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# MicroRNAs are tightly associated with RNA-induced gene silencing complexes in vivo

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## Abstract

Previous work has shown that synthesized siRNA/miRNA is tightly associated with RNA-induced Gene Silencing Complexes (RISCs) *in vitro*. However, it is unknown if the endogenous miRNAs are also stably bound to RISC complexes *in vivo* in cells under physiological conditions. Here we describe the use of the looped real-time PCR-based method to trace the location of endogenous miRNAs in intact cells. We found that most of the endogenous miRNAs are tightly bound to RISC complexes, and only a very small proportion of them are free in cells. Furthermore, synthesized single-stranded mature miRNA or hairpin miRNA precursor cannot replace endogenous miRNAs already present in RISC complexes. However, we found that modified 2-O-Methyl-ribonucleotides were able to dissociate the target miRNA specifically from the RISC complex. These findings have important implications for understanding the basis for the stability and metabolism of miRNAs in living cells.

## Introduction

MicroRNAs (miRNAs) are small non-coding RNAs, which have critical roles in normal mammalian development and diseases [1]. Based on previous work, it is known that the primary miRNAs (pri-miRNAs) are first cut by Drosha into 70-100nt miRNA precursors (pre-miRNAs), which are transported from the nucleus into the cytoplasm by exportin5 [2, 3]. Dicer then processes these pre-miRNAs into mature miRNAs, after which they enter into the RISC complexes to function [4–7]. It has been shown that RISCs bind siRNA/miRNA very stably *in vitro.* In fact, even 2.5M NaCl or 1M Urea cannot dissociate siRNA from RISC complex during purification of the RISC complex from 293T cells [8]. Martinez et al

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also showed that during affinity column purification of RISCs, siRNA bind tightly to them even when treated with 2.5M KCl [9]. Furthermore, Rivas et al showed that recombinant human Argonaute 2 and siRNA can form RISCs *in vitro* [10]. These *in vitro* constituted minimal RISCs exhibit the core functions that are attributed to RISCs.

At the moment there are 443 known mouse miRNAs in Sanger microRNA database, and their average copy number range from between 1,000 and 2,000 [11, 12]. If all the miRNAs are associated with RISC complexes in the cytoplasm, then the number of total RISC complexes in a single cell is likely to be around hundreds of thousands [12, 13].

Despite intensive biochemical analysis of miRNA and RISC, it is still not known what proportion of miRNA *in vivo* are associated with RISCs, and what proportion of miRNAs are 'free' from RISCs (not associated with RISCs) under physiological conditions in a cell. There are several difficulties that hinder investigations on this question. First, it is difficult to distinguish between Pre-miRNAs (double-stranded hairpin of 70-100nt) and mature miRNAs (single stranded of 18 –23nt) present in the cell *in vivo*. Second, biochemical analysis of small RNAs *in vitro* usually requires loading of radio-labeled double-stranded siRNAs or artificial miRNAs that are in the form of perfectly complement double-stranded RNAs. This procedure adopted *in vitro* may not correspond to the way in which miRNAs are integrated into RISCs from Pre-miRNAs that are partially complement hairpin RNAs. Thirdly, even if the radio-labeled pre-miRNAs used in the biochemical assay faithfully represents the real pre-miRNA *in vivo*, their concentration used is much higher than the endogenous levels, so that this analysis may not reflect the real situation under physiological conditions. Thus, even after several years of intensive research, it is still unknown if most miRNAs are associated with RISCs, and if so, whether they are tightly associated under physiological conditions in a cell. The answer to this question is critical to understand the stability and half-life of miRNAs *in vivo*. This should provide important indications concerning miRNA metabolism and disposal in living cells. It could also help to evaluate the effects and duration of siRNA knockdown, an approach that is being widely used for the analysis of gene functions [14].

To investigate this aspect, we have used a looped real-time PCR-based assay to detect endogenous miRNAs in a cell [12, 15]. It has been shown that this assay is specific to mature miRNAs, and it can easily discriminate between mature miRNA, genomic DNA (gDNA) from which miRNAs originate, primary miRNAs (Pri-miRNAs), and precursor miRNAs (Pre-miRNAs). Furthermore, the assay is very sensitive because it can detect miRNA in pg amounts of total RNA sample and it can also quantitatively detect mature miRNAs from whole cell lysate without any purification [12, 15]. This approach therefore offers a unique opportunity to detect endogenous levels of miRNAs directly and without modifications. In this report, we used this real-time PCR-based method to find out what proportion of miRNAs are associated stably with RISCs *in vivo* in a cell under physiological conditions.

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## Materials and Methods

### Reverse Transcription (RT)

One microlitre of cell lysate was used as template for a 10ul reaction. Briefly, 1μl of 10x cDNA Archiving Kit buffer, 0.5ul 100mM dNTP, 0.1ul of 500nM reverse primer, 0.13ul of 20U/ul RNase inhibitor, and 0.67ul of 50U/ul MMLV reverse transcriptase were added into each 10ul RT reaction. The reaction condition was as follows: 16°C for 30min then 42°C for 60min and finally 85°C for 5min to inactivate MMLV reverse transcriptase [12, 15].

### Real-time PCR reaction

This analysis was performed using 0.5ul of the RT product for a 10ul reaction. All reactions were duplicated. TaqMan universal PCR master mix (UMM) from ABI was used as instructed [12, 15].

### Isolation of total RNA

MirVana miRNA isolation kit (Ambion) was used to isolate total RNA as well as miRNAs according to manufacture's protocol.

### Cell lysate

ES cells (Or NIH/3T3 cells, Mouse Embryonic Fibroblasts) were digested by trypsin into single cell suspension. Then cell number was counted by using hemocytometer and the cells were resuspended in PBS at 5,000-30,000 cells/ul. These were subjected to 3 freeze-thaw cycles by being alternately placed on dry ice and room temperature to induce cell lysis. Visual inspection under a microscope confirmed that most cells were lysed following this treatment. All miRNA were released from cells by treating cell suspension at 95°C for 5min [12, 15].

## Results

Firstly, we established conditions that release all miRNAs from cells. After a series of tests, we found that treatment of cells at 95°C for 5min can release most if not all miRNAs from ES cells [12, 15] (Supplementary Fig 1A). MirVana is a well-known reagent that is highly efficient for the isolation of small RNAs, including miRNAs, from cells or tissues. It destroys RISC complexes and thus releases nearly all miRNAs present in cells. We found that treatment of whole cells at 95°C for 5min is as efficient as MirVana for releasing miRNAs from ES cells.

Secondly, we sought to establish a milder condition that can completely release RNA from cells, but without destroying stable protein complexes. From our investigations, we found that subjecting cells to 3 freeze-thaw cycles lysed ES cells (Supplementary Fig 1B), as confirmed by microscopic examination, which showed that most cells were lysed after this treatment. We then checked for the release of mRNA of a housekeeping gene, GAPDH that is highly expressed in cells, and found that most of the GAPDH mRNA was released from ES cells after freeze-thawing. Further treatment of the sample at 95°C for 5min did not release any further mRNA. To confirm that this was the case, we also checked for the

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release of 18S rRNA following this treatment, which confirmed the release of most of the 18S rRNA from ES cells (Supplementary Fig 1C). These data show that three freeze-thaw cycles can release most of the free RNAs present in cells.

Next, we examined the levels of endogenous miRNA that are associated with RISC complexes in cells by looped RT-PCR technique. MiR-16 and mir-20 are two highly expressed miRNAs in ES cells. When we compared the levels of miR-16 released following treatment at 95°C for 5min with the levels detected following 3 freeze-thaw cycles, we found at least 30 fold more miRNA following the first treatment (Fig 1A). Thus it seems that the levels of free miR-16 in ES cells is less than 3% of the total miR-16 present in ES cells. In other words, under physiological conditions, most of the miR-16 is stably associated with RISC complexes. We similarly checked the levels of miR-16 in another cell line, NIH/3T3 cells, and in primary mouse embryonic fibroblasts (MEFs). We found that the levels of free miR-16 accounted for at most only 1-2% of all miR-16 present in NIH/3T3 cells and MEFs (Fig 1B, 1C). To exclude any sequence specific bias in our analysis, we also examined the levels of miR-20, another highly expressed miRNA, and again found a similar ratio of free versus bound miRNA (Fig 1D-1F). Therefore we conclude that under physiological conditions *in vivo*, most of the miRNAs are tightly associated with RISC complexes, and possibly only 1-3%, if any, are free in cells.

To confirm that the miRNAs released by freeze-thaw cycles is indeed free miRNA, we treated the samples with RNase I. RNase I is known to degrade any RNAs into a mixture of mono-, di-, and trinucleotides (Ambion). Firstly we confirmed that RNase I treatment at 37°C for 5min can indeed completely degrade free miRNAs. RNase I treatment of miRNA obtained following treatment of cells at 95°C 5min, did indeed degrade all miRNAs to background levels (Fig 1G and 1H; Compare Column 3 and Column 4). Secondly, we found that the samples obtained after 3 freeze-thaw cycles when treated with RNase I for 5 min, did not degrade miRNAs associated with RISC dramatically (Fig 1G and 1H; Compare Column 1 and Column 2). Finally we compared cell lysate containing intact RISCs (following 3 freeze-thaw cycles), to samples that were treated at 95°C for 5 min that destroys the RISC complex. RNase I treatment of these samples showed that less than 1% miRNAs were degraded in samples following the first treatment compared to the latter samples (Fig 1G and 1H; Compare Column 1 and Column 3). This demonstrates that nearly all miRNAs are tightly associated with RISC complexes in cells and only a tiny fraction of miRNA are free in intact cells under physiological conditions.

We considered a possible argument that we may underestimate free miRNA in cells because any endogenous RNase in cell lysate (but not in living cells) may degrade free miRNAs dramatically, To exclude this possibility, we added purified total RNA (including mature miRNAs) into cell lysate obtained after 3 freeze-thaw cycles. Our analysis showed that incubating mature miRNA in cell lysate did not cause their degradation to significant degree (Supplementary Fig 2A). This provides evidence that the levels of free miRNA in cell lysate, is not underestimated in our assay.

Next, we set out to determine the stability of the miRNA association with the RISC complex. To test this, we treated ES cells at different temperatures and found that miR-16

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was stably associated with the RISC complex even after treatment at 60°C for 5min. It was only following treatment of the samples at 80°C for 5min that most of the miR-16 was released from the RISC complexes (Supplementary Fig 2B). These observations suggest that most of the endogenous miRNAs are very tightly associated with RISC complexes in cells.

It has previously been reported that siRNA can bind stably to RISC complexes even in the presence of 2.5M NaCl, 2.5M KCl, or 1M Urea and the re-constituted RISC complexes are biologically functional [10]. We found that the endogenous miR-16 was stably associated with RISC even following treatment with 2.5M NaCl or KCl of ES cell lysates. Treating ES cell lysates obtained following 3 freeze-thaw cycles with 2.5M NaCl or KCl did not show a dramatic increase in the release of miRNA (Supplementary Fig 2C). These data suggests that endogenous miRNAs are tightly bound to RISCs in cells, which resembles the stable association between siRNA/RISC *in vitro*.

Next we wanted to assess the dynamics of the association between miRNAs and RISC complexes. To test this, we loaded 0.5uM synthetic Pre-miRNA or mature miRNA into ES cell lysate and incubated the sample for 30min. We then checked the amount of miR-16 associated with RISC-in the cell lysate. We found that neither adding Pre-miRNA nor mature miRNA of Let-7a could release miR-16 from RISC complexes (Fig 2A). Adding 0.5 uM synthetic single-stranded mature miRNAs could not replace endogenous miRNAs in RISC complexes, which indicates that the relative abundance of miRNAs is determined by the amount of initial primary miRNA transcripts. This result is in agreement with that reported previously [9, 16]. Adding 0.5 uM synthetic hairpin precursor Let-7a (Pre-Let-7a) also could not replace endogenous miRNAs from their RISC complexes. These data suggest following possibilities: First, the replacement kinetic may be very slow and it may take hours or more to see an effect. Second, precursors may be ‘transported’ into RISC complexes by other protein complexes in living cells [17]. This suggests that the miRNA/RISC association is very stable and free miRNA cannot significantly replace miRNA that is already present in RISC complexes, at least under the experimental conditions we tested.

A chemically modified oligonucleotide, called ‘antagomirs’ has been shown to be able to knockdown complementary miRNA by promoting their degradation [18, 19]. One possibility is that antagomirs molecule may bind to target miRNA within RISC and dissociate it from this complex. RNases may subsequently degrade dissociated miRNA/antagomirs hybrid (the miRNA strand) released in the cell cytoplasm. To test this possibility, we incubated the ES cell lysate obtained following 3 freeze-thaw cycles described above, with 0.5nM to 50 nM of antagomirs of miR-16 (antagomir-16) for 30min. The sample was then treated with RNase I to degrade miR-16 that may have been released from the RISC complexes. Finally, RNase I was inactivated and the remaining RISC-bound miRNAs were released by treatment of the ES cell lysate at 95°C for 5min, and their levels were estimated by real-time PCR (Fig 2B). We found that incubation of the sample with 5 nM of antagomir-16 can degrade 90% of miR-16 in ES cell lysate (the antagomir-16 effect of blocking stem-loop RT-PCR reaction have been corrected by comparing to the same amount of synthetic miR-16 for 0.5-50 nM antagomirs data.). As a control for the antagomirs, we used antisense miR-16 RNA molecule or an unrelated antagomir-let-7a, but neither of them was able to promote the release and subsequent degradation of miR-16 from RISC in the freeze-thaw ES cell lysate.

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We also checked antagomirs effect in a similar lysate from mouse embryonic fibroblast cells (MEF), and obtained similar results (Fig 2C). The data are compatible with the notion that antagomirs promote dissociation of specific miRNAs from RISC, and this is followed by their subsequent degradation.

Next, we asked if the stability of miRNAs relies on RISC complexes. Argonaute proteins have been shown to be a core component of RISC complexes and there are four Argonaute genes-Ago1, 2, 3, and 4 [10]. We analyzed miR-16 expression in Ago2 knockout MEFs [20]. We found that in Ago2 knockout MEFs, miR-16 expression is reduced about three quarters compared to wildtype MEFs (Figure 2D). This further proved that endogenous miRNA are protected by RISC complex in vivo. This is also compatible with recently published data [20, 21].

Finally, we asked if looped RT-PCR technique is specific to mature miRNA and can discriminate between mature miRNAs from their precursors unequivocally. For this purpose, we measured miR-16 expression in MEFs that lack Dicer [22]. It is known that in such Dicer null cells, mature miRNAs are absent and there is an accumulation of pri-miRNAs and pre-miRNAs [23, 24]. We indeed found that compared to wildtype MEFs, there is virtually no detectable miR-16 (Fig 2E). These data prove that looped-RT PCR does specifically detect mature miRNA, and not the corresponding genomic locus, pri-miRNA, or pre-miRNA.

## Discussion

In general, our data shows that in cells under physiological condition, most miRNAs are tightly associated with RISC complexes and only a small fraction and possibly less than 3% is present outside RISCs. To our knowledge, this is the first time that endogenous miRNAs have been demonstrated to be tightly associated with RISCs *in vivo* under physiological conditions. It has been shown that *in vitro* the association between RISC complexes and siRNAs or miRNAs is very stable [8, 9]. This observation may explain why miRNAs are so stable in cells, and it also explains why they exhibit a very long half-life. These findings raise a number of interesting questions. Since miRNAs appear to be so tightly associated with RISCs *in vivo*, this requires an explanation as to how these molecules turn over in living cells. Furthermore, what does the relatively low abundance of free miRNAs represent? Are these miRNAs really free, or is this a reflection of a transient stage before mature miRNAs (single-stranded, 18-23nt) are integrated tightly into RISCs after they are processed from pre-miRNA hairpins (70-100nt)? Finally, since miRNA are so tightly associated with RISCs, does this imply that they are permanently ‘trapped’ in RISCs, or is there a dynamic exchange between individual RISCs [9, 16]? Further experiments are needed to answer some of these crucial questions. Our study also shows that antagomirs can effectively and specifically displace miRNA from RISC, which is followed by their degradation.

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## References


[1]. Ambros V. The functions of animal microRNAs. Nature. 2004; 431: 350–355. [PubMed: 15372042]

[2]. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003; 425: 415–419. [PubMed: 14508493]

[3]. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science. 2004; 303: 95–98. [PubMed: 14631048]

[4]. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Bio. 2005; 6: 376–385. [PubMed: 15852042]

[5]. Pham JW, Pellino JL, Lee YS, Carthew RW, Sontheimer EJ. A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila. Cell. 2004; 117: 83–94. [PubMed: 15066284]

[6]. Pham JW, Sontheimer EJ. Molecular requirements for RNA-induced silencing complex assembly in the Drosophila RNA interference pathway. J Biol Chem. 2005; 280: 39278–39283. [PubMed: 16179342]

[7]. Zamore PD, Haley B. Ribo-gnome: the big world of small RNAs. Science. 2005; 309: 1519–1524. [PubMed: 16141061]

[8]. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ. Argonaute2 Is the Catalytic Engine of Mammalian RNAi. Science. 2004; 305: 1437–1441. [PubMed: 15284456]

[9]. Martinez J, Tuschl T. RISC is a 5’ phosphomonoester-producing RNA endonuclease. Genes Dev. 2004; 18: 975–980. [PubMed: 15105377]

[10]. Rivas FV, Tolia NH, Song JJ, Aragon JP, Liu J, Hannon GJ, Joshua-Tor L. Purified Argonaute2 and an siRNA form recombinant human RISC. Nat Struct Mol Bio. 2005; 12: 340–349. [PubMed: 15800637]

[11]. Griffiths-Jones S. The microRNA Registry. Nucleic Acids Res. 2004; 32: D109–D111. [PubMed: 14681370]

[12]. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005; 33: e179. [PubMed: 16314309]

[13]. Shingara J, Keiger K, Shelton J, Laosinchai-Wolf W, Powers P, Conrad R, Brown D, Labourier E. An optimized isolation and labeling platform for accurate microRNA expression profiling. RNA. 2005; 11: 1461–1470. [PubMed: 16043497]

[14]. Dykxhoorn DM, Novina CD, Sharp PA. Killing the messenger: short RNAs that silence gene expression. Nat Rev Mol Cell Biol. 2003; 4: 457–467. [PubMed: 12778125]

[15]. Tang F, Hajkova P, Barton SC, Lao K, Surani MA. MicroRNA expression profiling of single whole embryonic stem cells. Nucleic Acids Res. 2006; 34: e9. [PubMed: 16434699]

[16]. Maniataki E, Mourelatos Z. A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev. 2005; 19: 2979–2990. [PubMed: 16357216]

[17]. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell. 2005; 123: 631–640. [PubMed: 16271387]

[18]. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. Silencing of microRNAs in vivo with ’antagomirs’. Nature. 2005; 438: 685–689. [PubMed: 16258535]

[19]. Krutzfeldt J, Kuwajima S, Braich R, Rajeev KG, Pena J, Tuschl T, Manoharan M, Stoffel M. Specificity, duplex degradation and subcellular localization of antagomirs. Nucleic Acids Res. 2007; 35: 2885–2892. [PubMed: 17439965]

[20]. O’Carroll D, Mecklenbrauker I, Das PP, Santana A, Koenig U, Enright AJ, Miska EA, Tarakhovsky A. A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. Genes Dev. 2007; 21: 1999–2004. [PubMed: 17626790]

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[21]. Diederichs S, Haber DA. Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. Cell. 2007; 131: 1097–1108. [PubMed: 18083100]

[22]. Yi R, O'carroll D, Pasolli HA, Zhang Z, Dietrich FS, Tarakhovsky A, Fuchs E. Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. Nat Genet. 2006; 38: 356–362. [PubMed: 16462742]

[23]. Kanellopoulou C, Muljo SA, Kung AL, Ganesan S, Drapkin R, Jenuwein T, Livingston DM, Rajewsky K. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev. 2005; 19: 489–501. [PubMed: 15713842]

[24]. Murchison EP, Partridge JF, Tam OH, Cheloufi S, Hannon GJ. Characterization of Dicer-deficient murine embryonic stem cells. Proc Natl Acad Sci USA. 2005; 102: 12135–12140. [PubMed: 16099834]

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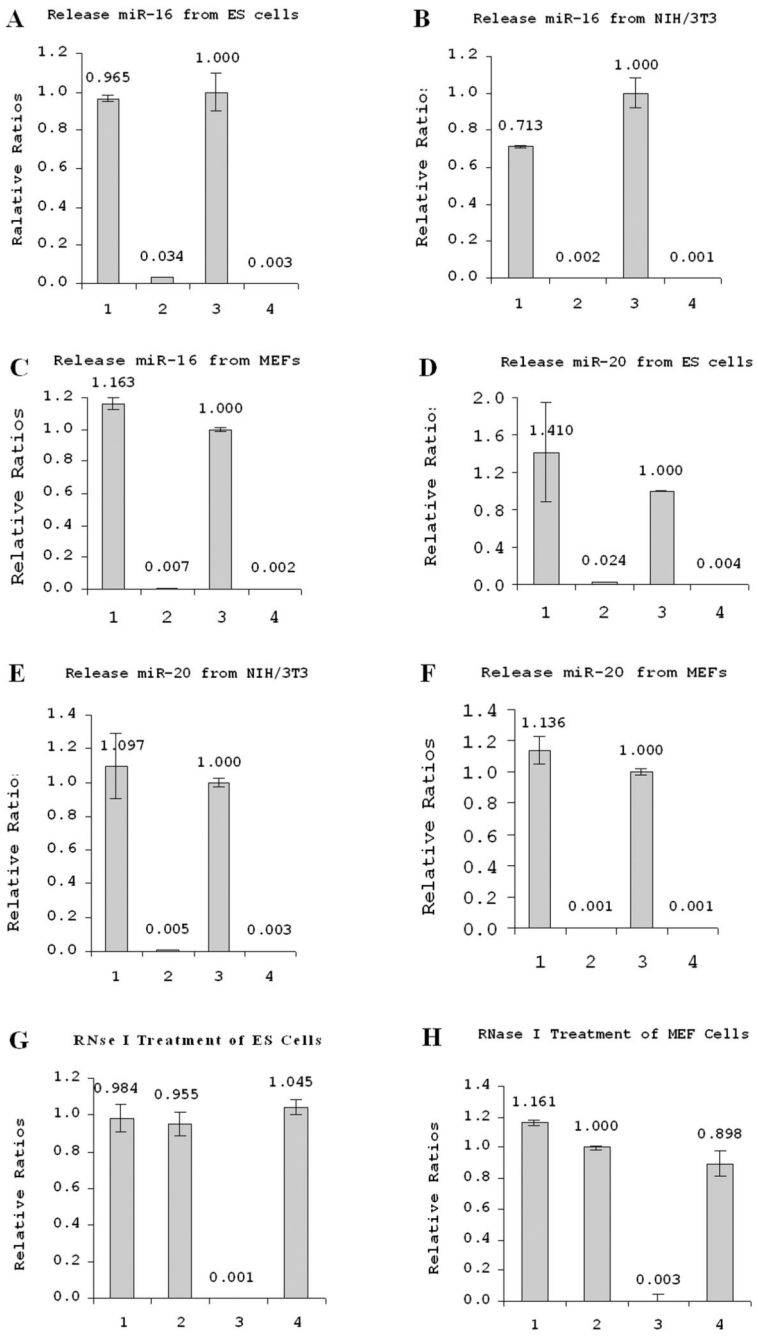


**Figure 1. Most miRNA are tightly associated with RISCs.**

**(A-C)** Measurement of miR-16 in ES cells, NIH/3T3 cells, and mouse embryonic fibroblasts (MEFs). (1) Three freeze-thaw cycles followed by 95°C for 5min; (2) Three freeze-thaw cycles only (3) Treatment at 95°C for 5min; (4) Treatment at 4°C only as a control. **(D-F)** Measurement of miR-20 level in ES cells, NIH/3T3 cells, and MEFs. Treatments 1, 2, 3 and 4 are as specified in A-C. **(G)** RNase I treatment of ES cell lysates. (1) ES cells after three freeze-thaw cycles, were treated with RNase I for 5min, and following exposure at 95°C for 5min to release all RISC-bond miRNAs. (2) Three freeze-thaw cycles followed by

incubation in the buffer for 5min, and at 95°C for 5min to release all RISC-bond miRNAs (as a control for RNase I treatment). (3) Incubation at 95°C for 5min to release miRNAs from RISC complex, followed by RNase I treatment for 5min, and further incubation of the cell lysate at 95°C for 5min. (4) ES cells were incubated at 95°C for 5min to release miRNAs from RISC complex, followed by treatment with buffer treatment for 5min, and incubation at 95°C for 5min (as a control for RNase I treatment). **(H)** RNase I treatment of MEF (Mouse Embryonic Fibroblast) lysates. (1) MEFs after three freeze-thaw cycles, were treated with RNase I for 5min, and following exposure at 95°C for 5min to release all RISC-bond miRNAs. (2) Three freeze-thaw cycles followed by incubation in the buffer for 5min, and at 95°C for 5min to release all RISC-bond miRNAs (as a control for RNase I treatment). (3) Incubation at 95°C for 5min to release miRNAs from RISC complex, followed by RNase I treatment for 5min, and further incubation of the cell lysate at 95°C for 5min. (4) MEFs were incubated at 95°C for 5min to release miRNAs from RISC complex, followed by treatment with buffer treatment for 5min, and incubation at 95°C for 5min (as a control for RNase I treatment).

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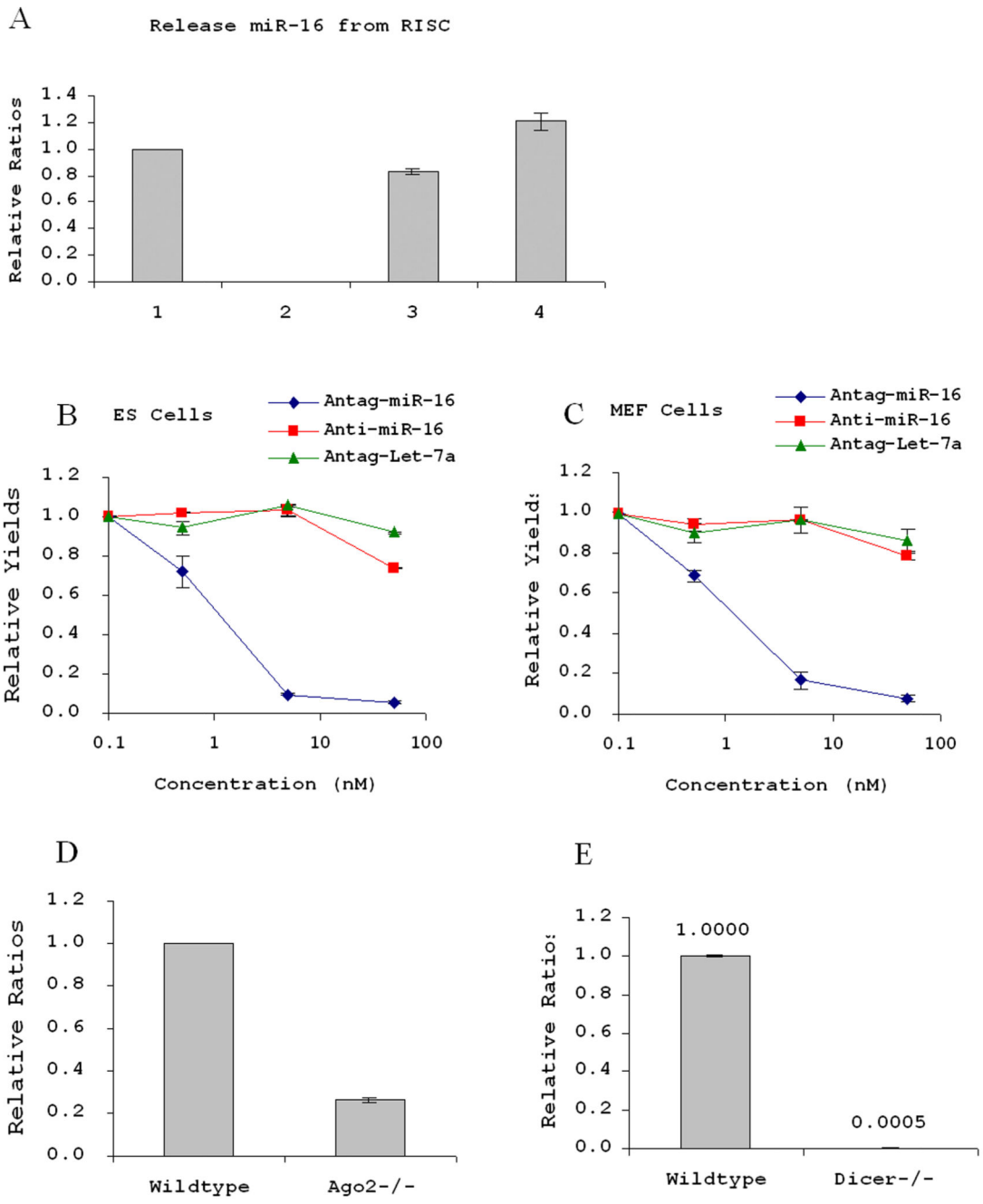


**Figure 2. The influence of antagomirs, Ago2, and Dicer on miRNA.**

**(A)** Excessive amounts of synthetic miRNA or its precursor cannot replace endogenous miRNAs-RISCs. (1) ES cells treated at 95°C for 5min to release miRNAs (positive control). (2) Treatment at 95°C for 5min followed by incubation with RNase I for 30min to degrade 'free' miRNAs, then 95°C for 5min to release RISC-bond miRNAs (as negative control). (3) ES cells subjected to freeze-thaw cycles, followed by incubation with 0.5uM synthesized let-7a for 30min, and then with RNase I for 30min to degrade 'free' miRNAs, and finally 95°C for 5min to release RISC-bond miRNAs. (4) ES cell lysate incubated with 0.5uM

synthesized pre-let-7a for 30min, and then with RNase I for 30min to degrade 'free' miRNAs, and finally at 95°C for 5min to release RISC-bond miRNAs. **(B-C)** Antagomirs effect on complementary miRNAs. Cells (ES or MEFs) were lysed by 3 freeze-thaw cycles. Then resulting cell lysate was incubated with 0.5nM-50nM of antagomirs of miR-16 (antagomir-16) for 30min, or as a control, with 0.5nM-50nM antisense miR-16 RNA or an unrelated antagomirs of Let-7a. RNase I was added subsequently to the samples to degrade miRNAs that were released from RISC complexes. Finally, RNase I was inactivated and the remaining RISC-bound miRNAs were released by treatment of the cell lysate at 95°C for 5min. **(D)** Measurement of mir-16 expression in wildtype and $Ago2^{-/-}$ mouse embryonic fibroblasts (MEFs). **(E)** Measurement of mir-16 expression in wildtype and $Dicer^{-/-}$ mouse embryonic fibroblasts (MEFs).

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