

Human Argonaute2 Mediates RNA Cleavage Targeted by miRNAs and siRNAs

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Summary

Argonaute proteins associate with small RNAs that guide mRNA degradation, translational repression, or a combination of both. The human Argonaute family has eight members, four of which (Ago1 through Ago4) are closely related and coexpressed in many cell types. To understand the biological function of the different Ago proteins, we set out to determine if Ago1 through Ago4 are associated with miRNAs as well as RISC activity in human cell lines. Our results suggest that miRNAs are incorporated indiscriminately of their sequence into Ago1 through Ago4 containing microRNPs (miRNPs). Purification of the FLAG/HA-epitope-tagged Ago containing complexes from different human cell lines revealed that endonuclease activity is exclusively associated with Ago2. Exogenously introduced siRNAs also associate with Ago2 for guiding target RNA cleavage. The specific role of Ago2 in guiding target RNA cleavage was confirmed independently by siRNA-based depletion of individual Ago members in combination with a sensitive positive-readout reporter assay.

Introduction

Sequence-specific gene silencing triggered by double-stranded RNA (dsRNA) is a fundamental gene regulatory mechanism present in almost all eukaryotes (Béclin et al., 2002; Denli and Hannon, 2003; Dykxhoorn et al., 2003; Finnegan and Matzke, 2003; Grewal and Moazed, 2003; Hannon, 2002; Plasterk, 2002; Tijsterman et al., 2002a; Ullu et al., 2004). Gene silencing mediated by dsRNA has been shown to act at the transcriptional and post-transcriptional level. Naturally produced dsRNAs may derive from several different sources. Molecules of dsRNA may emerge during viral infection and replication (Li et al., 2002; Mourrain et al., 2000; Pfeffer et al., 2004; Voinnet et al., 1999; Waterhouse et al., 1998, 2001) or after transposition of mobile genetic elements (Ketting et al., 1999; Sarot et al., 2004; Sijen and Plasterk, 2003; Tabara et al., 1999). Transcribed pseudogenes (Hirotsune et al., 2003), endogenous repetitive gene loci (Aravin et al., 2001, 2003; Hamilton et al., 2002; Mette et al., 2002; Schramke and Allshire, 2003; Verdel et al., 2004; Wu-Scharf et al., 2000; Xie et al., 2004; Zilberman et al., 2003) or microRNA genes (Bartel, 2004; Carrington and

Ambros, 2003; Lai, 2003) represent other endogenous sources of dsRNA.

Molecules of dsRNA are processed by Drosha and/or Dicer RNase III enzymes into small dsRNA molecules of specific length and structure (Bernstein et al., 2001; Elbashir et al., 2001a; Lee et al., 2002, 2003; Provost et al., 2002; Zamore et al., 2000; Zhang et al., 2002) and can enter into various gene silencing pathways that are collectively referred to as RNA silencing (Grishok et al., 2001; Hutvagner and Zamore, 2002; Lee et al., 2004; Olsen and Ambros, 1999; Slegger et al., 2002; Tijsterman et al., 2002a; Vastenhouw et al., 2003; Verdel et al., 2004; Xie et al., 2004). Depending on the source of dsRNA, the products of Dicer processing are termed small interfering RNAs (siRNAs), repeat-associated small interfering RNAs (rasiRNAs), or microRNAs (miRNAs). The siRNAs and rasiRNAs can be derived from any segment of the processed long dsRNA. In contrast, miRNAs are of uniquely defined sequence and are excised from shorter stem-loop-structured dsRNA in a non-random manner. Although it is not clear if these three types of small RNAs enter the same effector complexes, rasiRNAs, siRNAs, and miRNAs have all been shown to guide the cleavage of RNA substrates having a high level of sequence complementarity (Elbashir et al., 2001a; Hutvagner and Zamore, 2002; Sijen and Plasterk, 2003), a process typically referred to as RNA interference (RNAi). The effector complex targeting single-stranded complementary RNAs for degradation is known as the RNA-induced silencing complex (RISC) (Hammond et al., 2000). miRNAs can not only act to guide RNA cleavage (Llave et al., 2002; Palatnik et al., 2003; Pfeffer et al., 2004; Tang et al., 2003; Yekta et al., 2004), but are predominantly considered to act as translational repressors on partially complementary, evolutionary conserved sequences in the 3' UTR of the target mRNAs (Aukerman and Sakai, 2003; Olsen and Ambros, 1999; Slegger et al., 2002).

Dicer RNases process dsRNA and miRNA hairpin precursor molecules predominantly in the cytoplasm (Bernstein et al., 2001; Billy et al., 2001; Tang et al., 2003; Zamore et al., 2000). Dicer contains an N-terminal RNA helicase domain, a central RNA binding PAZ domain, two RNase III catalytic domains, one or two dsRNA binding domains (dsRBDs), and a domain of unknown function (Carmell and Hannon, 2004; Cerutti et al., 2000; Zhang et al., 2004). In *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and vertebrate animals, only a single Dicer gene has been identified (Bernstein et al., 2003; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Volpe et al., 2002; Wienholds et al., 2003). In *Drosophila melanogaster*, two Dicer paralogs operate with distinct functions: Dcr-1 is required for miRNA precursor processing while Dcr-2 is predominantly involved in processing of long dsRNA (Lee et al., 2004). In *Arabidopsis thaliana*, four Dicer paralogs have been identified, three of which have been implicated in processing of different sources of dsRNA (Schauer et al., 2002). DCL1 processes miRNA precursors (Park et al., 2002; Reinhart et al., 2002), DCL2 is

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required for production of siRNAs from plant viruses (Xie et al., 2004), and DCL3 is involved in the production of rasiRNAs (Xie et al., 2004). In the filamentous fungus *Neurospora crassa*, two Dicer genes of redundant function have been described (Catalanotto et al., 2004).

Small RNA duplexes undergo assembly into ribonucleo-protein effector complexes. (Elbashir et al., 2001a; Liu et al., 2003; Martinez et al., 2002; Mourelatos et al., 2002; Pham et al., 2004; Schwarz et al., 2003; Tang et al., 2003; Tomari et al., 2004; Tuschl et al., 1999; Zamore et al., 2000). Effector complexes contain only a single-stranded RNA that guides the sequence-specific recognition of complementary target nucleic acids (Hutvagner and Zamore, 2002; Martinez et al., 2002). Synthetic RNA duplexes or hairpin RNAs that resemble the processing products of the various RNase III enzymes in terms of structure and length also effectively enter RNA silencing pathways. In mammalian cells, short dsRNAs are commonly used to knock down gene expression since they generally do not trigger an interferon response (Dorsett and Tuschl, 2004).

All characterized RNA silencing effector complexes contain at least one Argonaute protein (Hammond et al., 2001; Hutvagner and Zamore, 2002; Martinez et al., 2002; Mourelatos et al., 2002; Tabara et al., 1999; Verdel et al., 2004). The Argonaute protein family constitutes the largest group of proteins specifically involved in dsRNA-triggered gene silencing. The number of Argonaute paralogs identified in different organisms ranges from one in *S. pombe* (Verdel et al., 2004) to more than twenty in *C. elegans* (Carmell et al., 2002; Grishok et al., 2001). Ten members have been identified in *A. thaliana* (Morel et al., 2002), five members in *D. melanogaster* (Williams and Rubin, 2002), and eight members in human (Sasaki et al., 2003).

Based on amino acid sequence alignments, the Argonaute protein family has been subdivided into two subfamilies referred to as the Ago and Piwi family (Carmell et al., 2002). Argonaute proteins contain a PAZ (Cerutti et al., 2000) and a PIWI domain (Carmell et al., 2002; Cerutti et al., 2000). The PAZ domain is a novel RNA binding module that specifically recognizes the characteristic 2-nucleotide (nt) 3' overhang and the base-paired terminus of siRNA duplexes (Lingel et al., 2003, 2004; Ma et al., 2004; Song et al., 2003; Yan et al., 2003). A segment of the human PIWI domain has been shown to mediate protein-protein interaction between Argonaute proteins and Dicer, which may facilitate the incorporation of the siRNA into the effector complex (Doi et al., 2003; Pham et al., 2004; Tahbaz et al., 2004).

Current evidence suggests that for a particular organism, different Argonaute proteins are not functionally redundant. Genetic experiments in *A. thaliana* showed that Ago1 is involved in both RNAi and miRNA-guided gene silencing mechanisms (Bohmert et al., 1998; Fagard et al., 2000; Kidner and Martienssen, 2004; Vaucheret et al., 2004), and Ago4 has been implicated in guiding chromatin modifications (Chan et al., 2004; Zilberman et al., 2003). In *C. elegans*, the Argonaute proteins RDE-1 and PPW-1 have been implicated in RNAi (Tabara et al., 1999; Tijsterman et al., 2002b). ALG-1 and ALG-2 associate with miRNAs and are required for translational repression and/or miRNA-guided target

RNA cleavage (Grishok et al., 2001; Hutvagner et al., 2004). In *D. melanogaster*, Ago1 and Ago2 have been copurified in association with miRNAs and have been implicated in playing a role in RNAi (Caudy et al., 2002; Hammond et al., 2001; Ishizuka et al., 2002; Williams and Rubin, 2002). Most recently, it was also shown that Ago1, but not Ago2, was required for accumulation of miRNAs, and that Ago2, but not Ago1, was required for dsRNA- and siRNA-triggered RNAi (Okamura et al., 2004). The *D. melanogaster* Argonaute aubergine (Aub) and piwi are also involved in RNA silencing (Aravin et al., 2001; Kennerdell et al., 2002; Pal-Bhadra et al., 2002; Tomari et al., 2004), and play an additional role in transcriptional gene silencing mechanisms and histone methylation (Pal-Bhadra et al., 2002, 2004). The mouse Argonaute proteins Mili and Miwi are expressed during different yet overlapping stages of spermatogenesis (Kuramochi-Miyagawa et al., 2004). Knockout of Mili (Kuramochi-Miyagawa et al., 2004) or Miwi (Deng and Lin, 2002) leads to failure of spermatogenesis.

The mammalian members of the Ago subfamily have, for the most part, been characterized biochemically. Human Ago1/eIF2C1 and/or Ago2/eIF2C2 have been identified in affinity-purified RISC that was reconstituted by incubation of biotinylated siRNA duplexes in HeLa cell cytoplasmic extract (Martinez et al., 2002). Endogenous miRNA-associated RISC has also been immunoprecipitated with the monoclonal antibody 8C7 raised against full-length Ago2 (Hutvagner and Zamore, 2002; Mourelatos et al., 2002). However, the epitope for the antibody was not mapped, and it is unclear if the antibody crossreacts with other coexpressed Ago-paralogs of almost identical molecular weight (Z. Mourelatos, personal communication). Ago2 has also been described as a component of a 15S ribonucleoprotein complex (miRNP), which contains miRNAs, Gemin3, and Gemin4 (Mourelatos et al., 2002). Geminins are proteins previously shown to be involved in the assembly of ribonucleoprotein particles (RNPs) (Meister et al., 2002). Fragile X mental retardation protein (FMRp) has also been reported to be associated with human Ago2 and miRNAs (Jin et al., 2004). Although there is little biochemical data regarding the mammalian Piwi subfamily, mouse Miwi has been shown to associate with mRNAs and is proposed to stabilize certain mRNAs during spermatogenesis (Deng and Lin, 2002).

In summary, depending on the dsRNA source and the cell-type-specific expression of RNA silencing proteins, RNA silencing pathways can be distinct, partially or extensively overlapping. Some of the conserved key components of the RNA silencing machinery, including the dsRNA-processing ribonuclease Dicer, the RNA-dependent RNA polymerase (RdRP) (Dalmay et al., 2000; Mourrain et al., 2000; Smardon et al., 2000; Yu and Kumar, 2003) (absent in fruitfly and mammals), and the small-RNA binding protein Argonaute (Carmell et al., 2002; Sasaki et al., 2003; Zilberman et al., 2003) have evolved into multi-protein families. The dissection of the function of the individual paralogs of RNA silencing proteins in each species has become an important task.

In order to study the function of human Ago1 through Ago4, we have established HeLa cell lines stably expressing FLAG/HA-tagged Ago proteins and monitored the mRNA expression of individual members in various

cell lines. We examined the specificity of miRNA association with the various tagged Ago proteins, and correlated it to miRNA-guided cleavage activity using in vitro target RNA cleavage reactions. Our results indicate that although Ago1 through Ago4 bound miRNAs indiscriminately of their sequence, only Ago2-containing miRNPs were able to guide cleavage of complementary target RNAs. The role of Ago2 in guiding cleavage was demonstrated independently by using a positive-readout reporter cell line that expresses EGFP protein when RNA silencing factors are transiently knocked down by siRNAs.

Results

Variation of miRNA-Guided Cleavage Activity in HeLa Cell Cytoplasmic Extract

In HeLa cell cytoplasmic extracts, it was previously observed that artificial target RNAs complementary to let-7a miRNA (Hutvagner and Zamore, 2002) or miR-21 (Meister et al., 2004) are subjected to degradation by a mechanism identical to siRNA-guided target RNA cleavage (Elbashir et al., 2001a). To examine if other endogenous miRNAs form RISCs in HeLa cells, we prepared cleavage substrates containing segments complementary to the high abundance let-7a, miR-16, 17, 19b, 21, 24, 27a, and 92 as well as the low abundance miR-25, 32, and 96. The relative abundance of miRNAs was determined by qRT-PCR from small RNA libraries (see also Figure 4). As negative control for a miRNA not expressed in HeLa cells, we selected the neuron-specific miR-124a (Dostie et al., 2003; Lagos-Quintana et al., 2002). 5' ³²P-Cap-radiolabeled single-stranded substrate RNAs cognate to the respective miRNA were incubated for 2 hr with HeLa S100 cytoplasmic extract, and cleavage products were analyzed by gel electrophoresis. The abundant miRNAs let-7a, miR-17, 19b, 21, and 92 guided cleavage of the respective targets at the expected positions (Figure 1, lanes 16, 18, 20, 22, 24) and the substrate complementary to neuronal miR-124a was unaffected (Figure 1, lane 12). However, substrates targeted by abundant miR-16, 24, and 27a were not cleaved to a significant extent (Figure 1, lanes 6, 8, 14). Furthermore, targets for the low abundant miR-25, 32, and 96 were not cleaved at a detectable level (lanes 2, 4, 10).

Nuclear and Cytoplasmic Distribution of miRNA-Guided Cleavage Activity

RNAi has been considered a cytoplasmic process, based on the evidence that *trans*-spliced polycistronic mRNAs could be silenced independently and that dsRNA directed against intronic sequences did not knock down the cognate mRNA (Fire et al., 1998; Montgomery et al., 1998). However, in at least one report, dsRNA directed against an intron did lead to specific gene silencing, suggesting that effector complexes could also be localized in the nucleus and target mRNAs that are not matured rapidly (Bosher et al., 1999). We therefore examined subcellular miRNP localization and cleavage activity in the HeLa cell system.

Cell nuclei were separated from cytoplasm using the Dignam protocol (Dignam et al., 1983), except that collected nuclei were subjected to an additional wash step

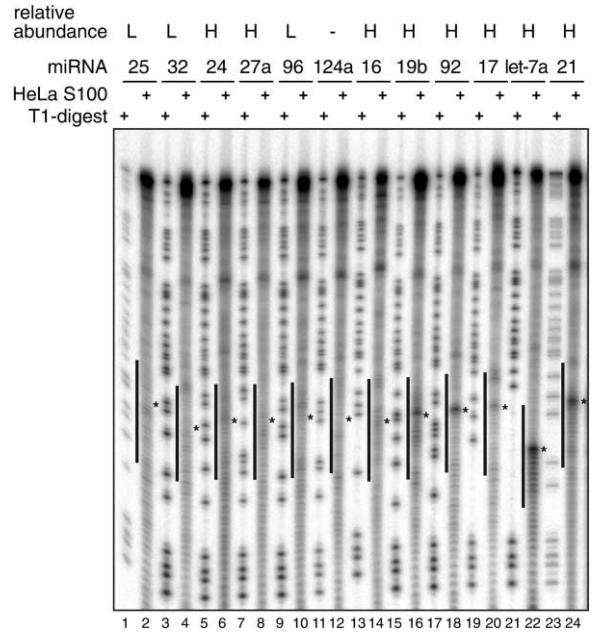


Figure 1. miRNA-Guided Cleavage of Sequence-Complementary Target RNAs in HeLa S100 Extract

³²P-Cap-labeled cleavage substrates were incubated for 2 hr in S100 extract and cleavage products were separated by 8% denaturing gel electrophoresis. The relative abundance of endogenous HeLa S3 miRNAs is indicated with L, low, and H, high was determined by small RNA cDNA library construction followed by semi-quantitative PCR (see Figure 4). The absence of neuron-specific miR-124a in HeLa cells is indicated by a dash. T1 refers to partial nuclease T1 digestion, which was performed for each substrate RNA to precisely locate the position of miRNA-guided cleavage. Black vertical lines next to the T1 ladder indicate the segment of the target RNA covered by the miRNA. The expected cleavage position, 10 nt upstream of the nucleotide across from the miRNA 5' end, is marked by an asterisk.

for more complete removal of cytoplasmic proteins. The separation of nuclear and cytoplasmic proteins was confirmed by Western blotting for the nuclear lamin A/C and the cytoplasmic β -tubulin (Figure 2A). Total RNA isolated from the nuclear and cytoplasmic fractions was probed for endogenous miR-21 by quantitative Northern blotting using a radiolabeled complementary oligodeoxynucleotide. Mature miR-21 was detected in both fractions, while the 70 nt miR-21 precursor was only detected in the nuclear fraction (Figure 2B). The concentration of miR-21 corresponds to approximately 2,500 molecules per cell, where about 20% of the mature form was localized to the nucleus. To assess if the matured form of miR-21 could target RNA cleavage in both compartments, nuclear and cytoplasmic extracts were incubated with ³²P-cap-labeled miR-21-complementary target RNA. Specific cleavage products were detected in both the cytoplasmic and nuclear incubation reaction, and the relative cleavage intensity reflected the amount of miR-21 present in both of the compartments (Figure 2C). Similar results were obtained when examining the cleavage reaction guided by let-7a miRNA (data not shown). These observations suggest that cytoplasmic miRNA-associated RISC is presumably able to translocate to the nucleus.

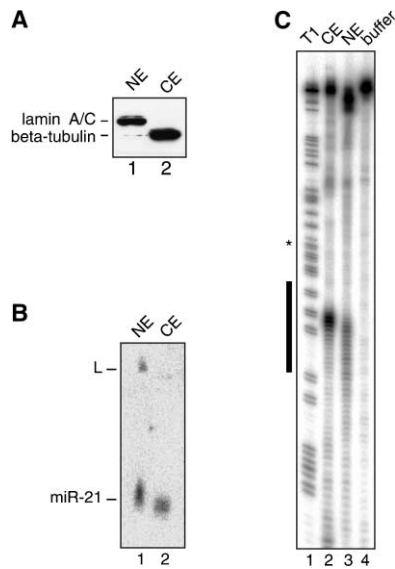


Figure 2. Mature miRNAs and miRNA-Guided Cleavage activity in Cytoplasmic and Nuclear Extracts

(A) Quality control of nuclear and cytoplasmic extracts. Aliquots containing 20 μ g of total protein from nuclear (NE) and cytoplasmic extracts (CE) were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane, and sequentially probed with antibodies specific for the nuclear proteins lamin A/C (lane 1) and for the cytoplasmic β -tubulin (lane 2).

(B) Nuclear and cytoplasmic localization of miR-21. Aliquots of 30 μ g of total RNA from nuclear and cytoplasmic HeLa extracts were separated by 15% PAGE, transferred to a nylon membrane, and probed for miR-21. The fraction of total RNA in the nucleus corresponded to 14% of total cell RNA. The miR-21 fold-back precursor (L) is indicated.

(C) miR-21-guided target RNA cleavage. 32 P-Cap-labeled cleavage substrate complementary to miR-21 was incubated with cytoplasmic or nuclear extract for 1 hr. Cleavage products were separated by 8% PAGE. T1 refers to partial nuclease T1 digestion of the miR-21 targeted RNA substrate. In lane 4, buffer was used instead of extract. The black bar to the left represents the region of the target RNA complementary to miR-21. Cleavage bands produced by T1 digestion appear as doublets because the template for transcription was amplified from a plasmid preparation that happened to contain two distinct clones, in which one clone had a 1 nt deletion or insertion at the position indicated by an asterisk.

The Ago Protein Subfamily Is Ubiquitously Expressed in Many Cell Lines

To obtain a comprehensive picture of transcript levels of all the 8 human Ago proteins (Sasaki et al., 2003) in cell lines amenable for biochemical studies, we measured mRNA levels using quantitative real-time RT-PCR. Total RNA was isolated from the cell lines of HPV-positive cervical carcinoma HeLa S3, embryonic kidney HEK 293, Burkitt's lymphoma BL-41, adrenal gland small cell carcinoma SW-13, hepatoma Hep G2, and Huh-7.5. Transcripts of all four members of the Ago subfamily could be detected in all cell lines (Figure 3). Ago2 and Ago3 mRNAs are generally transcribed at similar levels, while Ago1 and Ago4 mRNA are more variably transcribed. The transcript levels measured for the Ago members were 100- to 1000-fold lower than the GAPDH mRNA used for normalization, making mRNA quantification by Northern blotting difficult. The transcript levels of the Piwi family in these cell lines were drastically lower,

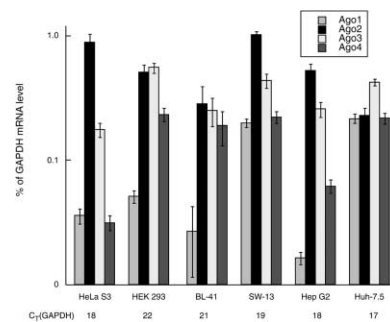


Figure 3. Ago Subfamily Genes are Transcribed in Different Cell Lines

Transcript levels of the Ago subfamily members were determined by qRT-PCR and are displayed in a logarithmic scale after normalized to GAPDH mRNA level of the respective cell line. The threshold cycle values (C_T) for GAPDH amplification are indicated to provide a measure of the cell-type specific relative abundance of GAPDH in the input total RNA.

at least 10,000-fold below GAPDH, and could not be quantified using qRT-PCR. We therefore concentrated on the functional analysis of Ago1 through Ago4 in HeLa cells.

The Members of the Ago Protein Subfamily Associate with miRNAs

In order to evaluate whether each of the Ago subfamily members associates with endogenously expressed miRNAs, 17 amino acid N-terminally FLAG/HA-tagged versions of the human Ago-subfamily were expressed in HeLa S3 and HEK 293 cells. Expression and isolation of the Ago-proteins carrying the same tag was useful in standardizing the isolation of the individual Ago RNPs. We established HeLa cell lines stably expressing FLAG/HA-tagged Ago1, Ago2, and Ago4, but could not obtain a stable line expressing Ago3. Tagged Ago3, however, was expressed when Ago3-encoding plasmids were transiently transfected into HEK 293 cells. HEK 293 cells were selected because they are, in contrast to HeLa cells, efficiently and cost effectively transfected with calcium phosphate at a large scale.

The FLAG/HA-tagged RNPs were captured from filtered cytoplasmic cell lysates with anti-FLAG antibody-conjugated resin and recovered by elution with a triple FLAG peptide. Western blot analysis of fractions of the eluate using anti-HA antibodies indicated that RNPs were effectively eluted (Figure 4A). The small RNAs found to be present in the FLAG peptide elution were isolated by digestion with proteinase K, phenol/chloroform extraction, and ethanol precipitation. miR-21 was detected by Northern blotting in RNPs isolated from HeLa FLAG/HA-Ago1, FLAG/HA-Ago2, and FLAG/HA-Ago4 cell lines (Figure 4B, lanes 3–5). No hybridization signal was detected in RNA isolated from a FLAG/HA-EGFP stable cell line (Figure 4B, lane 2), indicating that miR-21 was specifically associated with tagged Ago RNPs only. To examine miRNA association with an Ago3 complex, RNA was isolated from transiently transfected HEK 293 cells and subjected to Northern blotting for miR-16, which is more abundant than miR-21 in HEK 293 cells. A weak but specific signal was detected for

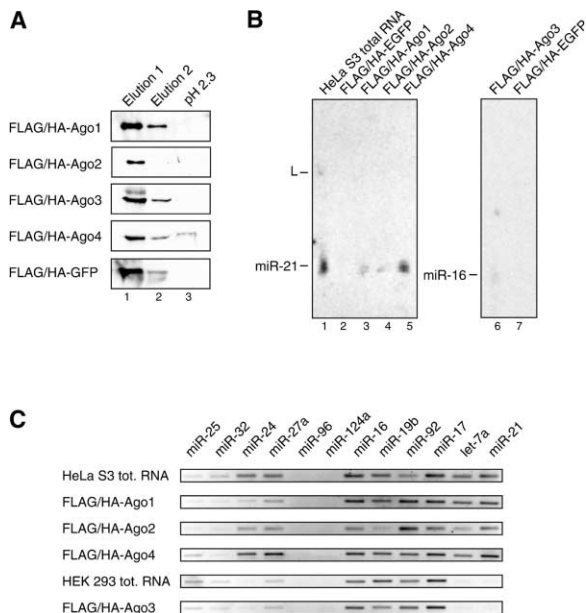


Figure 4. miRNAs Associate with FLAG/HA-Tagged Ago1 through Ago4 Protein Complexes

(A) Native elution of affinity-purified Ago protein complexes from the anti-FLAG resin. Cytoplasmic extracts, prepared from HeLa cells stably expressing FLAG/HA-tagged Ago1, 2, 4 and EGFP, or HEK 293 cells transiently expressing FLAG/HA-tagged Ago3, were loaded onto an anti-FLAG affinity column. The column was subsequently washed and bound proteins were eluted by sequential incubation of the beads in a buffered solution of 3x FLAG peptide. Finally, the resin was eluted with glycine (pH 2.3) to examine the residual bound proteins (lane 3). The eluates were separated by SDS PAGE and analyzed by Western blotting. Single bands specific to the tagged proteins were detected by a highly specific anti-HA antibody. (B) Detection of copurified miRNAs in RNA isolated from different tagged Ago complexes by Northern blot analysis. RNA isolated from eluate 1 was examined by Northern blotting with probes complementary to miR-21 (lanes 1 to 5) or miR-16 (lanes 6 and 7), respectively. The amount of total RNA isolated by immunoprecipitation was too low to be measured quantitatively by UV.

(C) Semi-quantitative PCR analysis of the miRNA content of small RNA cDNA libraries. PCR amplification on the library template was carried out using a miRNA-specific 3' primer in combination with the invariant 5' adaptor sequence primer. PCR was stopped when the most abundant amplification products were detectable yet still amplifying linearly. Aliquots of the PCR with equal cycle numbers were separated on an agarose gel and DNA was visualized by ethidium bromide staining. The image was inverted to better visualize the bands.

RNA isolated from the Ago3 RNP, and was absent from a preparation using FLAG/HA-EGFP control-transfected cells (Figure 4B, lanes 6 and 7).

To assess whether or not miRNAs associate with sequence preference with one of the Ago-containing RNPs, cDNA libraries were also prepared from the purified Ago-bound small RNAs as well as from total cellular small RNA. A modified small RNA cloning protocol was used (Pfeffer et al., 2003). In brief, 3' and 5' adaptor oligonucleotides containing non-palindromic restriction site sequences were ligated sequentially to the small RNAs. The 3' adaptor was chemically adenylated and ligated to the small RNAs using a truncated form of T4 RNA ligase 2 (Rnl2) (Ho et al., 2004). The 5' adaptor was

joined to the small RNA/3' adaptor ligation product using standard T4 RNA ligase (Rnl1) (Pfeffer et al., 2003). The final ligation product was reverse transcribed, PCR-amplified, and used as template for semi-quantitative PCR with miRNA-specific primers covering the 3' portion of the respective miRNA. In the library prepared from HeLa cell total RNA, let-7a, miR-16, 17, 19b, 21, 24, 27a, and 92 were highly abundant, while miR-25 and 32 were of low abundance, and miR-96 and 124a were not detectable (Figure 4C). Similar miRNA profiles were obtained for the libraries prepared from FLAG/HA-tagged Ago-specific complexes, indicating that Ago1, 2, and 4 bound to miRNAs with similar affinity. In the library prepared from HEK 293 cell total RNA, miR-16, 17, 19b, and 92 were highly abundant, while let-7a, miR-24, 25, 27a, and 32 were of low abundance, and miR-21, 96 and 124a were not detectable. The miRNA profile obtained for the library prepared from FLAG/HA-tagged Ago3 using HEK 293 cells appeared identical to the total RNA-derived profile, indicating that Ago3 also binds miRNAs indiscriminately of their sequence.

Given the association of miRNAs with the affinity-purified complexes, it is evident that tagging of these proteins did not interfere with their ability of RNP complex formation.

RISC Activity Requires Ago2 but Not Ago1, Ago3, or Ago4

After finding that all Ago subfamily proteins formed miRNPs, we wanted to elucidate the specific Ago proteins capable of the RISC cleavage reaction. The anti-FLAG-purified Ago complexes from HeLa cells were incubated with cap-labeled target RNAs to probe for miR-21-guided cleavage activity. Cleavage of the RNA substrate was detectable only for the Ago2 complex (Figure 5A, lane 6), but not for Ago1 or Ago4 (Figure 5A, lanes 4 and 7), or a control purification using FLAG/HA-EGFP (Figure 5A, lane 3). To address the function of Ago3 transiently expressed in HEK 293 cells, we first assayed for miR-21-guided cleavage activity. Cleavage of the RNA substrate was not detected (Figure 5A, lane 5); however, this result was ambiguous because miR-21 is significantly less abundant in HEK 293 cells than it is in HeLa cells. We therefore assayed for miR-16-guided cleavage. Again, we did not detect Ago3-associated cleavage, while the Ago2 complex isolated from HEK 293 cells cleaved the substrate effectively (Figure 5A, lanes 9 and 10). The control experiment with FLAG/HA-EGFP was devoid of cleavage activity (Figure 5A, lane 11). Further analysis of all purified complexes using let-7a substrate RNAs for cleavage confirmed that only Ago2-associated RNPs are able to guide cleavage (data not shown).

In order to address whether exogenously introduced siRNAs follow the same pattern of activity observed for endogenously expressed miRNAs, we also transiently expressed FLAG/HA-tagged Ago1 to Ago4 in HEK 293 cells, and then cotransfected synthetic siRNAs 24 hr later. Cell extracts were prepared another 36 hr later. The siRNA was directed against firefly luciferase mRNA, which was not expressed in those cells (Martinez et al., 2002). Equal amounts of anti-FLAG precipitates were incubated with cap-labeled luciferase target RNA cog-

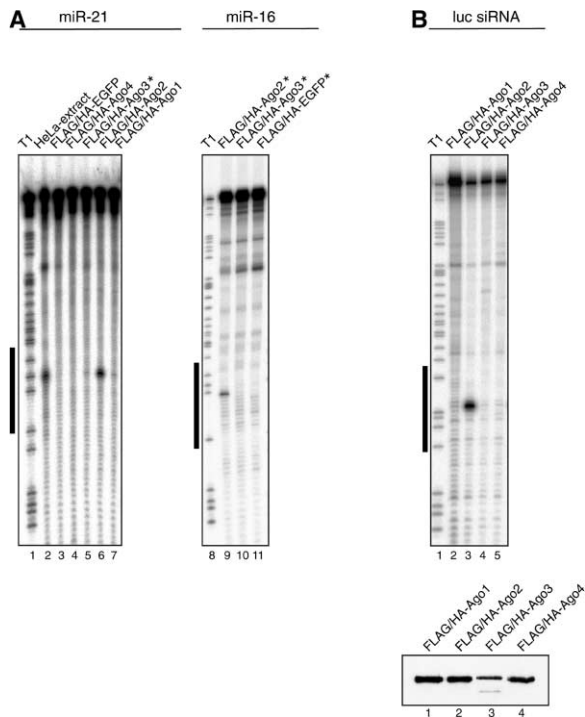


Figure 5. miRNA- and siRNA-Guided Cleavage Activity Is Mediated by the Ago2 Protein Complex In Vitro

(A) ³²P-Cap-labeled target RNAs containing miR-21 or miR-16 complementary sites were incubated with the FLAG-tag-purified Ago complexes. The cleavage products were resolved on 8% sequencing gels. The Ago complexes labeled with asterisk were purified from transiently transfected HEK 293 cells rather than stably transfected HeLa cells. miR-21 is abundant in HeLa cells but of low abundance in HEK 293 cells, miR-16 is abundant in both cell lines. Lanes 1 and 8 show the T1 hydrolysis ladder of the cleavage substrates. The black bars to the left side of the images represents the region of the target RNA complementary to miR-21 or miR-16.

(B) ³²P-Cap-labeled luciferase (luc) target RNA was incubated with FLAG-tagged Ago complexes purified from HEK 293 cells. Cells were first transiently transfected with FLAG/HA-Ago expression plasmids followed by luciferase siRNA transfection 24 hr later. Cells were harvested after another 36 hr incubation. The upper panel shows the target RNA cleavage, the lower panel the amount of FLAG/HA-tagged Ago protein used for the cleavage reaction.

nate to the transfected luciferase siRNA. Cleavage of the RNA substrate was detectable only for the Ago2 complex (Figure 5B, lane 3), but not for Ago1, Ago3, or Ago4 (Figure 5B, lanes 2, 4, and 5). Together with the miRNA-guided target RNA cleavage, this finding suggests that only Ago2 is associated with a complex containing an endonuclease.

Knock Down of Ago2 Interferes with miRNA-Guided Cleavage Activity in Cell Culture

In order to obtain independent evidence for the role of Ago proteins in guiding miRNA-specific target RNA cleavage, a cell-based positive readout EGFP reporter assay was applied (Meister et al., 2004). A fully complementary miR-21 sequence was inserted into the 3' UTR of an EGFP reporter plasmid and HeLa cells stably expressing the reporter were generated. A clone was se-

lected, in which the EGFP was repressed (Figure 6, panel 1) yet could be derepressed by transfection of an anti-miR-21 2'-O-methyl oligoribonucleotide (Figure 6, panel 3) (Meister et al., 2004). A control anti-miR-33 2'-O-methyl oligoribonucleotide was unable to de-repress EGFP (Figure 6, panel 5), indicating that the EGFP reporter gene was indeed under control of miR-21-specific RNA silencing. We then knocked down the individual Ago subfamily proteins with specific, validated siRNAs. The depletion of Ago1, 3, and 4 had no effect when compared to a control siRNA targeting a gene not expressed in HeLa cells (Figure 6, panels 7, 9, 13, and 15). However, the knock down of Ago2 resulted in upregulation of EGFP, indicating that Ago2 is required for miR-21-guided EGFP mRNA cleavage (Figure 6, panel 11). Despite the provision that the targeting of the RNAi machinery by siRNAs is only possible within a certain window, our positive readout system provides a robust and reliable tool for identifying genes implicated in miRNA-guided RNA silencing.

Discussion

Argonaute proteins constitute a family with a central role in RNA silencing processes (Carmell et al., 2002; Grishok et al., 2001; Martinez et al., 2002; Sasaki et al., 2003; Tabara et al., 1999; Tijsterman et al., 2002a; Vastenhouw et al., 2003; Vaucheret et al., 2004; Williams and Rubin, 2002; Xie et al., 2004). In mammals, Ago subfamily proteins appear to be expressed in all cell types, while Piwi subfamily proteins appear to be mostly restricted to hematopoietic stem cells (Sharma et al., 2001) and the germ line (Sasaki et al., 2003). Biochemical and structural studies (Hammond et al., 2001; Hutvagner and Zamore, 2002; Lingel et al., 2003, 2004; Ma et al., 2004; Martinez et al., 2002; Song et al., 2003; Yan et al., 2003) indicate that Ago proteins could be directly associated with the small RNAs residing in the RNA silencing effector complexes. To address the function of the Ago subfamily members in somatic cells, we have used biochemical and cell biological tools to investigate their specific association with endogenous miRNAs and their role in constituting RISC.

Our analysis indicated that, to a certain degree, miRNAs associate indiscriminately of their sequence with Ago1 through Ago4 protein complexes. The apparent expression of Ago1 through Ago4 in numerous tissues (Sasaki et al., 2003) and cell lines suggests that Ago proteins have a fundamental role in regulating gene expression. From the four different Ago complexes analyzed, only Ago2 RNPs guided target RNA cleavage. Endogenous miRNAs as well as transfected synthetic siRNAs enter Ago2 RNPs to guide target RNA cleavage.

It is presumed that the predominant function of miRNAs is translational repression rather than targeted RNA degradation. A current model of RNA silencing suggests that RISC is able to guide target RNA cleavage as well as translational repression depending on the extent of sequence-complementarity between small RNA and its target. Given that the Ago1, Ago3, and Ago4 do not guide cleavage and are therefore non-redundant with respect to Ago2, it is conceivable that these complexes are specific for translational regulation guided

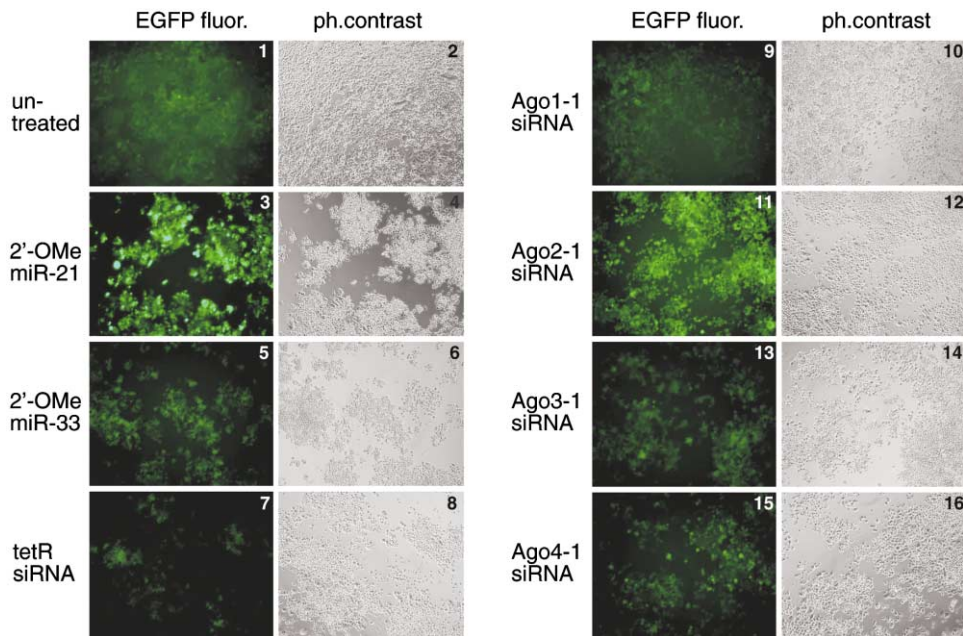


Figure 6. miR-21-Guided Cleavage Activity Is Mediated by Ago2 in HeLa Cell Culture

A stable HeLa cell line that expresses EGFP carrying a sequence with perfect complementarity to miR-21 in its 3' UTR was transfected with 2'-O-methyl oligoribonucleotides complementary to miR-21 or the control miR-33, Ago1 to Ago4-specific siRNA duplexes, or a control siRNA duplex cognate to the tetR sequence. Fluorescence and phase contrast images were recorded six days after transfection using a Zeiss Axiovert 200 inverted fluorescence microscope.

by miRNAs or another, yet-to-be-identified regulatory processes. Differential expression levels of Ago proteins as detected by qRT-PCR in different cell lines may point to further complexity of regulation of RNA silencing in a cell-type specific manner. Preliminary analysis of the protein levels for endogenous Ago1 to Ago4 in our HeLa cells indicate similar protein levels for Ago1 and Ago2, and an order of magnitude lower level for Ago3 and Ago4 (G.M., unpublished data). It is also conceivable that these Ago proteins are part of a more intricate assembly process ultimately yielding Ago2-containing RISC. Although our experiments cannot fully exclude this possibility, this is unlikely, because we were able to knock down Ago1, Ago3, and Ago4 without an effect on Ago2-guided target RNA cleavage. In contrast, *D. melanogaster* Ago1 (DmAgo1) and Ago2 (DmAgo2) may participate in a more hierarchical assembly of RISC. A recent study revealed that DmAgo1 was specifically required for miRNA accumulation and DmAgo2 was required for the dsRNA- and siRNA-triggered RNAi cleavage reaction (Okamura et al., 2004). Although mature miRNAs were shown to associate with both DmAgo1 and DmAgo2, the cleavage activity of isolated miRNA-associated Ago complexes was not tested, and it remains unclear if Ago1 associates with endonuclease activity. The *D. melanogaster* miRNA and siRNA pathways are unique because two specialized Dicer RNase III enzymes initiate the production of miRNAs and siRNAs (Lee et al., 2004).

Interestingly, not all Ago2-associated abundant miRNAs were able to guide cleavage of sequence-complementary target RNAs. Sequence-dependent cleavage activity of Ago2-associated miRNAs may follow some

of the same sequence preferences as those determining the efficiency of artificial siRNA duplexes designed to target RNA cleavage (Haley and Zamore, 2004; Reynolds et al., 2004). Given the apparent lack of fully or nearly fully complementary natural targets to mammalian miRNAs (Bartel, 2004; Carrington and Ambros, 2003), and the conviction that miRNAs act predominantly as translational repressors rather than mediating target mRNA cleavage, one would argue in favor of the possibility that Ago2 complexes have dual function in guiding both cleavage and translational repression. Depending on their RNA targets, some miRNAs may predominantly function as translational repressors since they may not have been under evolutionary selection for guiding effective mRNA cleavage, while others may have evolved to also use the degradative mechanism.

In previous reports, distinctions for which specific Ago family protein resided in RISC could not be made. Our laboratory had previously identified Ago1 and Ago2 in affinity-purified reconstituted complexes using biotinylated siRNAs (Martinez et al., 2002). Analysis of the role of Ago proteins in the current study contradicts an earlier study, which reported Ago1 as the predominant mediator of RNAi, as well as Ago2, 3, and 4 to a lesser extent (Doi et al., 2003). Competition between Ago-specific and reporter-gene-specific siRNAs, which were cointroduced into the cells, might have affected the amount of reporter-specific RISC for each experiment. Our positive readout system was less prone to such artifacts because the reporter was under control of a robustly expressed endogenous miRNA.

The distinct association of Ago2 complexes with cleavage activity suggests that either Ago2 itself is an

endonuclease or that it specifically associates with a yet-to-be-identified endonuclease. Given the strong sequence conservation of the Argonaute family PAZ and PIWI domains, the unique character of Ago2 must be defined by its residues at the N terminus or the spacer region between the PAZ and PIWI domain. Post-translational modifications specific for Ago2 may also play an important role in either activating Ago2 as an endonuclease or in regulating the association of Ago2 with a putative endonuclease protein. It has been demonstrated that modifications such as phosphorylation or methylation can create specific interaction platforms on proteins. Expressing recombinant Ago proteins and reconstruction of the cleavage activity will provide further insights into the mechanism of small RNA guided gene silencing.

Experimental Procedures

Oligonucleotide Synthesis and Enzymatic Preparation of Target RNAs

Oligodeoxyribonucleotides (ODNs) were synthesized at 0.2 μ mol scale using standard DNA phosphoramidite reagents (Proligo). The ODNs were deprotected for 16 hr at 55°C in 30% aqueous ammonia. After removal of the control pore glass support, the ODNs were precipitated by the addition of 12 ml absolute 1-butanol, dried, and dissolved in 0.5 ml water. ODNs longer than 60 nt were PAGE-purified. The following ODNs were prepared for constructing plasmids for run-off transcription of miRNA cleavage substrates: ODN1, 5' GGCATAAAGAATTGAAGAGAGTTTTCACTGCATACGACGATTCGTGATTTGTATTCAGCCCATATCGTTTCATAGCTTCTGCCAACCGA; ODN-miR-21, 5' GAACAATTGCTTTTACAGATGCACATATCGA GGTGAACATCAGTACGTCAACATCAGTCTGATAAGCTATCGGTTGGCAGAAAGCTAT; ODN-let-7a, 5' GAACAATTGCTTTTACAGATGCACATATCGAGGTGAAAACCTATACAACCTACTACCTCAAATGTCTTCGGTTGGCAGAAGCTAT; ODN-miR-16, 5' GAACAATTGCTTTTACAGATGCACATATCGAGGTGAACATCACACGCCAATATTTACGTGCTGCTACCGTTTCGGTTGGCAGAAGCTAT. To obtain ODN-miR-17, the miR-21 complementary sequence segment TAGCTTATCAGACTGATGTTGAC in oligonucleotide ODN-miR-21 was replaced by 5' ACTACCTGCACTGTAAGCACTTTG, for ODN-miR-19b by 5' TCAGTTTGCATGGATTGACACA, for ODN-miR-24 by 5' CTGTTCTGCTGCACTGAGCCA, for ODN-miR-25 by 5' CAGACCAGACAAAGTCAATG, for ODN-miR-27a by 5' AGCGGAACCTAGC CACTGTGAA, for ODN-miR-32 by 5' GCAACTAGTAATGTGCAATA, for ODN-miR-92 by 5' ACAGGCCGGGACAAAGTCAATA, for ODN-miR-96 by 5' GCAAAAATGTGCTAGTGCCAAA, and for ODN-miR-124a by 5' TGGCATTCCACCGCGTGCCTAA.

The miRNA cleavage substrates were prepared by PCR-based extension of ODN-1 and the miRNA-specific ODN. The dsDNA was then used as template for PCR with primers ODN-T7, 5' TAATACGACTACTATAGAACAATTGCTTTTACAG and ODN-SP6, 5' ATT TAGGTGACACTATAGGCATAAAGAATTGAAGA to introduce T7 and SP6 promoter sequences for in vitro transcription. The PCR product was ligated into pCR2.1-TOPO (Invitrogen). Plasmids isolated from sequence-verified clones were used as templates for PCR to produce sufficient template for run-off in vitro transcription reactions using phage RNA polymerases (Elbashir et al., 2001b). The luciferase cleavage substrate and siRNA was reported previously (Martinez et al., 2002).

Sequences of ODN primers for qRT-PCR were: Ago1, 5' GCACTGCCATTGGCAACGAA and 5' CATTGCGCCAGCTCACAAATGGCT; Ago2, 5' CGCGTCCGAAAGGCTGCTCTA and 5' TGGCTGTGCTTGTAAAACGCT; Ago3, 5' GGAATTAGACAAGCCAAATCAGCA and 5' AAGGTGGTCTATATCCTCTGGA; Ago4, 5' CTAACAGACTCCACAGC GTGTC and 5' GACTGGCTGGCCGTCTAGTCA; GAPDH, 5' CGC TCTCTGCTCCTCTGTT and 5' CCATGGTGTCTGAGCGATGT.

For semi-quantitative RT-PCR examining relative miRNA abundance in small RNA libraries, the invariant 5' primer 5' AGGGAGG CACCGATGCGG was combined with miRNA/3' adaptor-junction-specific 3' primers: miR-25, 5' TCTCGGTCTGATTAACCGC; miR-

32, 5' TTAAGTGTGCTTTAACCGC; miR-24, 5' AGCAGGAACAGT TTAACCGC; miR-27a, 5' CTAAGTCCGCTTTAACCGC; miR-96, 5' AGCACATTTTGTGCTTTAACCGC; miR-124a, 5' GGTGAATGCCATT AACCGC; miR-16, 5' GTAAATATTGGCTTTAACCGC; miR-19b, 5' CATGCAAAACTGATTTAACCGC; miR-92, 5' CCCGGCCTGTTTAA CCGC; miR-17, 5' AGTGCAGGTAGTTAACCGC; let-7a, 5' AGGTT GTATAGTTTAAACCGC; miR-21, 5' GACTGATGTTGATTAACCGC.

2'-O-Methyl oligoribonucleotides were synthesized using 5'-silyl, 2'-O-methyl phosphoramidites (Dharmacon Research) on 0.2 μ mol synthesis 3' C6 aminolinker synthesis columns, using a modified ABI394 synthesizer (Scaringe, 2001). The phosphate protection group was removed by flushing the column with 2 ml of 0.2 M 2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate in DMF/water (98:2 v/v) for 30 min at room temperature. The reagent was removed and the column rinsed with 10 ml water followed by 10 ml acetonitrile. Oligonucleotides were cleaved from the solid support by incubating the solid support with 1.9 ml of 40% aqueous methylamine over 1 hr in a screw-cap vial at 55°C. The 40% aqueous methylamine solution was removed from the solid support and dried down in an Eppendorf concentrator to remove methylamine and water. The residue was dissolved in 425 μ l of water and 75 μ l of 3 M sodium acetate (pH 6). The solution was filtered through a NANOSEP MF 0.45 μ m column filter (Pall Corp.) to remove residual solid support beads. Oligonucleotides were then precipitated by the addition of 1.2 ml of absolute ethanol. The collected pellets were dissolved in 400 μ l of water. The sequences of the 2'-O-methyl oligoribonucleotides were 5' GUCAACAUCAGUCUGAUAGCUAL (L, C6 3' aminolinker) for 2'-O-methyl antisense miR-21, and 5' GUGCAUUGUAGUUGCAUUGL for 2'-O-methyl antisense miR-33.

Four siRNA duplexes per target were designed in collaboration with Dharmacon, Inc. to specifically knock down individual Ago subfamily members. siRNAs were composed of 21-nt all-ribo sense and 21-nt 5' phosphorylated antisense RNAs. The sequences for sense and antisense strand were siAgo1-1, GAGAAGAGGUGCUC AAGAUU, pUUCUUGAGCACCUUCUCUCUU; siAgo1-2, GGAACA GUUCUACAAGUGGUU, pCCAUUGUAGAACUGUUUCUU; siAgo1-3, GCUGUUACCUCACUGGAUUAU, pUAUCCAGUGAGGUAACAG CUU; siAgo1-4, GGAGUUACUUCAUAGCAUUU, pAUGCUAUGAA AGUAACUCCUU; siAgo2-1, GCACGGAAGUCCAUCUGAAUU, pUU CAGAUUGGACUUCUGUCUU; siAgo2-2, GCAGACAAGAAUGUA UUAUU, pUAAUACAUCUUUGUCCUGCUU; siAgo2-3, GGGUCUGU GGUGAUAAUUAUU, pUAUUUAUACCCACAGACCCUU; siAgo2-4, GUAUGAGAACCACAAUGUCAUU, pGACAUUGGGUUCUCAUACUU; siAgo3-1, GAAAUUAGCAGAUUGGUAAUU, pUUAACAAUGUCUA AUUUCUU; siAgo3-2, CAAGAUACCUUACGCACAAUU, pUUGUGC GUAAGUUAUCUUGUU; siAgo3-3, GCAUCAUUAUGCAUAUGAUU, pUCAUUAUGCAUUAUGAUGCUU; siAgo3-4, GCACAUCUAUCUUU GCAAAUU, pUUUGCAAGAUAGUUGUUGCUU; siAgo4-1, GGCCAG AACUAAUAGCAUUU, pAUUGCUAUAUUGUUGGCUU; siAgo4-2, CCACUCGGAUCAUCUUAUUUU, pUAAUAGAUAGUCCGAGUGGUU; siAgo4-3, AGAUGAGAUUUCAGUAUGAUU, pUCAUACUGAAAUCU CAUCUUU; siAgo4-4, GAACCAGGAUGCUUCUUAUU, pAAAGGA AGCAUCCUGGUUCUU. siTetR, GGCCUUGAAUUGAUCAUUAUdT, pAUUAUGAUCAAUUAAGGCCUdT, were used as a negative control.

Plasmids and Antibodies

Reporter plasmid pcDNA4/GFPmir21 was generated by PCR amplification of pEGFP-N1 (Clontech) using primers 5' CGCTGGATCCAT GGTGAGCAAGGGCGAGGAG and 5' GCTCTAGAGGTACCTAGCT TATCAGACTGATGTTGAGTGCGGCGCTTACTTGTATC. The PCR product was digested with BamHI and XbaI and ligated into BamHI/XbaI-cut plasmid pcDNA4/TO (Invitrogen).

The open reading frames (ORFs) of human Ago1 through 4 were derived from analysis of the consensus sequences built from alignment of ESTs available in GenBank. Ago1 through 3 were PCR-amplified from HeLa Marathon cDNA using Advantage-HF PCR Kit (Clontech). The Ago4 cDNA clone IMAGE# 4373725 was ordered from ATCC. Clones were verified by sequencing. Site-directed mutagenesis was performed with the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) to remove the EcoR I site in Ago4. To generate FLAG/HA-tagged Ago and EGFP, the ORFs were cloned into a modified pIRENeo plasmid (Clontech) (Malik and Roeder, 2003) containing a N-terminal FLAG/HA tag. The Ago and EGFP cDNAs were cloned using Not I and EcoR I restriction sites.

The following antibodies were used in this study: monoclonal anti-HA antibody (Covance, Inc.), monoclonal 636 anti-lamin A/C antibodies (Rober et al., 1990), and monoclonal anti- β -tubulin antibodies (Sigma).

Cell Culture and Transfection

HeLa S3 and HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, and 100 μ g/ml streptomycin at 37°C in atmosphere containing 5% CO₂. HEK 293 cells were transfected using calcium phosphate. 1 to 2 hr prior to transfection, cells were plated at 50% confluency on a 10 cm dish. 5 μ g of plasmid DNA was diluted in 438 μ l water and 61 μ l 2 M CaCl₂. 500 μ l 2 \times HEPES-buffered saline (274 mM NaCl/1.5 mM Na₂HPO₄/54.6 mM HEPES-KOH [pH 7.1]) were added drop-wise under gentle agitation. The transfection solution was then sprinkled onto the cells.

To generate a stable cell line carrying EGFP under miR-21 regulation, HeLa cells were transfected with pcDNA4/GFPmir21 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Zeocin-resistant clones were tested for their ability to result in a EGFP-positive phenotype upon transfection with anti-miR-21 2'-O-methyl oligoribonucleotide. One day before transfection 10⁵ HeLa S3 cells, stably expressing pcDNA4/GFPmir21, were transfected with 60 pmol of siRNA duplexes and 60 pmol of 2'-O-methyl oligoribonucleotide using Lipofectamine 2000 according to manufacturer's protocol for a 24-well plate format. Fluorescent cell images were recorded on a Zeiss Axiovert 200 inverted fluorescence microscope (Plan-Apochromat 10 \times /0.45) equipped with Chroma Technology Corp. filter sets 41001 (EGFP) and AxioVision 3.1 software.

HeLa S3 cell lines stably expressing FLAG/HA-tagged human Argonaute proteins were generated as described (Malik and Roeder, 2003). About 10¹⁰ cells, corresponding to 40–60 liters of suspension culture, were harvested and used for extract preparation and biochemical purifications.

Cell Extracts

HeLa cell extracts were prepared as described (Dignam et al., 1983) with the following modifications. 5 \times 10⁹ suspension culture HeLa cells were collected by centrifugation and washed with PBS (pH 7.4). The cell pellet (approx. 15 ml) was resuspended in five pellet volumes of 10 mM KCl/1.5 mM MgCl₂/0.5 mM dithiothreitol/10 mM HEPES-NaOH (pH 7.9)/0.5 mM AEBSF and incubated for 10 min on ice and collected again by centrifugation. The cell pellet was resuspended in 2 pellet volumes of the buffer described above and homogenized by douncing. The cell nuclei were removed from the cell lysate by centrifugation at 1,000 g for 10 min. The supernatant was cleared further by ultracentrifugation for 30 min at 30,000 g to obtain the cytoplasmic extract. The concentration of KCl of the extract was subsequently raised to 100 mM by addition of 2 M KCl. The extract was then adjusted to a glycerol content of 10% and frozen in liquid nitrogen. For extract preparation using transiently transfected HEK 293 cells, 10 to 20 10 cm confluent plates were used.

Western Blotting and Immunopurification of FLAG/HA-Tagged Ago Complexes

For Western blotting, samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham-Biosciences) by semi-dry electroblotting. As primary antibodies, monoclonal anti-HA, anti-lamin A/C, or β -tubulin antibodies were used. As secondary antibody, peroxidase-conjugated anti-mouse antibodies (DAKO) was used. Bound antibodies were visualized by enhanced chemiluminescence system (ECL) according to the manufacturer's protocol (Amersham-Biosciences).

For purification of FLAG/HA-tagged Ago complexes, cytoplasmic extracts were cleared by centrifugation at 17,200 g for 30 min and a passage through a 0.45 μ m HT Tuffryn membrane filter (Pall Corp.). 300 μ l FLAG M2 agarose beads (Sigma) were washed once with 0.1 M glycine-HCl (pH 2.5) and equilibrated by washing with 1 M Tris-HCl (pH 8.0). The beads were then resuspended in 300 μ l buffer C (0.1 M KCl, 5 mM MgCl₂, 10% glycerol, 10% Tween20, 10 mM β -mercaptoethanol, 0.2 mM PMSF, and 20 mM Tris-HCl [pH 8.0]),

and incubated with approximately 10 ml cytoplasmic extracts for 4 hr at 4°C with rotation. The beads were collected and washed with 300 mM NaCl/5 mM MgCl₂/0.1% NP40/50 mM Tris-HCl (pH 7.5) followed by a wash with buffer C. Affinity-bound complexes were then eluted by shaking the beads in 300 μ l of 0.2 μ g/ml 3xFLAG peptide (Sigma) in buffer C for 2 hr at 10°C (eluate 1). The elution was repeated with another 300 μ l of 3x FLAG peptide solution to collect eluate 2. To isolate bound RNA, 100 μ l of the eluate 1 was treated with proteinase K (Tuschl et al., 1999). The RNA was precipitated after phenol/chloroform extraction, and resuspended in 20 μ l water.

miRNA Guided Cleavage Assay

In vitro transcribed cleavage substrates were ³²P-cap-labeled (Martinez et al., 2002), and cleavage was assayed as described previously (Meister et al., 2004) with the following modifications. 5 nM target RNA was incubated in a 25 μ l reaction solution containing 50% HeLa S100 extract, 1 mM ATP, 0.2 mM GTP, 10 U/ml RNasin (Promega) in 100 mM KCl/1.5 mM MgCl₂/0.5 mM DTT/10 mM HEPES-KOH (pH 7.9) at 37°C and the indicated time intervals. Cleavage reactions with immuno-precipitates used 12.5 μ l volume of beads in a 25 μ l reaction. The reaction was stopped by addition of 200 μ l proteinase K buffer followed by proteinase K treatment and RNA isolation as described above. The RNA was separated by 8% PAGE and radioactivity was detected by phosphorimaging.

Isolation of Total RNA from Cells, Northern Blotting, qRT-PCR, and siRNA Validation

Isolation of total RNA from cells and Northern blotting was performed as previously described (Lagos-Quintana et al., 2001; Meister et al., 2004; Pasquinelli et al., 2000). 30 μ g of total RNA or a 3 μ l aliquot of the 20 μ l of RNA isolated from the FLAG/HA-tagged Ago complexes was separated by 15% PAGE and transferred to a Hybond-N+ membrane (Amersham). For Northern blotting, the 5' ³²P-radiolabeled probe for miR-21 was 5' TCAACATCAGTCTGA TAAGCTA and for miR-16 was 5' GCCAATATTACGTGCTGCTA. The amount of miR-21 per HeLa cell was determined by quantitative Northern blotting (Meister et al., 2004).

For qRT-PCR, 1 μ g of DNase-I-treated total RNA was reverse transcribed using SuperScript III (Invitrogen) with (dT)₁₈ for 50 min at 50°C. qPCR was performed using SYBR Green I (Molecular Probes) and HotStar Taq (Qiagen) and a final concentration of 10% (v/v) DMSO, 4 mM MgCl₂, 1 \times ROX reference dye (Invitrogen) and primers at 0.3 μ M final concentration. Taq polymerase was activated according to the manufacturer's protocol followed by 40 cycles of 30 s 95°C/1 min 60°C/30 s 72°C. PCR was performed on a Stratagene Mx3000P PCR cycler supplied with analytical software.

To verify the siRNA-mediated knock down of Ago mRNA, siRNA-transfected cells were collected 24 hr post transfection, total RNA was isolated using RNeasy columns (Qiagen), and analyzed by qRT-PCR as described below. Using GAPDH mRNA levels for normalization, the siRNA pool for Ago1, 2, and 4 reduced the cognate mRNA levels by more than 90%, while the pool for Ago3 reduced levels by 75%. Individual siRNA duplexes targeting the ORF of Ago1 to 4 were tested by transfecting the siRNA duplex together with plasmids encoding the FLAG/HA-tagged Ago and FLAG/HA-tagged EGFP proteins. Cells were harvested 2 days after transfection and the tagged proteins were examined by Western blotting using anti-HA antibodies. Complete knock down of the FLAG/HA-tagged cognate target was observed for siAgo1-1, siAgo1-2, siAgo2-1, siAgo4-1, and more than 90% reduction of target was observed for siAgo2-2 and siAgo3-1.

Cloning of Small RNAs and Semi-Quantitative RT-PCR

Total RNA (80 μ g) isolated from HeLa S3 or HEK 293 cells was separated on a 15% denaturing polyacrylamide gel and 19–24 nt small RNAs were recovered from the gel and used as input for adaptor ligation. 8 μ l of the 20 μ l of RNA isolated from the FLAG/HA-tagged Ago complexes was used for library construction without further gel purification. Adaptor ligation and RT-PCR of the ligation product was performed as described (Pfeffer et al., 2003) with the following modifications. The 5' adenylated 3' adaptor oligodeoxynucleotide (5' AppTTTAACCGCGGCCACAGL; Ap, adenylate; p,

phosphate; L, C7-3'-aminolinker (ChemGenes)) was ligated to the small RNA fraction in the absence of ATP using T4 RNA ligase Rnl2(1-249) ligase (Ho et al., 2004) kindly provided by Stuart Shuman. The gel-purified ligation product was then joined to the 5' adaptor oligoribonucleotide (5' AGGGAGGCACCGATGCGG) using standard T4 RNA ligase (NEB) and followed by gel-purification. RT-PCR was performed using primers 5' TGCTGGTGCCCGGGTAAA and 5' AGGGAGGCACCGATGCGG. Semi-quantitative PCR was performed using the 70 bp PCR-amplified library and primer pairs specific for the individual miRNAs at 50°C annealing temperature steps.

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