Focusing on RISC assembly in mammalian cells

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Received 11 January 2008. Available online 4 February 2008.

Abstract

RISC (RNA-induced silencing complex) is a central protein complex in RNAi, into which a siRNA strand is assembled to become effective in gene silencing. By using an in vitro RNAi reaction based on Drosophila embryo extract, an asymmetric model was recently proposed for RISC assembly of siRNA strands, suggesting that the strand that is more loosely paired at its 5′ end is selectively assembled into RISC and results in target gene silencing. However, in the present study, we were unable to establish such a correlation in cell-based RNAi assays, as well as in large-scale RNAi data analyses. This suggests that the thermodynamic stability of siRNA is not a major determinant of gene silencing in mammalian cells. Further studies on fork siRNAs showed that mismatch at the 5′ end of the siRNA sense strand decreased RISC assembly of the antisense strand, but surprisingly did not increase RISC assembly of the sense strand. More interestingly, measurements of melting temperature showed that the terminal stability of fork siRNAs correlated with the positions of the mismatches, but not gene silencing efficacy. In summary, our data demonstrate that there is no definite correlation between siRNA stability and gene

silencing in mammalian cells, which suggests that instead of thermodynamic stability, other features of the siRNA duplex contribute to RISC assembly in RNAi.

Keywords: siRNA; RISC assembly; Silencing activity; Thermodynamic stability

RNAi is a potent gene silencing technology whereby introduction of double-stranded RNA molecules into cells results in degradation of homologous mRNA transcripts [1], [2] and [3]. However, the gene silencing efficacies of siRNAs differ widely [4], [5] and [6]. To identify specific and effective siRNAs, various parameters have been investigated for their contributions to RNAi [3], [7], [8], [9], [10] and [11]. While some studies emphasize the importance of sequence and structural features of the siRNA duplex [3], [4], [5] and [6], others suggest that structural features of the mRNA target site are critical; these could obstruct gene silencing by an otherwise effective siRNA [4], [9], [12] and [13]. More recently, unwinding of the siRNA duplex was suggested as another important issue in RNAi. To investigate the contribution of siRNA terminal stability to duplex unwinding and gene silencing, Schwarz et al. performed in vitro RNAi reactions based on Drosophila embryo extracts, and proposed an asymmetric model for RISC assembly of the siRNA strand [14]. They showed that the two siRNA strands are not equally eligible for RISC assembly due to their different terminal stabilities. While the strand with higher free energy at its 5′ end is selectively assembled into RISC, the other strand is excluded from RISC and destroyed. Further studies by this group showed that protein R2D2 senses the thermodynamic difference between the two siRNA ends, binds to the end with a greater double-stranded character, and thereby determines the siRNA strand to be assembled into RISC [15]. Taken together, their studies suggest that an effective siRNA duplex is likely to have asymmetric terminal stability; the 5′ end of an effective siRNA strand has a higher free energy and is preferred to the other strand. This model was immediately and widely accepted as a major principle in siRNA design, and integrated into most siRNA selection algorithms. However, it is mainly established on in vitro experimental data, and has not been extensively validated in mammalian cells, in which most RNAi research and applications are carried out.

To determine the contribution of siRNA stability to gene silencing in mammalian cells, we performed cell-based assays and large-scale RNAi database analyses. RISC assembly was further investigated by using fork siRNAs. In addition, the influence of siRNA terminal mismatch on thermodynamic stability was investigated by melting temperature measurements.

Materials and methods

Calculation of terminal free energy. The terminal free energy values of the first four base pairs at each end of a siRNA duplex were calculated as described by Schwarz and coworkers by using the nearest-neighbor method and the mfold algorithm, assuming that the helix is fully formed. Energy parameters

from the Turner lab were used in calculations. Software is available at http://sirna.cgb.ki.se/EnergyCalculator for free energy calculation of siRNA sequences.

Spectroscopy. UV–vis melting curves (absorbance versus temperature profile) of matched and mismatched RNA duplexes were obtained on a Varian Cary 300 Bio UV–visible spectrophotometer (Varian, USA) using a 1.0 cm path-length cell and 0.2 OD RNA duplex. Absorbance was measured in RNase-free buffer (10 mM phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, pH 7) at 260 nm from 20 to 90 °C with a heating rate of 0.5 °C/min. Prior to the measurements, annealing of RNA duplexes was carried out by heating to 90 °C for 5 min followed by slowly cooling to 4 °C overnight. Melting temperatures were calculated by the Carry WinUV software package.

Results

Effective siRNAs show diverse terminal stability patterns

To study RISC assembly of siRNA in mammalian cells, a panel of 16 siRNAs, which included both effective and ineffective siRNAs targeting 11 endogenous genes, was randomly selected from our siRNA resource (siRNA sequences are listed in Table 1). The gene silencing efficacy of siRNA sense and antisense strands were individually evaluated using a siRNA validation assay, which measured gene silencing efficacy at the protein level [16]. To this end, an isolated siRNA target site complementary to one of the two siRNA strands was cloned in-frame with the firefly luciferase gene in a mammalian expression vector (Fig. 1). When expressed in culture cells, the fused reporter transcript functions as an artificial target for the complementary siRNA strand. Therefore, the gene silencing efficacy of the siRNA strand of concern was determined by measuring reporter gene silencing.

Table 1. Sequences of siRNAs used in RNAi study

siRNA	Gene	Sense target sequence
1	CD46-1	5′-CTTATTGGAGAGAGCACGA-3′
2	CD46-2	5′-GCCTCCAGTCTCAAATTAT-3′
3	Cyclin-G1	5′-GATCTACTTAGTCTAACTC-3′
4	Dusp6	5′-GAGTTTGGCATCAAGTACA-3′
5	NPY305	5′-TGAGAGAAAGCACAGAAAA-3′
6	Fas679	5′-CCATACCAATGAATGCCTC-3′

7	Fas873	5′-TCTCATGGGAAGAGTGATG-3′
8	Fas297	5′-GGGAAGGAGTACATGGACA-3′
9	Cdc2-1	5′-AAGGGGTTCCTAGTACTGC-3′
10	Cdc2-2	5′-TCGGGAAATTTCTCTATTA-3′
11	CDK2-1	5′-AAGGCAGCCCTGGCTCACC-3′
12	CDK2-2	5′-GACGGAGCTTGTTATCGCA-3′
13	Lamin A/C	5′-CAGAACTGCAGCATCATGT-3′
14	LIMK1	5′-CACCATGGACTTTGGCCTC-3′
15	BCL2	5′-ACGCTTTGCCACGGTGGTG-3′
16	Caveolin2	5′-TGCCCTCTTTGAAATCAGC-3′

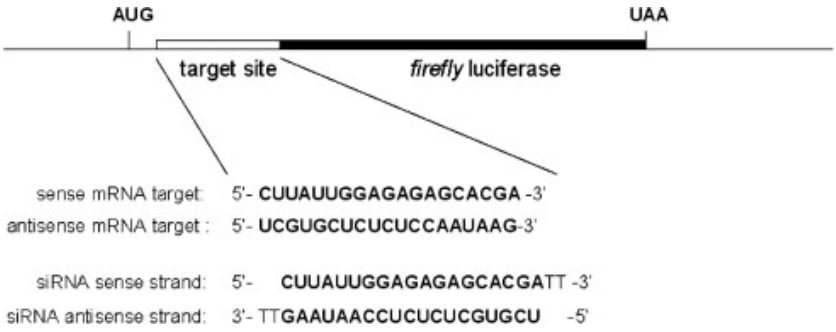

Fig. 1. Schematic diagram of fusion firefly luciferase reporter. The fusion luciferase reporter contains an inserted in-frame AUG start codon, and a box representing the siRNA target site, followed by the luciferase gene.

For each siRNA, two target reporters were constructed, each complementary to one of the siRNA strands (Fig. 1). In total, 32 target reporters were generated for 16 siRNAs. By using these reporters, the silencing efficacy of each siRNA strand was individually determined in HEK293 cells (Fig. 2). To facilitate analysis, 60% reduction of reporter gene expression was set as the cutoff to divide the siRNA strands into effective and ineffective groups. Out of 32 siRNA strands, 16 were effective and the others were ineffective. Among the 16 effective siRNAs, four were composed of two effective strands (Fig. 2, siRNA-1, 3, 5, and 10), nine were composed of one effective strand (siRNA-2, 4, 6, 8, 9, 12, 14, 15, and 16), and the remaining three had no effective strands (siRNA-7, 11, and 13).

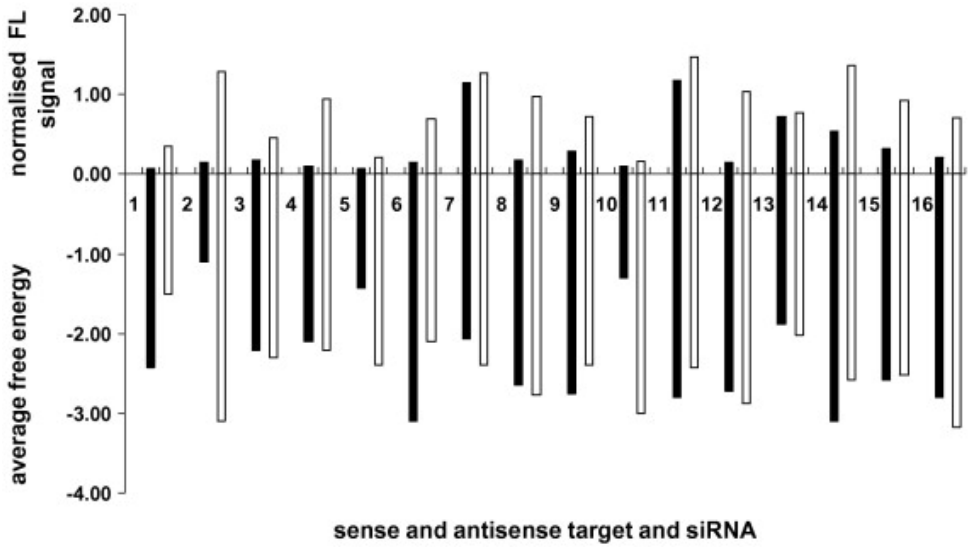

Fig. 2. Terminal stability and silencing activity of siRNA. Silencing activity of siRNA strands is plotted in the positive area of the vertical axis, and the average free energy (G) of the first four base pairs at the 5′ and 3′ ends (refer to the orientation of the siRNA sense strand) of siRNAs are plotted in the negative area of the vertical axis. The data for siRNA antisense strands are presented as black bars, and for siRNA sense strands as white bars.

Individual measurement of the silencing efficacy of each siRNA strand enabled us to study the correlation between siRNA stability and gene silencing. The thermodynamic stability of the four terminal base pairs at each end of the siRNA duplex was first calculated as described in the literature [14]. Terminal stability is represented by the average free energy (G), and is referred to as 5′ G or 3′ G according to the orientation of the siRNA sense strand. A stability ratio, obtained by dividing the absolute value of the difference between 5′ G and 3′ G by the absolute value of the sum of 5′ G and 3′ G, was calculated for each siRNA and used to describe the stability difference at the two ends. A ratio of 0.05% was taken as the standard to divide the siRNAs into symmetric and asymmetric stability groups. The symmetric group (siRNA-3, 4, 8, 12, 13, and 15) was composed of siRNAs with stability ratios less than 0.05, indicating that the stability at both siRNA ends is essentially the same. The asymmetric group (siRNA-1, 2, 5, 6, 7, 9, 10, 11, 14, and 16) was composed of siRNAs with stability ratios equal to or greater than 0.05, indicating that the stability at each siRNA end was significantly different. In the latter case, siRNAs were further divided into two subgroups with higher 5′ G (siRNA-1, 6, 9, 11, and 14) or higher 3′ G (siRNA-2, 5, 7, 10, and 16).

The terminal stabilities of siRNAs were aligned with silencing efficacies (Fig. 2). If RISC assembly is governed mainly by the relative terminal stability of siRNA, more effective siRNAs are expected to have a greater 5′ G, as proposed by the asymmetric RISC assembly model. However, such correlation was not found (Fig. 2). For example, in the asymmetric stability group, five siRNAs had higher 5′ G, and the other five had higher 3′ G. Although half of the siRNAs had much lower 5′ free energy than 3′ free energy, their antisense strands were able to assemble into RISC, passed through all downstream process, and finally repressed target gene expression. In addition, five out of six siRNAs of the symmetric stability group showed asymmetric silencing activity. These results indicate that the terminal stabilities of siRNA is not the major determinant of its silencing activity.

siRNA database analyses

To further study this issue, two recently reported large-scale siRNA databases (HuSiDa and siRNAdb), which contain most of the experimentally validated siRNAs in the public domain, were analyzed [17] and [18]. The HuSiDa database has 1021 siRNAs with more than 50% silencing efficacy, and siRNAdb has both high and low efficacy siRNAs. In RNAi, it is well established that the characteristics of the siRNA duplex and the structural features of the mRNA target are the two determinants of silencing efficacy [3], [4], [5], [6], [9], [12] and [13]. To focus our study on siRNA stability and gene silencing, we had to exclude the potential influence of mRNA target structure. To do this, only highly effective siRNAs, with silencing efficacies of more than 75%, were selected. Potent target gene silencing was experimentally confirmed for these siRNAs, indicating that their target structures do not interfere with RNAi. The terminal thermodynamic stability of siRNA duplexes was calculated and compared with their silencing efficacy. The results showed that while 52% of the siRNAs had higher free energy at the 5′ end of the antisense strand, 46% had higher free energy at the 5′ end of the sense strand, and the remaining siRNAs had equivalent free energy at both ends.

It worth noting that most, if not all, siRNAs collected in these databases had been pre-selected by various criteria. To exclude the influence of pre-selection, 180 systemically designed and validated siRNAs were included [19]. These siRNAs target two 197-base coding regions of firefly luciferase and the human cyclophilin B gene, systematically covering 50% of all possible siRNA target sites in these two regions. In our analysis, 42% of these siRNAs had higher free energy at the 5′ end of the antisense strand, and the rest had higher free energy at the 5′ end of the sense strand. In addition, another randomly selected dataset containing 2431 siRNAs was also studied [20]. While 53% of the siRNAs had higher free energy at the 5′ end of the antisense strand, 45% had higher free energy at the 5′ end of the sense strand. Therefore, no correlation was found between siRNA stability and gene silencing. To our knowledge, this is the most comprehensive analysis on this issue to date.

RISC assembly of terminally mismatched siRNA

So, we were unable to establish the correlation proposed by the asymmetric RISC assembly model. To further pursue this problem, experiments were designed using a special form of siRNA (fork siRNA). Following the principle of the asymmetric RISC assembly model, fork siRNA was designed to have a mismatch at the 5′ end of the antisense strand [21] and [22]. This design greatly increases the free energy at the 5′ end, presumably making the antisense strand favored for assembly into RISC. Although the gene silencing efficacy of fork siRNA is dramatically improved, the sequence variation between the fork and the native siRNA is only one terminal nucleotide. Therefore, increased silencing efficacy is mainly caused by more efficient RISC assembly of the fork siRNA strand. This unique feature was utilized in our study to examine RISC assembly of the siRNA strand.

Based on the Fas679 siRNA sequence, four terminally mismatched fork siRNAs were synthesized. Each had a single-base mutation at position 18 or 19 of the siRNA antisense strand, resulting in a C:C or C:A mismatch (Fig. 3a). Two additional matched siRNAs, Fas679-As1A1U (terminal C:G changed to U:A) and Fas679-As1C1G (terminal C:G changed to G:C), were also included (Fig. 3a). In order to evaluate the influence of the terminal mismatch on siRNA thermodynamic stability, melting temperatures were measured (Fig. 3c). Tm decreased by 3.01 °C for the C:A mismatch and 2.41 °C for the C:C mismatch when they were at position 19, and decreased by 4.94 °C for C:A and 4.98 °C for C:C mismatch when they were at position 18. For the two matched siRNAs, Tm decreased by 0.94 °C when the terminal base pair was changed from C:G to U:A, and increased by 2.03 °C when it was changed from C:G to G:C. Interestingly, the thermodynamic stability of fork siRNAs was correlated with the position of the mismatch, but not its identity. In addition, more striking drops of melting temperature were seen in the fork siRNAs than in the matched siRNAs, indicating that a mismatch greatly destabilizes siRNA duplex structure.

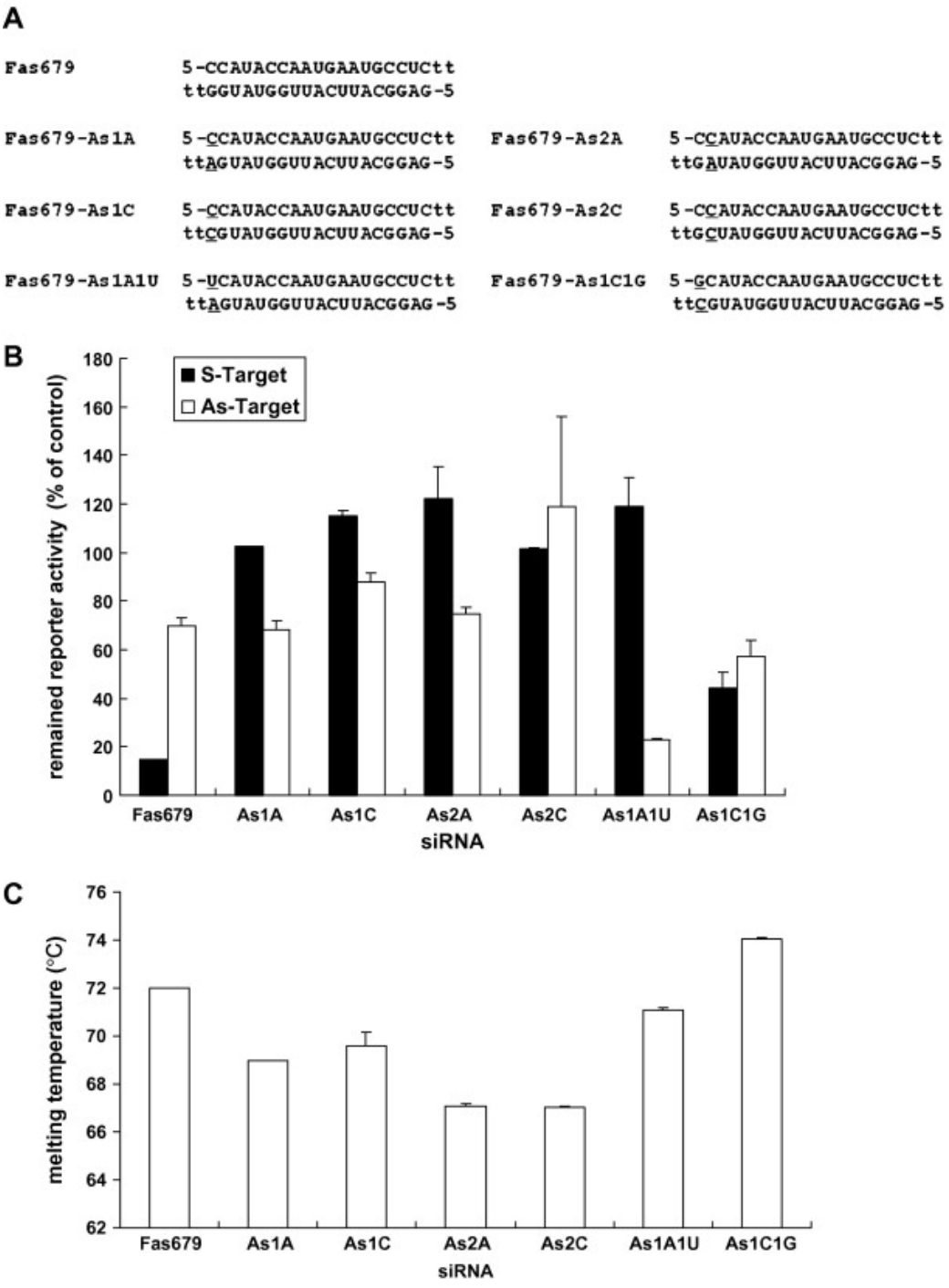

Fig. 3. Silencing activity and melting temperatures of Fas679 siRNAs. (a) Sequence of matched and mismatched RNA duplexes. (b) Silencing activity of Fas679 siRNA antisense or sense strands measured by using a siQuant vector incorporating the antisense or sense target site. Data for siRNA antisense strands targeting a sense target are presented as black bars, and sense strands targeting an antisense

target as white bars. (c) Melting temperatures of RNA duplexes. All results presented in bar graphs were repeated at least twice.

According to the asymmetric RISC assembly model and published studies [21] and [22], when a mismatch is incorporated at the 3′ end of a siRNA antisense strand, RISC assembly of the sense strand increases and that of the antisense strand decreases. To assess the gene silencing of these siRNAs, the silencing efficacy of the sense and antisense siRNA strands listed in Fig. 3a were evaluated. In agreement with the predictions, the silencing efficacy of fork siRNA antisense strands decreased, indicating that the terminal mismatch decreases its RISC assembly. However, the silencing efficacy of the sense strands did not increase as predicted. One possibility is that the RISC assembly of the sense strand does not increase. Another possibility is that the sense strand is ineffective in gene silencing even when it is loaded into RISC. Interestingly, when the terminal base pair was changed from C:G to U:A in Fas679-As1A1U, which only marginally decreased the melting temperature, the silencing efficacy of the sense strand greatly increased, showing that the sense strand was effective when loaded into RISC (Fig. 3b). Our data indicated that the RISC assembly of siRNA sense strands does not increase in fork siRNAs. Therefore, we were able to show that, at the cellular level, increasing terminal free energy at the 5′ end of the siRNA sense strand did not increase its RISC assembly. For Fas679-As1C1G, changing the terminal base pair from C:G to G:C unexpectedly increased Tm by 2.03 °C. However, the silencing efficacy of the antisense strand decreased and that of the sense strand increased compared with native siRNA. Taken together, these data indicate that the terminal free energy of siRNA is not a major determinant of silencing efficacy, and further suggest that other structural features of the siRNA duplex, such as duplex conformation, play a role in RNAi.

Discussion

To gain more insight into RISC assembly of siRNA, cell assays were performed to assess the contribution of siRNA terminal stability. Although most siRNA antisense strands in our test siRNA set were effective in silencing their respective target genes, half of them did not follow the asymmetric RISC assembly model. To further characterize this problem, bioinformatics analyses were carried out on siRNA databases, including a pre-designed siRNA database, a systematically designed siRNA database, and a randomly designed siRNA database. To our knowledge, this is the most comprehensive study on this issue to date. Again, no correlation was found in the database analyses. Taking these findings together, we conclude that there is no correlation between siRNA stability and silencing activity. Further experiments on siRNA thermodynamic stability showed that the melting temperatures of fork siRNAs were correlated with the positions of the introduced mismatches, but not with the gene silencing activity. In contrast to previous studies, our data indicate that there is no definite correlation between siRNA stability and gene silencing. Experiments on fork siRNAs further demonstrated that the influence of siRNA terminal base

pairs on silencing efficacy may be mediated by structural features of the siRNA duplex, but not by thermodynamic stability.

It was noted that while we measured siRNA silencing efficacy mainly in cultured cells, the asymmetric RISC assembly model is mainly based on in vitro RNAi reactions. Although RNAi is established as an evolutionarily conserved process, significant difference in RNAi occurs between species. For example, diverse Argonaute family members, which are the core components of RISC, have been identified in different species [24] and [25]. Drosophila contains four characterized Argonaute proteins (Piwi, Aubergine, dAgo1, and dAgo2), plus one predicted from genomic DNA (dAgo3), in which dAgo1 is required for efficient RNAi in Drosophila embryos. Eight family members have been identified in human cells, distributed between two subfamilies. The Ago subfamily contains Ago1, Ago2, Ago3, and Ago4, and the Piwi subfamily contains PIWI1, PIWI2, PIWI3, and PIWI4. We speculate that these and other differences in RNAi may account for the controversial findings of our studies.

Acknowledgments

We appreciate Drs. Iain Bruce and Tong Zhang for critically reading of this manuscript. This work was supported by a grant from Ministry of Education of China (20070001011), grants from the National 863 High-Tech R&D Program (2006AA02Z104 and 2007AA02Z165), and a grant from the NSFC of China (30771085).

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