Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Wistar Institute

2. **Reporting Period (start and end date of grant award period):** 01/01/09 – 06/30/10

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Russel E. Kaufman, M.D.

4. **Grant Contact Person's Telephone Number:** 215-898-3926

5. **Grant SAP Number:** SAP #4100047657

6. **Project Number and Title of Research Project:** Project 3: Frequency, Rate and Significance of MicroRNA Editing

7. **Start and End Date of Research Project:** 01/01/09 – 06/30/10

8. **Name of Principal Investigator for the Research Project:** Kazuko Nishikura, Ph.D.

9. **Research Project Expenses**.

   9(A) Please provide the amount of health research grant funds spent on this project for the entire duration of the grant, including any interest earned that was spent:

   $ 288,988.44

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost (Salary & FB)
Nishikura	Principal Investigator	55%	$ 172,795

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Iizasa	Postdoctoral fellow	50% (Yr 1-2)
Ota	Postdoctoral fellow	50% (Yr 2)

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____X____ No_____

If yes, please indicate the source and amount of other funds:
NIH; $200,000

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes____X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Control of Cardiogenesis by MicroRNA Editing	☒ NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)	April 2009	$1,000,000	$1,000,000
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$
	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes _____X____ No_____

If yes, please describe your plans: I plan to submit a new NIH grant in October 2010.

12. Future of Research Project. What are the future plans for this research project?

We will continue to investigate the significance of microRNA editing. More recently we discovered that viral miRNAs are also subject to A-to-I RNA editing. We will investigate the significance of editing in virus replication and latency.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes ____X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male	3			2
Female	1			
Unknown				
Total	**4**			**2**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic	4			2
Unknown				
Total	**4**			**2**

	Undergraduate	Masters	Pre-doc	Post-doc
White	3			
Black				
Asian	1			2
Other				
Unknown				
Total	**4**			**2**

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No ____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes ____X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

By securing PI's salary, we could bring more postdoctoral fellows and undergraduate students into this project, which in turn greatly improved the overall project performance.

16. **Collaboration, business and community involvement.**

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No ____X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No ____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No _____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant application's strategic plan). Summarize the progress made in achieving these goals, objectives and aims for the entire grant award period. Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project Title and Objectives

Frequency, Fate and Significance of MicroRNA Editing - Within the last decade a new type of ribonucleic acid (RNA) was discovered, microRNA (miRNA), which plays a major role in the regulation of gene expression. miRNAs are produced as larger gene transcripts (pri-miRNA) which are subsequently processed to mature miRNA. We found that pri-miRNAs are subject to editing by conversion of adenosine residues to inosine residues (A→I editing). Editing of pri-miRNAs results in inhibition of their processing and alters the genes targeted by the unedited miRNAs, resulting in naturally occurring "off-targeting effects". The overall goal of this project was to gain a better understanding of the biological significance of miRNA editing.

Project Overview

The broad, long-term goal of this project was to increase our understanding of ribonucleic acid interference (RNAi) biology and improve applications of the microRNA (miRNA)-based RNAi

technology. Our recent studies have revealed that primary transcripts of certain miRNA genes (pri-miRNAs) are subject to one type of RNA editing that converts adenosine residues into inosine in double-stranded RNA (dsRNA). Editing of pri-miRNAs results in inhibition of their processing to mature miRNAs or expression of edited mature miRNAs and silencing of a set of genes different from those targeted by the unedited miRNAs, resulting in naturally occurring "off-targeting effects" of miRNA-based RNAi gene silencing. It is currently not known how widely pri-miRNA editing takes place. In this project, therefore, we would first estimate the A→I editing frequency of mammalian and viral pri-miRNA, determine its biological significance, and assess its "off-targeting effects". A search through an miRNA database identified a large number of mammalian and viral candidate pri-miRNAs containing a "preferred" A→I editing site within a consensus sequence predicted from a set of pri-miRNAs proven to have editing sites. These candidate pri-miRNAs were tested experimentally in order to determine what fraction of mammalian and viral pri-miRNAs are edited. The effects of editing on miRNA processing or expression of the edited miRNAs would be evaluated by subjecting these newly identified pri-miRNAs containing specific A→I editing sites to an *in vitro* miRNA processing assay. We conducted targeted cloning and identification of edited mature miRNAs that would be processed from edited pri-miRNAs.

The specific aims of this project were:

Specific Aim 1. Survey miRNA gene transcripts for A→I RNA editing and identify edited miRNAs in order to determine the overall frequency of mammalian and viral pri-miRNA editing.

Specific Aim 2. Determine the effects of the pri-miRNA editing *in vitro* by miRNA processing assay using recombinant Drosha-DGCR8 and Dicer-TRBP complexes.

Summary of Research Completed

Overview:
We completed both specific aims.

Specific Aim 1. Determination of the overall frequency of pri-miRNA editing. We conducted a large scale survey of human pri-miRNAs containing the UAG triplet sequence. By direct sequencing of RT-PCR products corresponding to pri-miRNAs, we examined 209 pri-miRNAs and identified 43 UAG and also 43 non-UAG editing sites in 47 pri-miRNAs, which were highly edited in human brain. Our studies predict that approximately 16% of human pri-miRNAs are subject to A→I editing; and, thus, miRNA editing could have a large impact on the miRNA-mediated gene silencing.

Specific Aim 2. Determination of the effects of the pri-miRNA editing *in vitro* by miRNA processing assay using recombinant Drosha-DGCR8 and Dicer-TRBP complexes. We found that a majority of pri-miRNA editing is likely to interfere with the miRNA processing steps. In addition, four new edited miRNAs with altered seed sequences were identified by targeted cloning and sequencing of the miRNAs that would be processed from edited pri-miRNAs. Thus, miRNA editing could have a large impact on the miRNA-mediated gene silencing.

Results

Survey for A→I RNA editing of UAG triplets of pri-miRNA sequences.
A→I editing of select pri-miRNAs was reported by several groups. By examining the editing frequency of the miRNA editing sites previously investigated by us, we noted that high frequency editing often occurred at the adenosine residue within the UAG triplets. The preference of the UAG triplet sequences for editing was reported previously in a small-scale survey of pri-miRNA editing sites as well as statistical analysis of editing sites present in non-coding repetitive element sequences such as Alu and SINE. We reasoned that the adenosine in the UAG triplet of pri-miRNA sequences may be preferentially edited *in vivo*. The pri-miRNA sequences containing the UAG triplet were identified in 474 human pri-miRNAs registered at the Sanger Center miRBase site (miRBase 9.1). Since the UAG triplets in single stranded regions are unlikely to be edited by ADARs, we selected 494 triplets in dsRNA regions of 291 pri-miRNAs. Among these candidates, UAG triplets that are mismatched at both U and G are also unlikely to be edited; therefore, 257 pri-miRNAs that contain 341 UAG triplet sequences in the dsRNA region were investigated. We attempted to amplify all of the 257 pri-miRNAs by RT-PCR, and 209 pri-miRNAs (~80%) were successfully amplified from the total RNAs derived from human brain where pri-miRNAs are edited frequently. Direct cDNA sequencing of RT-PCR products identified 43 UAG triplets in 38 pri-miRNAs which were edited with frequency higher than 10% (Table 1.1, 1.2). The 38 pri-miRNAs identified to contain one or more UAG editing sites accounted for 18.2% of 209 pri-miRNAs examined (Table 1.2). In addition, 43 non-UAG triplet editing sites were also identified while examining editing of UAG triplets (Table 2.1). Among those sites, 28 sites were detected in 11 pri-miRNAs which also contained at least a separate UAG triplet editing site, whereas 15 non-UAG triplet sites were found in 9 pri-miRNAs that did not contain the UAG triplet editing site (Table 1.2).

Most preferred UAG triplet editing sites
While analyzing the sequence and dsRNA structure of the UAG triplets, we noted that the editing frequency varies dependent on the complementary strand sequence that form the duplex structure with the UAG triplet. Therefore, we subgrouped 341 UAG triplets of 209 pri-miRNAs into three groups based on the nucleotide sequence complementary to U and G residues of the UAG triplet. a) Both U and G are Watson-Crick base-paired. b) Either one of U and G, or both, are wobble base-paired. c) Either one of U and G is mismatched (Table 1.1, 1.2). We detected editing of 34 sites (19.8%) of 31 pri-miRNAs (23.7%) with both U and G residues base-paired with A and C, respectively, in the Watson-Crick base-pairing, *i.e.*, group (a) (Table 1.1, 1.2), 4 sites (4.1%) of 4 pri-miRNAs (4.9%) with either U and G residues, or both base-paired in the wobble pairing, *i.e.*, group (b) (Table 1.1, 1.2), and 5 sites (6.8%) in 5 pri-miRNAs (7.7%) with either U and G residues in a mismatched base pairing, *i.e.*, group (c) (Table 1.1, 1.2). The results revealed that the two neighboring nucleotides of the UAG triplet need to be stably base-paired preferentially in the Watson-Crick base-pairing in order to be edited efficiently. Finally, the editing frequency was plotted against the effect of the nucleotide complementary to the adenosine residue of the UAG triplet. The presence of cytosine in the opposite strand resulted in the most frequent (15/27 or 55.6%) and highest mean of the editing efficiency (57.3%) followed by uridine (27/275 or 9.8%) and absence of the complementary nucleotide (1/15 or 6.7%) (Table 1.3). The preference of A·C mismatched pairs for editing was noted through mutagenesis of known protein coding pre-mRNA. Furthermore, analysis of non-coding Alu and SINE sequences

once again revealed much higher frequency for A·C mismatched editing sites, revealing its common preference for editing of pre-mRNAs, non-coding RNAs containing inversely oriented repeat elements, and also miRNAs.

The effects of the -2 and +2 positions

We then examined the effects of base-pairings at the -2 and +2 positions on editing efficiency at UAG triplets that already had Watson-Crick base-pairings at the -1 and +1 sites (Table 3). When both the -2 and the +2 positions were stabilized by Watson-Crick base-pairings, editing efficiency was 29%. By contrast, the replacement of even a single Watson-Crick base-pairing with a U-G wobble base-pairing or a mismatched base-pairing reduced the overall editing efficiency to 10.3% and 8.0%, respectively, indicating the importance of the stable dsRNA structure locked by Watson-Crick base pairings not only at the -1 and +1 positions but also at the -2 and +2 sites (Tables 1.1, 1.2, and 3). We also examined the influence of the -3 and +3 positions, which had no significant effects on the editing efficiency.

Effects of a specific nucleotide at the -2 and +2 positions on the editing efficiency were also investigated. No significant effects were detected for the editing frequency of UAG triplets with regard to the nucleotide at the -2 position, although the average editing efficiency was slightly higher for the triplets with A or G compared to those U or C at the -2 position (Table 4). By contrast, the presence of the adenosine residue at the +2 position resulted in the highest rate of the UAG triplet editing (59.3%), followed by guanosine (20.0%) or cytidine (21.4%), and uridine (11.1%) (Table 5). The +2 nucleotide appeared to influence also the mean editing frequency; adenosine the highest (69.1%) followed by uridine (50.0%), guanosine (24.0%), and cytidine (10.0%) (Table 5). Together, our statistical analysis revealed the most preferred sequence and structure of the UAG triplet editing site. The adenosine residue of the UAG triplet with a partner nucleotide of cytidine stably locked with four Watson-Crick base-pairings and the presence of adenosine at the +2 position appears to be the most frequently and highly edited site of pri-miRNAs in human brain.

Non UAG editing sites

In this study, 43 non-UAG triplet editing sites were also identified in 209 pri-miRNAs examined (Table 2.1). Among 43 non-UAG triplets, 32 sites had Watson-Crick base-pairings at both the -1 and the +1 sites, indicating once again the importance of the stable structure surrounding the adenosine residue to be edited. The presence of cytidine in the opposite strand resulted in most frequent editing (10/137 sites, 7.3%) in comparison to the presence of other partner nucleotides, *e.g.,* uridine (32/2273 sites, 1.4%), indicating that mismatched A·C pairs are also the most favored editing sites even among non-UAG triplets (Table 2.2). AAG, AAA, UAU, UAC, and UAA were frequently edited. By contrast, editing of triplets having guanosine as the 5' neighbor, *e.g.*, GAU, was extremely rare. Although these non-UAG triplet editing sites identified were in some cases highly edited, 10 AAG editing sites out of a total of 231 AAG triplets present in 209 pri-miRNAs, among the most frequently edited non-UAG triplets, form a marked contrast to 43 UAG triplet sites (Table 1.3 and 2.2). The 209 pri-miRNAs were selected unbiased for non-UAG triplet sites. According to non-UAG triplets located within the dsRNA region of 209 pri-miRNAs (examined), we anticipate proportionally that an additional 11 AAG triplet editing sites might be identified among 253 AAG sites (data not shown) present in 217 pri-miRNAs not examined in this study, or a total of 21 AAG editing sites, far less than the 43 UAG triplets. Thus, the results

of our survey indicate that the UAG triplet is likely the most favored triplet of pri-miRNAs for A→I editing. A statistical analysis of a large number of editing sites identified in Alu and mouse SINE repeat sequences also revealed the preference of U and C as the 5' nearest neighbor and G preference for the 3' nearest neighbor and favored UAG triplet sequences. *In vitro* and *in vivo* editing analysis of 16 sites located within intron 4 of ADAR2 pre-mRNAs had previously revealed the 5'-nearest neighbor preference (U=A>G), again consistent with our analysis of pri-miRNA editing sites.

Frequency of pri-miRNA editing

A small scale survey for A→I editing of pri-miRNAs in human brain was conducted before, revealing 6 out of 99 pri-miRNAs examined and thus the estimate of 6% of pri-miRNAs to be subject to editing. In this study, we noted that many pri-miRNAs reported previously not subject to A→I RNA editing were determined to be in fact highly edited, *e.g.*, pri-miR-368, pri-miR-376a-2, and pri-miR-411. This is likely due to the relatively low frequency of successful amplification of pri-miRNA sequences (99 pri-miRNAs amplified from 231 investigated) in the previous study. For analysis of UAG triplet editing sites, we preselected 257 out of 474 human pri-miRNAs (miRBase 9.1) that contained 341 UAG triplets in the dsRNA region. In this study, 209 pri-miRNAs out of the 257 pri-miRNAs were successfully RT-PCR amplified from human brain RNAs, revealing 47 pri-miRNAs that indeed contained UAG and/or non-UAG editing sites. Among the 47 pri-miRNAs, 20 pri-miRNAs contained non-UAG editing sites regardless of the presence or absence of UAG editing sites. Although 209 pri-miRNAs were selected for those containing UAG triplets, they were unbiased for non-UAG triplets. Therefore, we proportionally anticipate an additional 21 pri-miRNAs to contain the non-UAG triplet site in 217 pri-miRNAs excluded in this study. Together, we expect 68 (47 + 21) out of 426 (209 + 217) pri-miRNAs, or ~16% of all human pri-miRNAs to contain UAG and/or non-UAG editing sites. Our updated estimate of ~16% certainly changes the global impact of A→I editing on the miRNA-based silencing of mammalian genes.

Table 1.1. Editing of UAG sites present in human pri-miRNAs

	W-C group (a)	G-U group (b)	Mismatch group (c)	Total of 3 groups (a+b+c)
UAG sites examined	172	96	73	341
Edited at UAG sites	34	4	5	43
Editing frequency (%)	19.8	4.1	6.8	12.6

Table 1.2. Editing of pri-miRNAs containing UAG triplets

	W-C group (a)	G-U group (b)	Mismatch group (c)	Total of 3 groups (a+b+c) [a]
miRNAs examined	131	82	65	209
Edited at UAG sites	31	4	5	38
Edited at non-UAG sites	4	3	4	9
All edited pri-miRNAs	35	7	9	47
Frequency (%)	23.7 (26.7) [b]	4.9 (8.5) [b]	7.7 (13.8) [b]	18.2 (22.5) [b]

[a] Many pri-miRNAs contained multiple UAG triplets of different types, leading to the total pri-miRNA number that is less than the summation of three types.

[b] Frequency for all pri-miRNAs edited at both UAG and non-UAG sites.

Table 1.3. Effects of the nucleotide complementary to the UAG editing site

	The complementary nucleotide					
	C	U	G	A	None	Total
Edited/total (%)	15/27 (55.6)	27/275 (9.8)	0/11 (0.0)	0/13 (0.0)	1/15 (6.7)	43/341 (12.6)
Mean editing efficiency (%)	57.3	50.3	-	-	20.0	52.0

Table 2.1. Non-UAG editing sites

	The number	The mean editing efficiency (%)
AAG	10	49.5
AAA	8	50.0
UAU	6	23.3
UAC	5	20.0
UAA	4	43.8
CAG	2	75.0
CAC	2	20.0
CAU	2	22.5
CAA	1	40.0
AAC	1	20.0
AAU	1	10.0
GAG	1	30.0
Total	43	38.3

Table 2.2. Effects of the nucleotide complementary to the non-UAG site

	The complementary nucleotide					
	C	U	G	A	None	Total
AAG	0/13	10/185	0/11	0/16	0/6	10/231
AAA	3/15	5/205	0/31	0/33	0/12	8/296
UAU	1/13	4/240	0/8	1/20	0/5	6/286
UAC	0/10	5/157	0/8	0/17	0/9	5/201
UAA	0/6	4/164	0/10	0/27	0/11	4/218
CAG	1/19	1/238	0/16	0/15	0/7	2/295
CAC	2/10	0/170	0/6	0/9	0/9	2/204
CAU	1/5	1/221	0/4	0/15	0/13	2/258
CAA	1/7	0/166	0/10	0/18	0/7	1/208
AAC	0/9	1/135	0/13	0/10	0/7	1/174
AAU	0/16	1/190	0/21	0/17	0/10	1/254
GAG	1/14	0/202	0/11	0/17	0/9	1/253
Total[a]	10/137 (7.3%)	32/2273 (1.4%)	0/149 (0.0%)	1/214 (0.5%)	0/105 (0.0%)	43/2878 (1.5%)

[a]Edited and potential non-UAG sites identified in the 209 pri-miRNAs were counted (edited/potential).

Table 3. The effects of the base-pairing at the -2 and +2 positions

	W-C group	G-U group	Mismatch group	Total
UAG sites examined	93	29	50	172
Edited at UAG sites	27	3	4	34
Mean editing efficiency (%)	29.0	10.3	8.0	19.8

Table 4. The effect of the nucleotide -2 position

	The nucleotide at -2 position				
	A	G	U	C	Total
Editing frequency, edited/total (%)	10/27 (37.0)	7/21 (33.3)	4/16 (25.0)	6/29 (20.7)	27/93 (29.0)
Mean editing efficiency (%)	69.5	58.6	30.0	30.0	52.0

Table 5. The effect of the nucleotide +2 position

	The nucleotide at +2 position				
	A	G	U	C	Total
Editing frequency, edited/total (%)	16/27 (59.3)	5/25 (20.0)	3/27 (11.1)	3/14 (21.4)	27/93 (29.0)
Mean editing efficiency (%)	69.1	24.0	50.0	10.0	52.0

Expression of edited mature miRNAs

Many pri-miRNA editing sites were located within the mature miRNA sequence or corresponding partner miRNA sequence in the opposite strand, which could be processed to edited mature miRNAs as we previously reported for miR-376 cluster RNAs. We applied the targeted cloning strategy as described previously, attempting to isolate cDNA clones corresponding to all of these potential edited mature miRNAs. Only four mature miRNAs containing UAG and/or non-UAG editing sites not previously reported were identified; miR-379-5p, miR-411-5p, miR-607-3p, and miR-99b-3p (Figure 1). Interestingly, both miR-379 and miR-411 are members of the miR-379 cluster. Simultaneous expression of several edited mature miRNAs of the miR-376 cluster has been also reported, indicating the possibility of simultaneous editing of clustered miRNAs. The A→I editing sites identified in these mature miRNAs were located in the seed sequence known to play a major role in the hybridization of a miRNA with the target site, thus affecting selection of the target genes. As we have demonstrated for edited miR-376 RNAs, application of target prediction programs is likely to reveal that these newly identified edited mature miRNAs target genes are different from those targeted by the unedited miRNAs.

A recent global survey for miRNAs from various human and mouse tissues identified a large number of miRNAs that had nucleotide changes within the mature miRNA sequence, including those already reported previously as edited miRNAs such as miR-368, miR-376a1, miR-376a2. However, four edited miRNAs identified in the present study as well as those identified previously (hs-miR-376b, mu-miR-376a, mu-miR-376b, and mu-miR-376c) by the targeted

cloning strategy were not included in the list of the miRNAs with A→G changes determined by the unbiased cloning strategy, indicating a limitation of the unbiased cloning strategy for detection of certain miRNA editing sites. Nevertheless, this deep sequencing study for unbiased expression profiling identified a few new miRNAs that had A→G nucleotide changes (potential A→I editing) at a frequency higher than 5%, *i.e.*, hs-19b-2, hs-miR-512-1 and hs-miR-512-2, mu-miR-17, mu-miR-450b, and mu-miR-483. We tested some of these miRNAs containing the A→G sequence changes independently to see whether they are A→I edited miRNAs. Through analysis of the pri-miRNA sequence corresponding to the mature miRNAs with A→G changes, we found that at least the reported sequence heterogeneity of hs-miR-19b-2 and mu-miR-17 is not due to A→I editing, indicating that they are most likely sequencing errors. Together, expression of edited mature miRNAs appears to be relatively rare, although a large fraction of human pri-miRNAs undergoes editing at the sequence corresponding to mature miRNAs. Thus, processing of a majority of edited pri-miRNAs must be suppressed as we previously reported for miR-142 and miR-151.

Processing of edited pri-miRNAs

We then investigated the effects of miRNA editing on processing of pri- to pre- or pre- to mature miRNAs by subjecting unedited and edited pri-miRNAs to an *in vitro* processing assay using purified Drosha-DGCR8 or Dicer-TRBP complexes as described previously. We randomly selected six pri-miRNAs, *i.e.* pri-let-7g, pri-miR-33, pri-miR-133a2, pri-miR-197, pri-miR-203, and pri-miR-379, among 43 pri-miRNAs that have at least one UAG triplet editing site (Figure 2). None of the edited mature miRNAs expected to be processed from pri-let-7g and pri-miR-33 were detected, although editing sites were located within the mature miRNA sequences of these pri-miRNAs. Although the edited miR-379 RNAs were detected (Figure 1B), the frequency of detection of edited mature miR-379 (15%) versus unedited miR-379 was much lower than that expected from the editing frequency of pri-miR-379 (60%), indicating that processing of edited pri-miR-379 RNAs may not be as efficient as that of unedited pri-miR-379 RNAs. We also noted this proportionally less frequent detection of edited mature miR-411 RNAs (Figure 1C). Furthermore, neither unedited nor edited miR-203 was detected in human brain (Table 5), perhaps due to their low expression levels. We tested a set of unedited and "pre-edited" pri-miRNAs for *in vitro* processing assays. In the "pre-edited" pri-miRNA used for the assay, the adenosine residue of the editing site was replaced with guanosine. We previously demonstrated that the A→G alteration of pri-miRNA is treated the same as A→I editing by Drosha-DGCR8 complexes and also by the Dicer-TRBP complexes.

Both +11 and +14 sites of pri-let-7g were edited at 10 and 30% *in vivo* in human brain. The effects of editing at the major +14 site were tested for *in vitro* processing (Figure 2A). Although the editing at the +14 site did not affect the Drosha cleavage step, it suppressed the Dicer cleavage step, leading to 2-fold reduction in the mature miRNA levels. By contrast, editing of pri-miR-33 at the +15 site, pri-miR-133a2 at the +20 site, and pri-miR-379 at the +10 site inhibited the Drosha cleavage step and reduced the generation of their precursor forms (pre-miRNAs) down to a 50% level as compared to their unedited controls (Figure 2B, C, F). This is likely the reason for less frequent detection of edited mature miR-379 RNAs (Figure 1B). Editing of these three pri-miRNAs had no effect on the Dicer cleavage step (Figure 2B, C, F). Interestingly, editing of pri-miR-203 at the +85 site enhanced its processing by the Drosha-

DGCR8 complex, leading to 2-fold increase in generation of pre-miR-203 RNAs (Figure 2E). This is the first example of pri-miRNA editing that results in promotion of processing. Similarly,

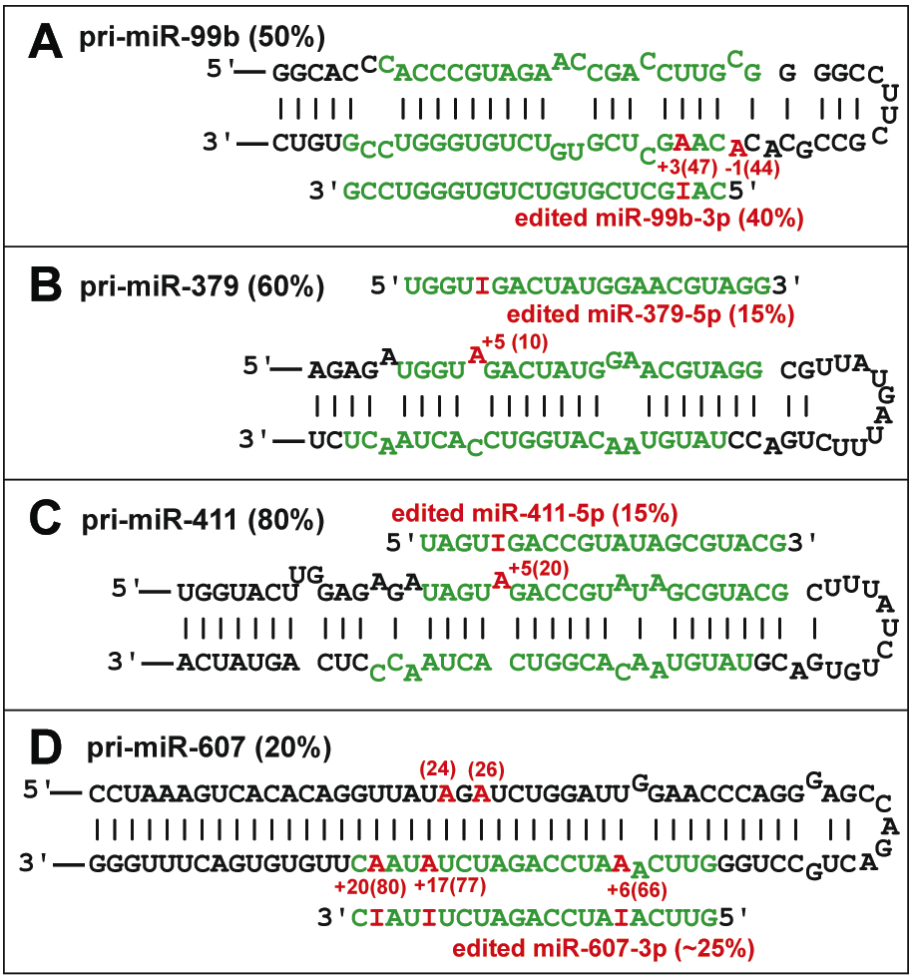

Figure 1. Identification of edited mature miRNAs. Four pri-miRNA structures and edited mature miRNAs are presented (A, B, C, D). Editing efficiency of pri-miRNA and mature miRNAs is indicated in parentheses in black and red, respectively. The 5' end of the mature miRNA is counted as +1, and the editing site is indicated by the number highlighted in red. The position of the pri-miRNA editing site is also indicated in parenthesis. The 5' end of the pri-miRNA structure registered at the Sanger Center miRBase site is counted as 1.

a slight increase in processing of pri-miR-197 edited at the +14 site by the Drosha-DGCR8 complex was detected (Figure 2D). Editing of both pri-miR-203 and pri-miR-197 did not affect the Dicer cleavage step (Figure 2D, E). The editing site of pri-miR-203 is located within the mature miRNA sequence (Figure 2E). Thus, although not detected in human brain investigated in this study, it is possible that edited miR-203 RNAs may be detected in the tissues where they are highly expressed. Interestingly, it has been recently reported that blockage of pri-let-7g processing by Lin28 RNA binding protein plays an important role in the miRNA-mediated

differentiation in stem cells. Furthermore, a critical role played by miR-203 and miR-133a2 in differentiation and proliferation of skin progenitor cells and cardiomyocytes, respectively, has been reported. It remains to be investigated whether A→I editing of let-7g, miR-133a2, and miR-203 affect the cellular process proposed to be regulated by these miRNAs. Although only a limited number of pri-miRNA editing sites were tested, all six examples investigated were found to affect the processing of miRNAs, indicating that the major function of pri-miRNA editing is modulation of miRNA biogenesis and expression levels rather than generation of edited mature miRNAs and redirection of target gene selection. Finally, editing of pri-mRNAs may affect the processing steps not tested in this study such as nuclear export as well as degradation of edited pri-miRNAs by the ribonuclease specific to inosine-containing RNAs.

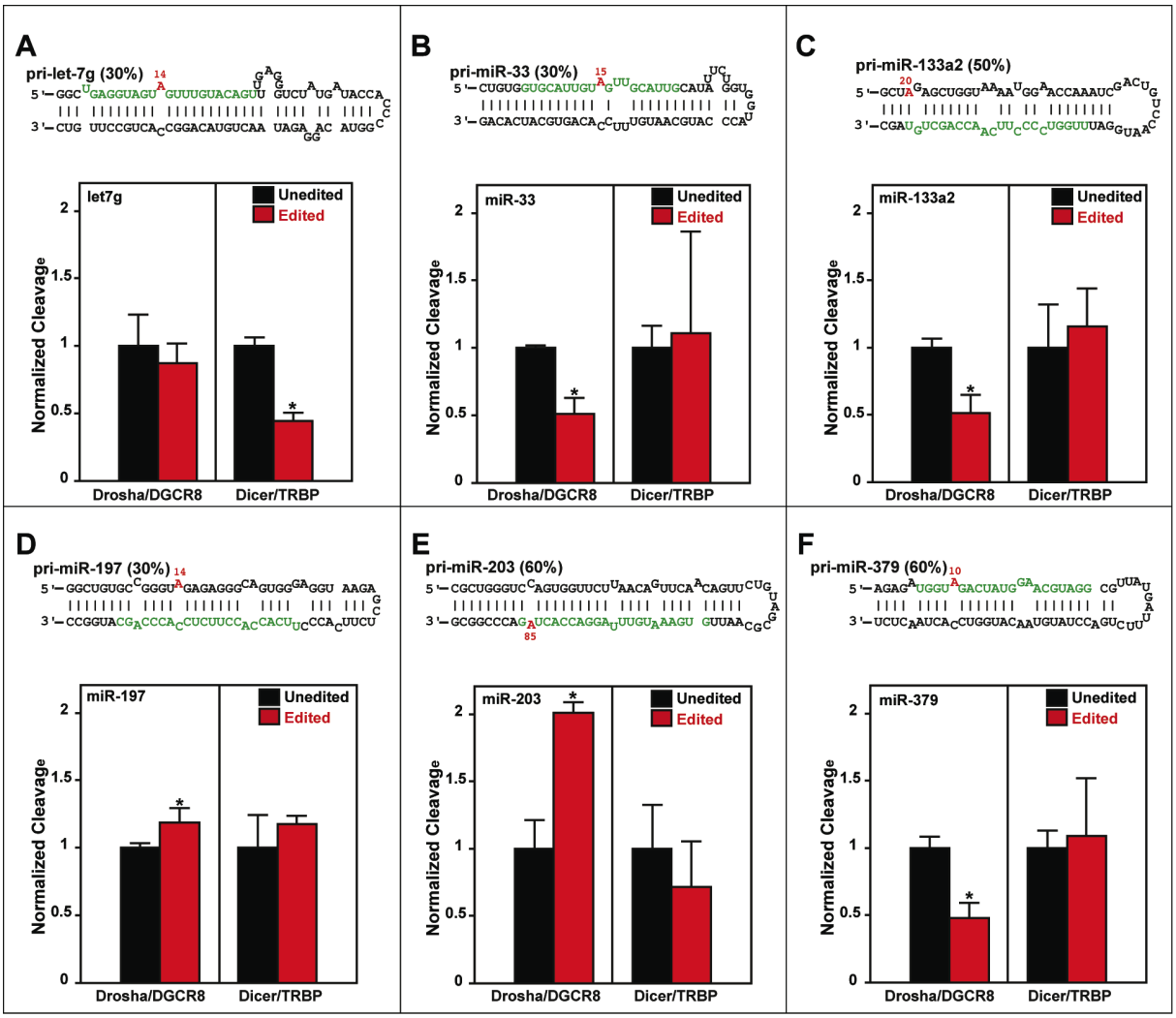

Figure 2. *In vitro* processing of edited pri-miRNAs. *In vitro* cleavage of pri-miRNAs by Drosha/DGCR8 complexes and pre-miRNAs by Dicer/TRBP were determined separately (A, B, C, D, E, F). Editing efficiency of pri-miRNA is indicated in parentheses. The editing site is indicated by the number highlighted in red. The 5' end of the pri-miRNA structure registered at the Sanger Center miRBase site is counted as 1. Three independent assays were done (n=3). Mann-Whitney *U*-test, *p<0.05.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__ No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X___ No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian

_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X___ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication, listed in the table, in a PDF version 5.0.5 format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI

Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer
If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
Functions and Regulation of RNA Editing by ADAR Deaminases.	Kazuko Nishikura	Annu. Rev. Biochem.	January 2010	☐Submitted ☐Accepted ☒ Published
				☐Submitted ☐Accepted ☐ Published
				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X_____ No_____

If yes, please describe your plans: I plan to submit a manuscript to *Nucleic Acids Res.* sometime late this year or early next year.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention,

diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____X____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

NAME **NISHIKURA, Kazuko** eRA Commons Username: **KNISHIKURA**	POSITION TITLE **Professor**

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Kanazawa University, Japan	B.Sc.	1972	Biochemistry
Kanazawa University, Japan	M.Sc.	1974	Biochemistry
Osaka University, Japan	Ph.D.	1979	Medical Science
MRC Lab. Mol. Biol., Cambridge, England	Postdoc	1979-1980	Molecular Biology
Stanford Univ. Sch. Medicine, Stanford, CA	Postdoc	1980-1982	Molecular Biology

A. Personal Statement:
Over the past two decades, we pioneered in the field of A→I RNA editing. ADAR activities were originally identified as a mysterious new dsRNA-unwinding activity in *Xenopus* eggs and embryos. Soon after, however, we discovered that ADAR is in fact a dsRNA-specific adenosine deaminase. This opened the previously unrecognized A→I RNA editing field. Our group isolated for the first time human ADAR1 cDNA clones, following the biochemical purification and microsequencing of bovine ADAR1 protein, which led to cloning of human and rodent ADAR2 and ADAR3 genes and eventually to identification of other vertebrate and invertebrate ADAR family genes. We conducted extensive biochemical studies on the molecular mechanism of ADAR action, and demonstrated the requirement of homodimerization of ADAR1 and ADAR2 for their enzymatic activities. We created and characterized *ADAR1 null* mutant mice, and demonstrated that inactivation of ADAR1 results in massive apoptosis and death of *ADAR1 null* embryos. More recently we have been investigating A→I RNA editing of miRNAs. We have shown that primary transcripts of miRNA are subject to A→I RNA editing and demonstrated that miRNA editing results in inhibition of miRNA processing at Drosha or Dicer cleavage steps or leads to expression of edited mature miRNAs that silence genes different from those targeted by unedited miRNAs. Our past and current work have contributed greatly to understanding ADAR genes and proteins and the A→I RNA editing mechanism. I am regarded as one of the leading investigators in the RNA editing field as demonstrated, for instance, by my recent election to the Chair (2011) position of the Gordon Research Conference on RNA editing.

B. Positions and Honors:

1976-1977	Graduate student in the laboratory of Dr. M.F. Perutz at the MRC Laboratory of Molecular Biology, Cambridge, England. Identification of residues contributing to the Bohr effect of human hemoglobin.
1977-1979	Lecturer, Department of Biochemistry, Tsukuba University School of Medicine, Ibaraki, Japan. Identification of residues contributing to the Bohr effect of human hemoglobin.
1979-1980	Postdoctoral Fellow in the laboratory of Dr. E.M. De Robertis. Studies of eukaryotic gene expression using *Xenopus* oocyte injection method. MRC Laboratory of Molecular Biology, Cambridge, England.
1980-1982	Postdoctoral Fellow in the laboratory of Prof. R.D. Kornberg. *In vitro* transcription and RNA processing of Ig heavy chain genes. Department of Structural Biology, Stanford University School of Medicine, Stanford, CA. Recipient of Anna Fuller Fund Postdoctoral Fellowship.
1983-1989	Assistant Professor, The Wistar Institute, Philadelphia, PA
1989-1994	Associate Professor, The Wistar Institute, Philadelphia, PA
1987-present	Adjunct Faculty, University of Pennsylvania, Department of Human Genetics, Philadelphia, PA
1995-present	Professor, The Wistar Institute, Philadelphia, PA

Honors:

1985-1988	American Cancer Society, Junior Faculty Award
2009-2013	Ellison Medical Foundation Senior Investigator Award

Study Sections:

1993-1997	Regular reviewer for NIH Cellular Biology and Physiology Study Section II
2004	Ad hoc reviewer for NIMH conferences and scientific Meeting Applications ZMH1 CNF-A Study Section

Scientific Advisory Committee:

2007-present	Specialized Neuroscience Research Program (SNRP), University of Puerto Rico, San Juan, PR

Invited Speaker:

2008	"RNA and the Etiology of Disease", Rome, Italy
2009	Gordon Research Conferences on "RNA Editing," Vice-chair, Galveston, TX
2009	The IIIe Cycle Meeting on "The Central Dogma and Beyond," Villars-sur-Ollon, Switzerland
2009	13th International Symposium on "Purine and Pyrimidine Metabolism", Stockholm, Sweden
2009	The Oligonucleotide Therapeutics Society Symposium on "Multileveled Oligonucleotide Approaches in Neuroscience Research", Fukuoka, Japan
2010	BMB2010 Joint Meeting of Biochemistry and Molecular Biology. Plenary Lecturer, Kobe, Japan
2011	Gordon Research Conferences on "RNA Editing," Chair, Galveston, TX

C. Selected Peer-reviewed Publications (selected from 77):

1. Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., **Nishikura, K.**, and Shiekhattar, R. 2005. TRBP links the RNase III Dicer to Argonaute 2, the catalytic engine of RNA-induced silencing complex. Nature. 436: 740-744.
2. Yang, W., Chendrimada, T.P., Wang, Q., Higuchi, M., Seeburg P.H., Shiekhattar, R., **Nishikura K.** 2006. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat. Struct. Mol. Biol. 13: 13-21.
3. **Nishikura, K.** 2006. Editor meets silencer: crosstalk between RNA editing and RNA intereference. Nat. Rev. Mol. Cell. Biol. 7: 919-931.
4. Valente, L. and **Nishikura, K.** 2007 RNA-binding yndependent dimerization of ADAR and dominant negative effects of nonfunctional subunits on dimer functions, J. Biol. Chem. 282: 16054-16061.
5. Kawahara, Y., Zinshteyn, B., Sethupathy, P., Iizasa, H., Hatzigeorgiou, A.G., and **Nishikura, K.** 2007. Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. Science 315: 1137-1140.
6. Kawahara, Y., Zinshteyn, B., Chendrimada T.P., Shiekhattar, R., and **Nishikura K.** 2007. RNA editing of microRNA-151 blocks cleavage by the Dicer-TRBP complex. EMBO Reports. 8: 763-769. PMCID: PMC1978079
7. Kawahara, Y., Megraw, M., Kreider, E., Iizasa, H., Valente, L., Hatzigeorgiou, A.G., and **Nishikura, K.** 2008. Frequency and fate of microRNA editing in human brain. Nucleic Acids Res. 36: 5270-5280. PMCID:

PMC2532740
8. Kawahara, Y., Grimberg, A., Teegarden, S., Mombereau, C., Sui, L., Bale, B.L., Blendy, J.A., **Nishikura, K.** 2008. Dysregulated editing of serotonin 2C receptor mRNAs results in energy dissipation and loss of fat mass. J. Neurosci. 28: 12834-12844. PMCID: PMC2615198
9. Iizasa, H. and **Nishikura, K.** 2009. A new function for the RNA-editing enzyme ADAR1. Nat. Immunol. 10: 16-18.
10. **Nishikura, K**. 2010. Functions and regulation of RNA editing by ADAR deaminases. Annu. Rev. Biochem. 79: 321-349.
11. Mombereau, C., Yukio Kawahara, Y., Gundersen, B.B., **Nishikura, K.** and Blendy, J.A. 2010. Functional relevance of serotonin 2C receptor mRNA editing in antidepressant- and anxiety-like behaviors. Neuropharmacol., in press.