeutically complexed RNA, cannot cross the capillary endothelium and will remain in circulation until filtered by the kidneys. Local delivery of therapeutic RNA by injection increases its bioavailability in target tissue and minimizes uptake in non-target tissues, but is limited to eye, skin, mucous membranes and tumors. Systemic delivery into the bloodstream is challenged by phagocytic immune cells such as macrophages and monocytes, which remove complexed RNAs from the body. Most ASOs delivered to muscle, heart and bone end up not in the cytoplasm, where they can find their target mRNAs, but in phagolysosomes. Cells of the liver, spleen and some tumors

allow molecules up to 200 nm in diameter to enter and so the liver is among the most successful organs for delivering therapeutic RNAs.

In cases where localized delivery is not possible, delivery using PEGylated liposomes, lipidoids and biodegradable polymers are alternatives (Figure A.5).

To avoid being filtered by the kidneys and enhance intracellular delivery nucleic acids can be encapsulated in lipids forming vesicles between 50 and 500 nm.

Liposomes are lipid bilayers with an aqueous core that contains the nucleic acid cargo. Lipoplexes are liposomes that contain cationic lipids that drive the interaction between the lipid bilayer and the negatively charged nucleic acid molecules. The anionic charge of the nucleic acids and their hydrophilicity is counterbalanced by the cationic lipids, resulting in a net positive charge, enabling the liposomes to bind to anionic cell surface molecules. The composition of these lipid particles can be tailored to facilitate fusion with the cytoplasmic, endosomal or nuclear membrane, as well as to promote endosomal release once inside the cell. The lipid head group, for example, can be pH sensitive, so that the liposomes interact with anionic phospholipids in the endosome, generating non-bilayer structures that disrupt the endosomal membrane, liberating the RNA.

In a screen of a library of ionizable cationic lipids with superior siRNA delivery capacity, a lipid nanoparticle (LNP) formulation was identified that substantially improved delivery. To test this delivery formulation, an siRNA that targets the hepatocyte mRNA transthyretin (TTR) was used because this protein has a short half-life and TTR protein levels can be easily measured in serum. The LNP delivery strategy in rodents and nonhuman primates was effective at 0.01 mg per kg and 0.1 mg per kg, respectively, administered as a single dose of TTR siRNA by 15 minute, intravenous, cephalic vein infusion at 5 ml per kg⁴⁴. Forty-

eight hours later, the siRNA reduced TTR mRNA levels by 30% in the livers of three Cynomolgus monkeys. Another potent delivery formula, the lipidoid, contains lipids with an amine-containing polar head group and nonpolar alkyl tails that are 12 carbons in length. When tested in nonhuman primates, this delivery formulation was effective at 0.03 mg per kg of siRNA. Forty-eight hours after infusion, the 0.03 mg per kg dose of the TTR siRNA in the lipidoid formulation reduced TTR mRNA levels by 70%⁴⁵. These advanced delivery formulations are almost 100 times more effective than typical lipid-based delivery carriers, which require doses of at least 1 mg/kg of siRNA to achieve 50% gene silencing. We anticipate that such advanced lipid systems will be useful in delivering anti-miRNA ASOs to specific tissues and organs in humans.

“Decoy” transcripts can compete for miRNAs, blocking their function

ASOs act, at least in part, as competitive inhibitors of miRNAs, suggesting that miRNA-binding RNA transcripts may also be designed to sequester and thereby inhibit specific miRNAs. Such miRNA decoys could provide an inexpensive alternative to proprietary oligonucleotide chemistries and delivery formulations, enabling research laboratories to examine the consequence of inhibiting each known miRNA in a particular cultured cell⁴⁶ or model animal⁴⁷ or plant⁴⁸. Moreover, miRNA-binding transcripts can be expressed from viral vectors, allowing the development of anti-miRNA gene therapy approaches. Ironically, the first demonstration of such transcripts, miRNA “decoys”⁴⁹ or “sponges”⁴⁶, preceded the discovery that plants naturally use such miRNA-binding transcripts to reduce the activity of specific miRNAs⁵⁰.

The first miRNA decoy consisted of an adenoviral vector with two sites for the muscle-specific miRNA-133 inserted in the 3' UTR of a GFP reporter gene,

under control of an RNA polymerase II promoter from cytomegalovirus (CMV). This viral vector was used to confirm that loss of miRNA-133 expression, in mouse and human disease models, leads to cardiomyocyte hypertrophy⁴⁹. Unlike chemically modified anti-miRNA oligonucleotides, miRNA decoys that include GFP allow one to determine the tissues or cell types where a miRNA is produced, as GFP will be repressed where the miRNA is present. “miRZips” and “TuD RNAs” (tough decoy RNAs) are microRNA decoy targets transcribed by RNA polymerase III (H1 or U6)^{51,52}. Their nuclear export has been optimized to achieve high cytoplasmic expression. miRZips use the RNA polymerase III H1 promoter to express a single microRNA decoy hairpin with one arm that is perfectly complementary to the microRNA. This strategy causes degradation of the microRNA. TuD RNAs use the RNA polymerase III U6 promoter to express an RNA that contains multiple microRNA binding sites between 18 bp stem regions that help prevent nuclease degradation of the RNA decoy targets. For the TuD RNAs, the binding site is perfectly complementary to the miRNA, but contains 4 nt inserted at the site of Ago2 cleavage to prevent the TuD RNA from being inactivated. Such adaptations of the miRNA sponge concept allow longer term inhibition of microRNAs than can be achieved by transient transfection of RNA polymerase II sponge reporter vectors or modified RNA oligonucleotides.

miRNA sponges can be stably integrated into chromosomes, designed to be drug-inducible or controlled by promoters whose expression is restricted to a desired cell type, tissue, or developmental stage. For example, in *Drosophila*, a miRNA sponge for miR-8 revealed that post-synaptic expression of miR-8 is required for proper development of the neuromuscular junction⁴⁷. Further modification of the sponge concept is underway to create separate sponge-

expressing lines of transgenic fruit flies for each fly miRNA. Each line can be crossed with a second fly strain producing a transcription factor that promotes sponge expression in a specific cell type or developmental time, allowing discovery of the contribution of a miRNA to development, physiology or behavior⁴⁷. In the future, we anticipate that transgenic sponges will be designed to permit their expression in mice at particular developmental stages or in specific tissues, perhaps by using the well established Cre-*loxP* system^{53,54}.

miRNA replacement therapy seeks to reintroduce a missing miRNA

Some diseases may be due to loss or reduced expression of a particular microRNA. Interestingly, expression of most miRNAs in cancer is lower than normal. For example, the miRNA *let-7* represses expression of the oncogenes Ras, Myc and HMGA-2, and *let-7* levels were found to be low and HMGA2 mRNA high in primary tumors derived from 100 patients diagnosed with ovarian cancer. *let-7* expression was also reduced in mammosphere-derived cancer stem cells when compared with normal breast or non-selected tumor cells, indicating that *let-7* may prevent proliferation of cancer-initiating stem cells^{25,55}. p53 expression caused by DNA damage promotes transcription of the miRNA-34 miRNA family, which is deleted in some cancers. miRNA-15 and 16 are frequently deleted in B-cell lymphocytic leukemia, and their expression is reduced by 80% in prostate cancer. Other miRNA genes, including *let-7*, reside at fragile sites where chromosomes often break, leading to cancer⁵⁶. Thus, many miRNAs meet the classical definition of tumor suppressor genes. Replacement of such tumor suppressor miRNAs might augment traditional cancer chemotherapy.

miRNAs whose expression is lost or reduced can be replenished by adding back the miRNA. Adding the miRNA back in a single dose may not allow sustained target regulation due to inefficient delivery or degradation, but data from multiple doses of siRNAs suggest that three-to-five doses of replacement miRNA, modified or formulated for optimal delivery, might provide sufficient miRNA for 20 to 30 days. Alternatively, cells can be infected with viral vectors encoding short hairpin RNAs (Figure 3) that are processed in the cell into mature miRNAs^{26,27,56}. Viral delivery of miRNAs can be optimized to achieve a specific and continuous level of expression.

miRNA replacement therapy must be both effective and safe. Over expression of shRNA in rats caused hepatotoxicity, organ failure and death⁵⁷. Argonaute proteins and the pre-miRNA export protein, Exportin-5 limit the amount of exogenous siRNA or miRNA that a cell can tolerate⁵⁷⁻⁶². shRNAs that are more pre-miRNA-like or authentic pre-miRNAs themselves will likely minimize toxicity while retaining potency for their intended targets^{60,63}.

miRNA-directed regulation can improve traditional gene therapy approaches

Gene therapy holds great promise to replace defective protein-coding genes underlying many genetic diseases. However, ensuring expression of the therapeutic transgene in the correct tissue while minimizing its expression elsewhere remains challenging because even tissue-specific promoters can be leaky. Combining miRNA regulation with gene therapy allows targeted and potent expression of transgenes. Such “de-targeting” strategies incorporate miRNA target sites in the 3' UTR of the therapeutic transgene, preventing its expression in cells that express the corresponding miRNA. The transgene will be expressed in the intended cell-type, where the miRNA is not expressed. For

example, miRNA-122 is specific to the liver, so systemically delivered transgenes containing binding sites for miRNA-122 will be silenced in hepatocytes, but not cells elsewhere. This strategy was used to restrict the expression of a transgene in a lentiviral vector to astroglial cells⁶⁴. Starting with a lentivirus engineered to preferentially infect neurons and glia, miRNA-124 target sites were inserted in the 3' UTR to prevent transgene expression in neuronal cells, which express miRNA-124, and allow transgene expression in glial cells, which do not express miRNA-124. Injection of the vector into the hippocampus in mice produced transgene expression in astrocytes and Bergmann glial cells, but not in pyramidal neurons or Purkinje cells⁶⁴. Since each site is only 21 nt long, binding sites for multiple, tissue-specific miRNAs can be incorporated in the 3' UTR, extinguishing transgene expression in many different tissues simultaneously.

miRNA-mediated transgene detargeting has also been used to promote immune tolerance of a transgene-encoded antigen. Annoni and colleagues exploited the tissue specificity of miRNA-142, which is expressed only in hematopoietic cells, to prevent a lentiviral vector from producing transgenic protein in antigen presenting cells⁶⁵. By blocking transgene expression in immune cells, they avoided the common problem of T-cells detecting and eliminating cells expressing the foreign transgenic protein. Interestingly, a control experiment to prevent expression in the liver using miRNA-122 binding sites revealed that liver expression of the transgene was required to induce antigen tolerance.

Replication-selective oncolytic viruses—genetically engineered adenoviruses that selectively infect and kill tumor cells—have been proposed as alternatives to standard chemotherapy. Avoiding expression in the liver is

particularly important as adenovirus-based therapies cause liver toxicity. Since neuroendocrine tumors of the ileum can metastasize to the liver⁶⁶, a key challenge is to produce the transgenic protein in the cancer cells residing in the liver, but not in untransformed hepatocytes. Whyte and colleagues proposed a clever solution to this problem. They used the chromogranin-A promoter, which is active in neuroendocrine tumors, to specifically express the E1A protein, a viral protein that activates viral and cellular genes critical for viral infection, while adding miRNA-122 binding sites to the 3' UTR of the E1A mRNA to prevent viral replication in hepatocytes⁶⁷. In a mouse model, the miRNA-regulated, oncolytic adenovirus killed tumor cells without detectable liver toxicity.

Unique miRNA expression patterns in stem cells can be exploited to select for a specific cell type from a mixed cell population, before adding cells back to the patient and as a strategy for monitoring lineage-specific differentiation of induced pluripotent and embryonic stem cells. In stem cell therapy applications, where cells are engineered to express normal genes that are mutated in the patient, it is critical to remove the pluripotent cell population from the therapeutic differentiated cells before transplanting them back into the patient to prevent unwanted proliferation and tumor development. Expression of a suicide gene or fluorescent reporter can be controlled by miRNAs whose expression is specific to a differentiated cell type in a population of pluripotent stem cells⁶⁸. Differentiation-induced miRNA expression could turn off the reporter gene to allow separation of the differentiated cells from the pluripotent cells. A suicide gene can also be turned off by cell type specific miRNAs to allow differentiated cells of a specific lineage to proliferate. Additionally, by combining multiple

miRNA target sites, expression of a transgene can potentially be suppressed in multiple cell types or tissues. Such a strategy requires calibrating miRNA expression and target site affinity so that the desired level of regulation of the transgene is achieved.

Prospects

The realization that the inappropriate production of individual miRNAs contributes to disease has reinvigorated antisense oligonucleotide drug development. ASOs readily inhibit miRNAs—far more reliably than they do mRNAs, and the unique properties of Argonaute proteins permits the use of remarkably short ASOs: 15 nt oligonucleotide ASO are now in clinical trials and 8 nt versions show promise in non-human primates. Many new roles for individual miRNAs in disease, aging and cancer are likely to emerge over the next five years. Once the role of a specific miRNA in disease pathogenesis is established, selecting specific anti-miRNA inhibitor chemistries and delivery strategies promises to be straightforward. Nonetheless, effective and safe delivery of anti-miRNA drugs remains difficult for many cell types such as brain and muscle. Thus, treating diseases with anti-miRNA oligonucleotides will require the development of novel modification, conjugation or formulation strategies. It is our hope that the anti-miRNA therapeutics field will soon converge on a small number of “platform” technologies that allow a rapid and safe development path from academic discovery to effective drug.

Figures

Figure A.1. microRNA biogenesis pathway.

Broderick and Zamore, Figure 1

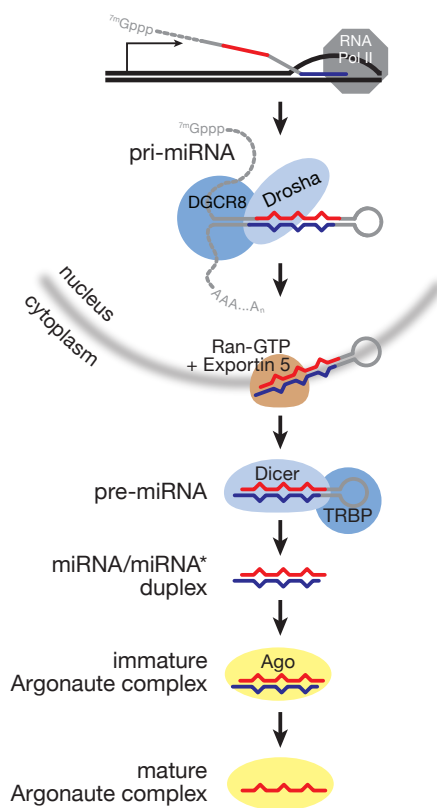


Figure 1. miRNA biogenesis in mammals.

Figure A.2. miRNAs bind target mRNAs via their seed sequence.

Broderick and Zamore, Figure 2

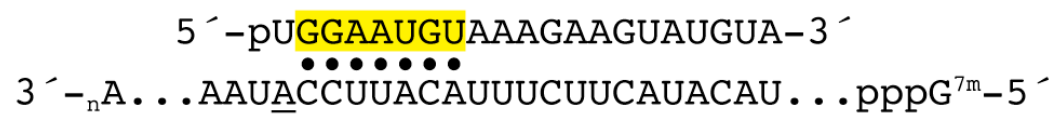


Figure 2. miRNAs bind target mRNAs via their seed sequence. Typical miRNA-binding sites also feature an adenosine (underlined) across from the first nucleotide of the miRNA, even though the structure of a miRNA bound to an Argonaute protein precludes base pairing at this position.

Figure A.3. miRNA replacement strategies.

Broderick and Zamore, Figure 3

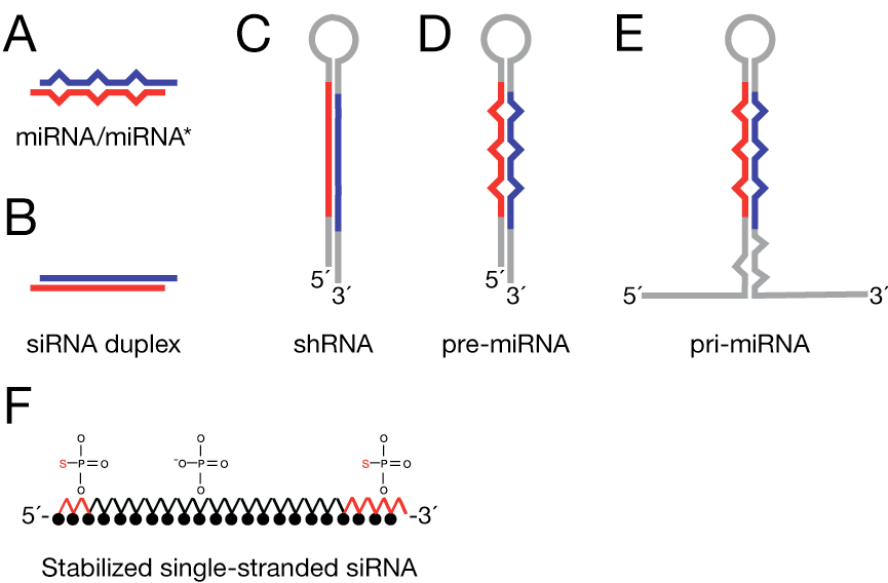


Figure 3. miRNA replacement strategies: (A) mature miRNA/miRNA* duplex; (B) small interfering RNA duplex; (C) small hairpin RNA; (D) pre-miRNA; (E) pri-miRNA; (F) modified single stranded RNA.

Figure A.4. Strategies for delivery of anti-miRNA oligonucleotides to cells in vivo.

Broderick and Zamore, Figure 4

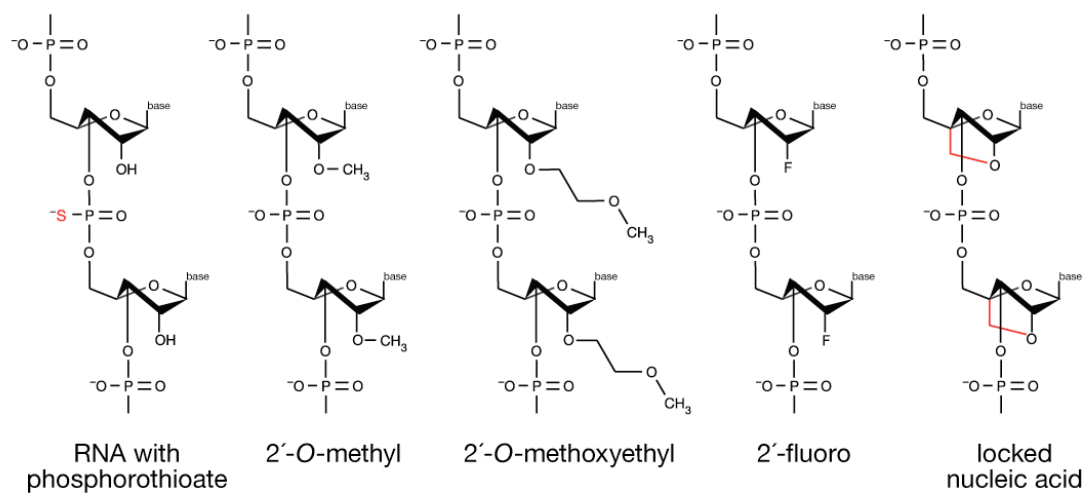


Figure 4. Strategies for delivery of anti-miRNA oligonucleotides to cells in vivo.

(A) Modification. Black filled circles represent 2'-*O*-methyl, 2'-*O*-methoxyethyl, or 2'-fluoro modified nucleotides. (B) Conjugation. Antagomirs are 2'-*O*-methyl oligonucleotides conjugated to cholesterol at their 3' ends, and contain phosphorothioate linkages between nucleotides at both ends in place of natural phosphate linkages. (C) Formulation. Lipid nanoparticles are lipid vesicles containing therapeutic RNA. The formulated lipid bilayer encapsulates the therapeutic RNA, delivering it to cells and promoting fusion with the phospholipid bilayer of cell membranes. Individual lipids within the vesicle bilayer can contain ionizable head groups that will disrupt the endosome at low pH to release the therapeutic RNA to the cytoplasm.

Figure A.5. Chemical modifications that improve the stability, biodistribution, and delivery of ASOs.

Broderick and Zamore, Figure 5

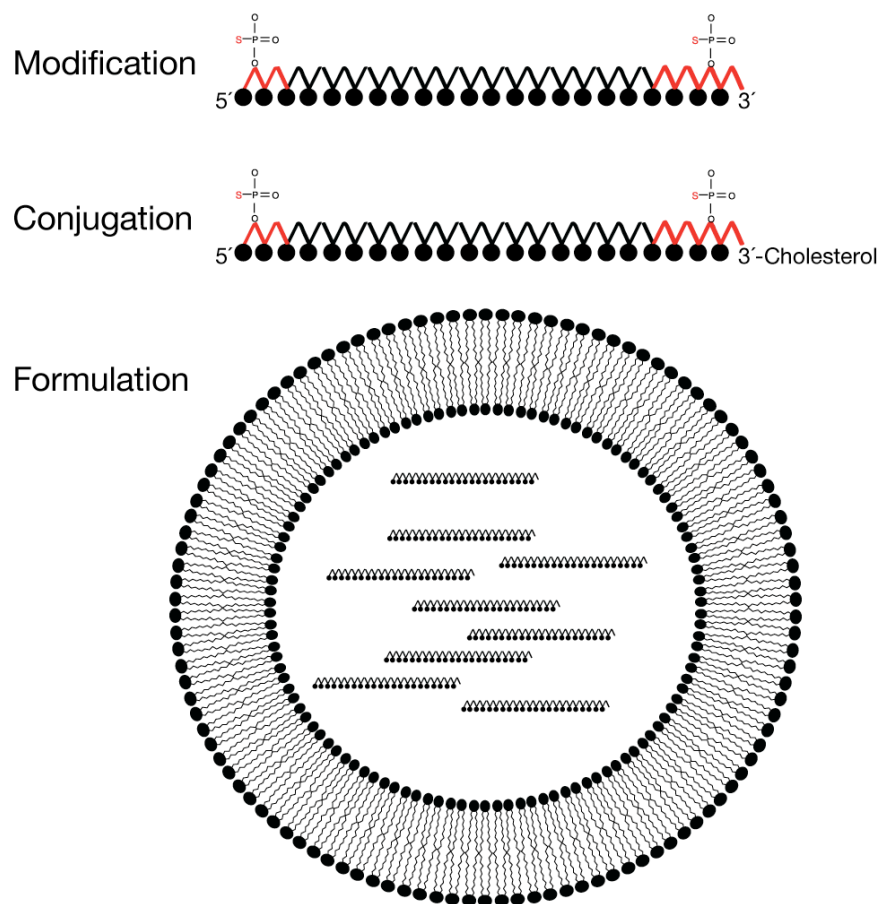


Figure 5. Chemical modifications that improve the stability, biodistribution, and delivery of ASOs. RNA (red; S indicates sulfur substitution of a non bridging oxygen to make a phosphorothioate linkage between nucleotides), 2'-O-methyl RNA contains a methyl group bound to the 2' oxygen of the ribose; 2'-O-methoxyethyl RNA contains a methoxy group bound to the 2' oxygen of the ribose; 2'-fluoro RNA contains fluorine molecule bound to the 2' oxygen of the ribose; and locked nucleic acid (red) introduces a 2',4' methylene bridge in the ribose to form a bicyclic nucleotide).

Table A.1. miRNA therapeutics in commercial development.

Company	Diseases	Chemistries	Stage	References
Regulus Therapeutics	immuno-inflammatory, cardiovascular, metabolic disease, oncology, fibrosis, and hepatitis C infection	miRNA inhibitors using 2'-methoxyethyl , 2'- fluoro RNA, bicyclic ribose modifications	pre-clinical	Esau et al., 2006 Esau et al., 2007 Krutzfeldt et al., 2005 Krutzfeldt et al., 2007 www.regulusrx.com
Santaris Pharma A/S	cancer and inflammatory diseases, hepatitis C infection	miRNA inhibitors using locked nucleic acid chemistry	miR-122 inhibitor: Phase I completed, Phase	Ørom et al., 2006 Elmén et al., 2008 www.santaris.com
miRagen Therapeutics	cardiovascular and muscle diseases	miRNA inhibition and replacement	pre-clinical	www.miragentherapeutics.com
Mirna Therapeutics	non-small cell lung cancer and prostate	miRNA replacement using siRNAs	pre-clinical	www.mirnatherapeutics.com

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