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):** 01/01/2011 – 6/30/2012

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:** 4100054879

6. **Project Number and Title of Research Project:** 3 - Regulation of EBV Infection and Latency by Editing of Viral MicroRNAs

7. **Start and End Date of Research Project:** 01/01/2011 – 6/30/2012

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:

   $ $153,349.35

   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
Nishikura	PI	35%	$90,409.20

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
Ariyoshi	Postdoctoral fellow	50% (Yr 1-2)

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 breast cancer metastasis by EBV microRNA	X NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	June 2012	$2,863,965	$ Pending

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 research grant to Department of Defense in August 2012.

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

We will continue to investigate the significance of EBV microRNA BART6 editing. More recently we obtained a line of evidence indicating that the viral miRNA may control Epitherial-to-Mesenchymal transition and contribute metastasis of EBV-positive tumors. We will investigate the significance of our new findings.

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	2			2
Female				
Unknown				
Total	**2**			**2**

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

	Undergraduate	Masters	Pre-doc	Post-doc
White	1			
Black				
Asian	1			2
Other				
Unknown				
Total	**2**			**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 posdocs 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 period that the project was funded (i.e., from project start date through end date). 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.

We completed both specific aims originally proposed.

Specific Aim 1; *determine the significance of pri-miR-BART6 RNA editing in viral latency and lytic replication.* Primary transcripts of ebv-miR-BART6 (pri-miR-BART6) were edited at the +20 site in latently EBV-infected cells. Editing of wild-type pri-miR-BART6 RNAs dramatically reduced loading of miR-BART6-5p RNAs onto the miRNA-induced silencing complex. We found that editing of pri-miR-BART6 inhibit expression and function of miR-BART6, which consequently affected viral replication and also transition of the viral latency.

Specific Aim 2; determination *the in vivo function of miR-BART6 RNAs in viral latency and lytic replication.* Our previous studies indicated that miR-BART6-5p RNAs silence Dicer through multiple target sites located in the 3'UTR of Dicer mRNA. We investigated the Dicer silencing function of miR-BART6 in the regulation of viral replication and and latency. We found that miR-BART6-5p RNAs suppress the EBNA2 viral oncogene required for transition from immunologically less responsive type I and type II latency to the more immunoreactive type III latency as well as Zta and Rta viral proteins essential for lytic replication, revealing the regulatory function of miR-BART6 in EBV infection and latency.

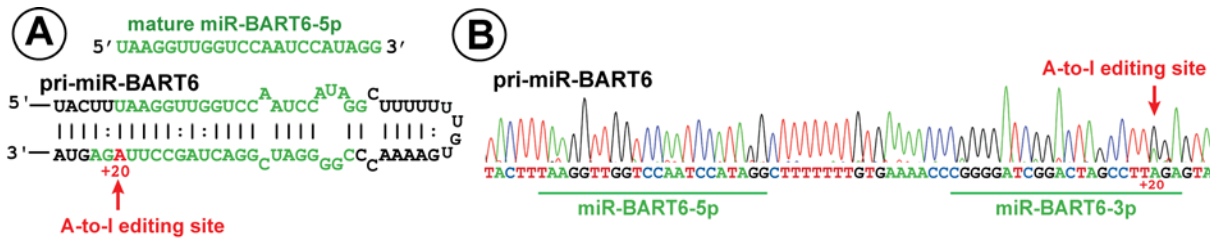

Fig. 1. A-to-I RNA editing of pri-miR-BART6 RNAs. (**A**) The hairpin structure of pri-miR-BART6 is shown. The edited adenosine (+20 site, highlighted in red) is indicated by a number with the 5' end of the mature miR-BART6-3p sequence counted as +1. The regions to be processed into mature miRNAs (5p sense and 3p antisense strands) are highlighted in green. Mature miR-BART6-5p is also shown. (**B**) The DNA sequencing chromatogram of RT-PCR products is shown. The RNA-editing site (+20) is detected as an A-to-G change in the cDNA sequencing chromatogram (red arrow).

Results:

Identification of an A-to-I editing site in EBV pri-miR-BART6 and targeting of Dicer by miR-BART6. Our previous studies revealed that the +20 site of pri-miR-BART6 was highly edited by ADAR1 in Daudi and GM607 cells (Fig. 1). Furthermore, *in silico* prediction identified human Dicer as a high-score target for miR-BART6-5p. All four target sites identified were unique to the human Dicer 3'UTR and not evolutionarily conserved, even for the chimpanzee Dicer 3'UTR. It seems that miR-BART6-5p evolved to target Dicer specifically in human during the establishment of the EBV-host relationship. Validation experiments were conducted, which confirmed that miR-BART6 indeed targets Dicer *in vivo*.

Suppression of miRISC loading of miR-BART6-5p RNAs by editing.
We thought that editing might affect the selection and loading of the "effective" strand onto the miRISC complex (1). We therefore examined the assembly of functional miRISC from recombinant FLAG-tagged Ago-2 complexed with Dicer and TRBP and either unedited or "edited" wild-type pre-miR-BART6
RNAs (Fig. 2A). We found that formation of the functional miRISC and consequent silencing (cleavage) of the 5p target RNA was indeed much more efficient with unedited pre-miR-BART6 than with edited pre-miR-BART6 (Fig. 2B, 2C). Together, our results demonstrated that editing of pri-miR-BART6 inhibits the overall silencing effects of miR-BART6 RNAs.

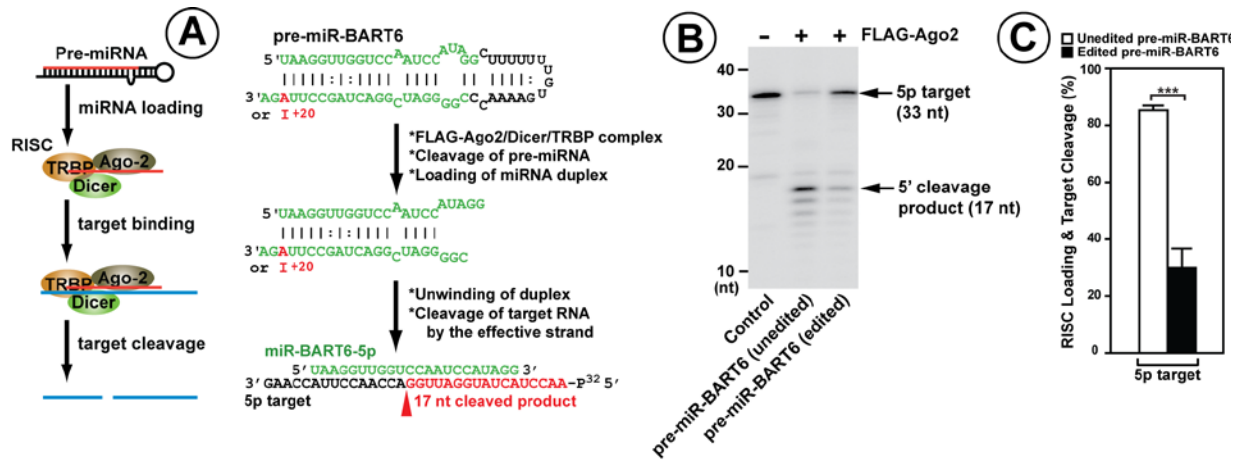

Fig. 2. RISC loading of miR-BART6-5p is inhibited *via* editing of the +20 site by ADAR1. (**A**) A RISC loading assay of pre-miR-BART6-5p. Cleavage of the cognate target for miR-BART6-5p RNAs is schematically shown. The target RNA was 5'-^{32}P-labeled. (**B**) Cleavage of the cognate target product (17 nt) guided by miR-BART6-5p was substantially more efficient with unedited pre-miR-BART6 RNAs than with "edited" pre-miR-BART6 RNAs containing an A-to-G substitution at the +20 site. (**C**) Quantitative summary of RISC loading experiments is presented. The cleavage efficiency was estimated by the ratio of the radioactivity of the correctly cleaved band over that of the uncleaved control band.

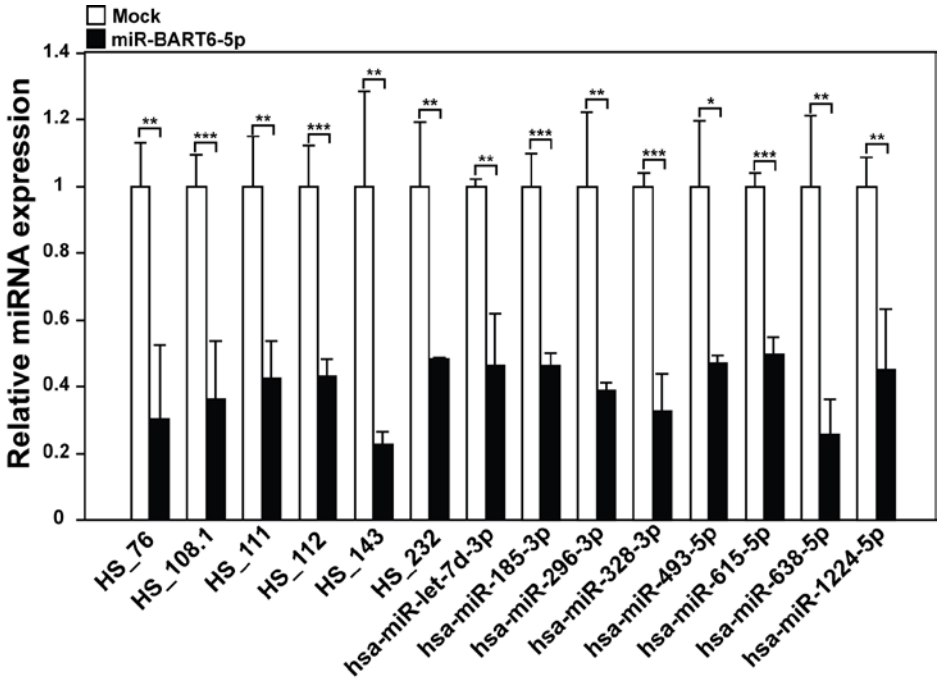

Fig. 3. Expression of miR-BART6-5p affects the expression of many miRNAs through repression of Dicer. Decreased expression of select miRNAs. The miRNA array analysis revealed fourteen miRNAs for which expression levels were decreased more than 2-fold in HeLa cells transfected with miR-BART6-5p RNAs. Certain miRNAs (e.g., HS-76) published previously have been not yet registered in the miRBase miRNA database site. The three miRNAs for which expression levels increased by more than 2-fold. Three independent assays were done. Mann-Whitney *U*-test, *p<0.05, **p<0.005, ***p<0.001. Error bars, SEM (n=3).

Suppression of many miRNAs by miR-BART6-5p—The dramatic reduction in the Dicer expression mediated by miR-BART6-5p suggests that it may affect the biogenesis of miRNAs globally. We therefore examined the effects of Dicer suppression on expression of other miRNAs by miRNA array analysis. The miRNA levels were examined in HeLa cells with substantially reduced Dicer levels following transfection with miR-BART6-5p RNAs. This study revealed that levels of at least 69 miRNAs were significantly reduced and 14 of these miRNAs showing more than 2-fold suppression (Fig. 3). Synthesis of these miRNAs may be particularly sensitive to the Dicer concentration. Our results demonstrated that suppression of Dicer mediated by miR-BART6-5p RNAs affects the expression of a large number of miRNAs.

Modulation of the EBV latency state by miR-BART6-5p RNAs.
We then asked whether Dicer silencing by miR-BART6-5p RNAs could control the EBV infection state. To examine this possibility, we first examined the relative expression levels of miR-BART6-5p strand RNAs and Dicer among GM607, Daudi, and C666-1 cells by qRT-PCR. Much higher levels of miR-BART6-5p were detected in C666-1 cells which have much less editing than GM607 and Daudi cells

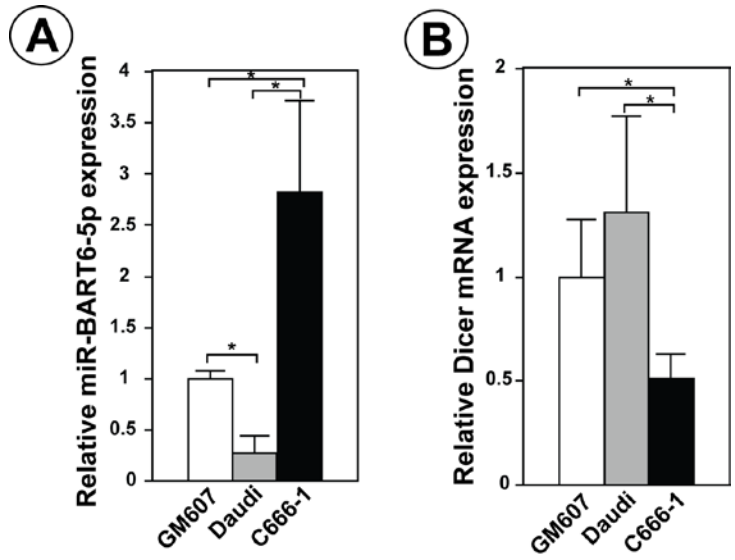

Fig. 4. Relative expression levels of miR-BART6-5p and Dicer in different cell lines.
(**A**) miR-BART6-5p RNA levels were examined by qRT-PCR and normalized to β-actin mRNA level. Three independent assays were done. Mann-Whitney *U*-test, *p<0.05. Error bars, SEM (n=3). (**B**) Dicer mRNA levels were monitored by qRT-PCR and normalized to β-actin mRNA levels. Three independent assays were performed. Mann-Whitney *U*-test, *p<0.05. Error bars, SEM (n=3).

(Fig. 4A). As expected, Dicer levels were lowest in C666-1 cells, in inverse relation to the miR-BART6-5p levels (Fig. 4B). Accordingly, we decided to explore the significance of Dicer repression by miR-BART6-5p RNAs in C666-1 cells. We first attempted to antagonize the miR-BART6-5p RNAs expressed in C666-1 cells by transfection of a miR-BART6-5p antagomir. As expected, the miR-BART6-5p antagomir substantially decreased the miR-BART6-5p level (~20-fold) with a concomitant increase in the Dicer levels (~2-fold), indicating that miR-BART6-5p RNAs constantly suppress and maintain Dicer at the reduced levels in C666-1 cells (Fig. 5A). We then examined the relative expression levels of several genes known to be important for either lytic infection or the state of latency: EBNA1, EBNA2, LMP1, Zta, and Rta (2,3). EBNA1 is detected in type I, II, and III latency, whereas EBNA2 and LMP1 are usually detected in type III latency (2,3). EBNA2 is essential for the transformation of B lymphocytes and plays a central role in type III latency by up-regulating promoters of all latent EBV genes. Deficiency of the EBNA2 expression is known in type I and II latency (2,3). A weak expression of LMP1 in type II latency and its deficiency in type I latency have been reported (2,3). By contrast, Zta and Rta are essential for the initiation of the lytic EBV infection cycle (2,3). By antagonizing miR-BART6-5p, Zta and Rta increased by 2- to 3-fold, indicating that miR-BART6-5p keeps these gene products under control. Furthermore, we noticed substantial upregulation of EBNA2 oncogene expression (~5-fold) and LMP1 (~2-fold) by suppression of miR-BART6-5p RNAs, whereas no effects on EBNA1 were observed (Fig. 7*A*).

The activities of the three viral promoters Cp, Wp, and Qp were also monitored (Fig. 6B). Transcription from Cp and Wp is characteristic of type III latency (4), whereas the Qp promoter is used in EBV-infected cells undergoing type I or II latency (5,6). We used qRT-PCR primers specific for RNAs initiating at Wp, Cp, or Qp (7). Significant upregulation of type III latency-specific Cp and Wp promoter activities (5.4- and 11-fold, respectively) were detected in C666-1

cells transfected with miR-BART6-5p antgomir. On the other hand, Qp promoter activities associated with type I and type II latency were completely abolished, not detectable in comparison to control.

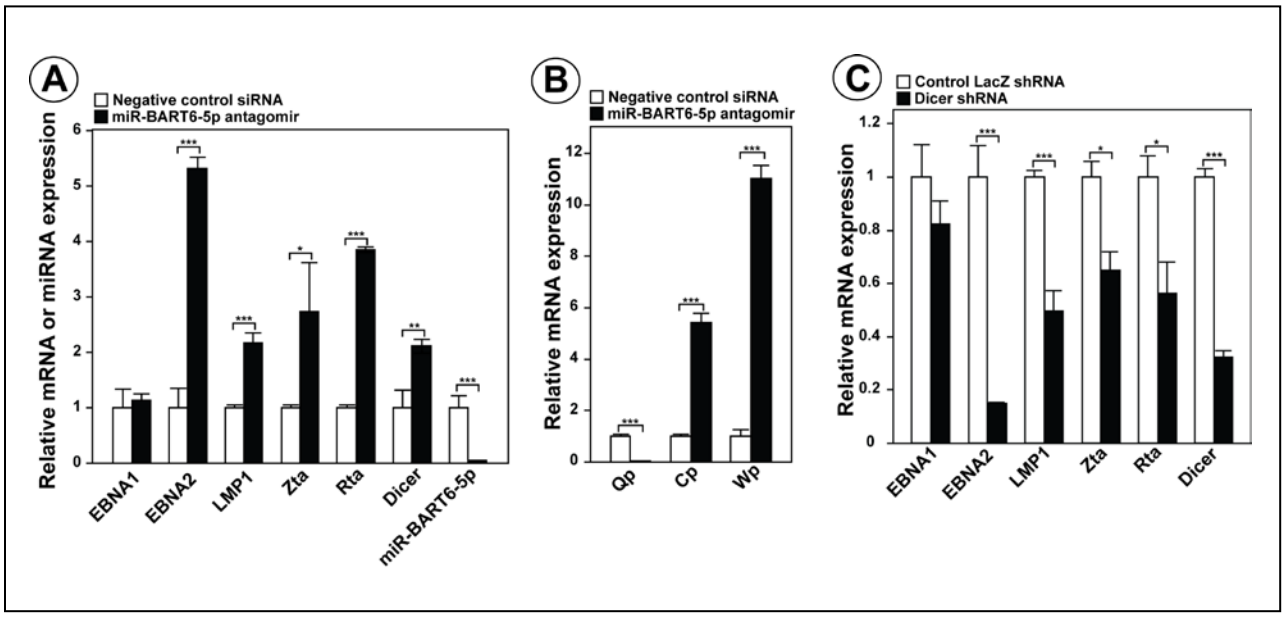

Fig. 5. Control of viral genes critical for the state of latency and lytic viral replication. (**A**) Upregulation of EBV genes critical for latency and viral replication by the miR-BART6-5p antagomir. Expression of select viral genes including miR-BART6-5p in C666-1 cells transfected with the miR-BART6-5p antagomir or control (sequence unrelated Qiagen AllStars Negative Control siRNA) was examined by qRT-PCR. Three independent assays were done. Mann-Whitney *U*-test, *p<0.01, **p<0.005, ***p<0.001. Error bars, SEM (n=3). (**B**) Changes induced by the miR-BART6-5p antagomir in the viral promoters Qp, specific for the type I latency, and Cp and Wp, specific for the type III latency. Transcripts initiated from Qp, Cp, and Wp were determined by qRT-PCR and compared to β-actin transcripts. Three independent assays were done. Mann-Whitney *U*-test, **p<0.005, ***p<0.001. Error bars, SEM (n=3). **(C)** Repression of EBV genes following Dicer knock-down by shRNA. Expression of viral genes in C666-1 cells transfected with the Dicer targeting shRNA expression plasmid or control vector containing shRNA against LacZ was monitored by qRT-PCR. Three independent assays were conducted. Mann-Whitney *U*-test, *p<0.01, ***p<0.001. Error bars, SEM (n=3).

We then attempted to silence Dicer directly by transfecting a Dicer targeting short hairpin RNA (shRNA) expression vector in C666-1 cells (Fig. 6C). This reduced Dicer levels by ~70%, indicating the efficiency of this Dicer targeting siRNA expression vector. As expected, changes in the marker genes were completely opposite to those noted in C666-1 cells transfected with the miR-BART6-5p antagomir; ~7-fold reduction in the EBNA2 expression as well as substantial downregulation for LMP1, Zta, and Rta (Fig. 6C), further confirming that control of these critical viral genes by miR-BART6-5p is mediated

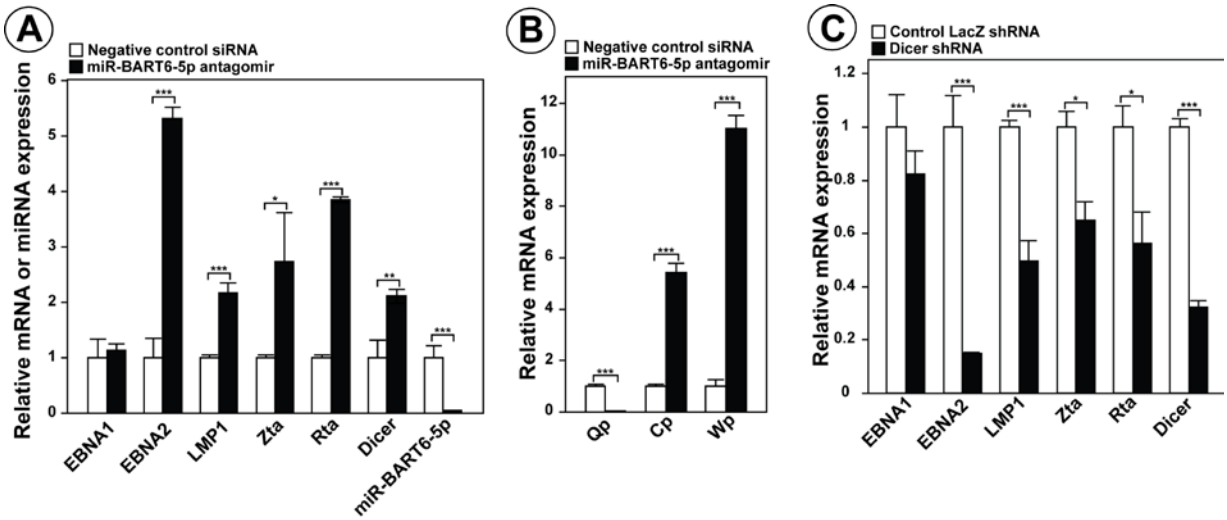

Fig. 6. Control of viral genes critical for the state of latency and lytic viral replication. (**A**) Upregulation of EBV genes critical for latency and viral replication by the miR-BART6-5p antagomir. Expression of select viral genes including miR-BART6-5p in C666-1 cells transfected with the miR-BART6-5p antagomir or control (sequence unrelated Qiagen AllStars Negative Control siRNA) was examined by qRT-PCR. Three independent assays were done. Mann-Whitney *U*-test, *p<0.01, **p<0.005, ***p<0.001. Error bars, SEM (n=3). (**B**) Changes induced by the miR-BART6-5p antagomir in the viral promoters Qp, specific for the type I latency, and Cp and Wp, specific for the type III latency. Transcripts initiated from Qp, Cp, and Wp were determined by qRT-PCR and compared to β-actin transcripts. Three independent assays were done. Mann-Whitney *U*-test, **p<0.005, ***p<0.001. Error bars, SEM (n=3). **(C)** Repression of EBV genes following Dicer knock-down by shRNA. Expression of viral genes in C666-1 cells transfected with the Dicer targeting shRNA expression plasmid or control vector containing shRNA against LacZ was monitored by qRT-PCR. Three independent assays were conducted. Mann-Whitney *U*-test, *p<0.01, ***p<0.001. Error bars, SEM (n=3).

directly via its silencing effects on Dicer.

Finally, we examined genetically identical pairs of Burkitt's lymphoma Mutu I and Mutu III cell lines which are in type I and type III latency, respectively, in order to access the function of miR-BART6-5p and Dicer silencing in B lymphoma cells (non-NPC cell lines). We found that the miR-BART6-5p level is higher in Mutu I than in Mutu III, whereas the Dicer level was lower in Mutu I than in Mutu III as expected (data not shown). We next transfected Mutu I cells with miR-BART6-5p antagomir. As we observed in C666-1 cells, the antagomir effectively reduced miR-BART6-5p levels (~10-fold) and increased Dicer levels (1.9-fold). Furthermore, significant upregulation of EBNA2, LMP1, Zta, and Rta was detected (Fig. 7A). Downregulation of Qp activities and upregulation of Cp and Wp activities were observed (Fig. 7B). We then transfected Mutu III cells with the Dicer shRNA expression plasmid, which successfully repressed Dicer levels (~5-fold). Opposite effects of the miR-BART6-5p antagomir were detected; suppression of EBNA2, LMP1, Zta, and Rta (Fig. 7C). Upregulation of Qp activities and downregulation of Cp and Wp activities were also observed (Fig. 7D).

Together, these results suggest that Dicer suppression mediated via miR-BART6-5p RNAs maintains not only the type II latency of C666-1 cells, but also the type I latency of Mutu I cells by suppressing lytic replication and also inhibiting transition of these cell lines to type III latency, a more immunoresponse-prone state of the viral infection cycle.

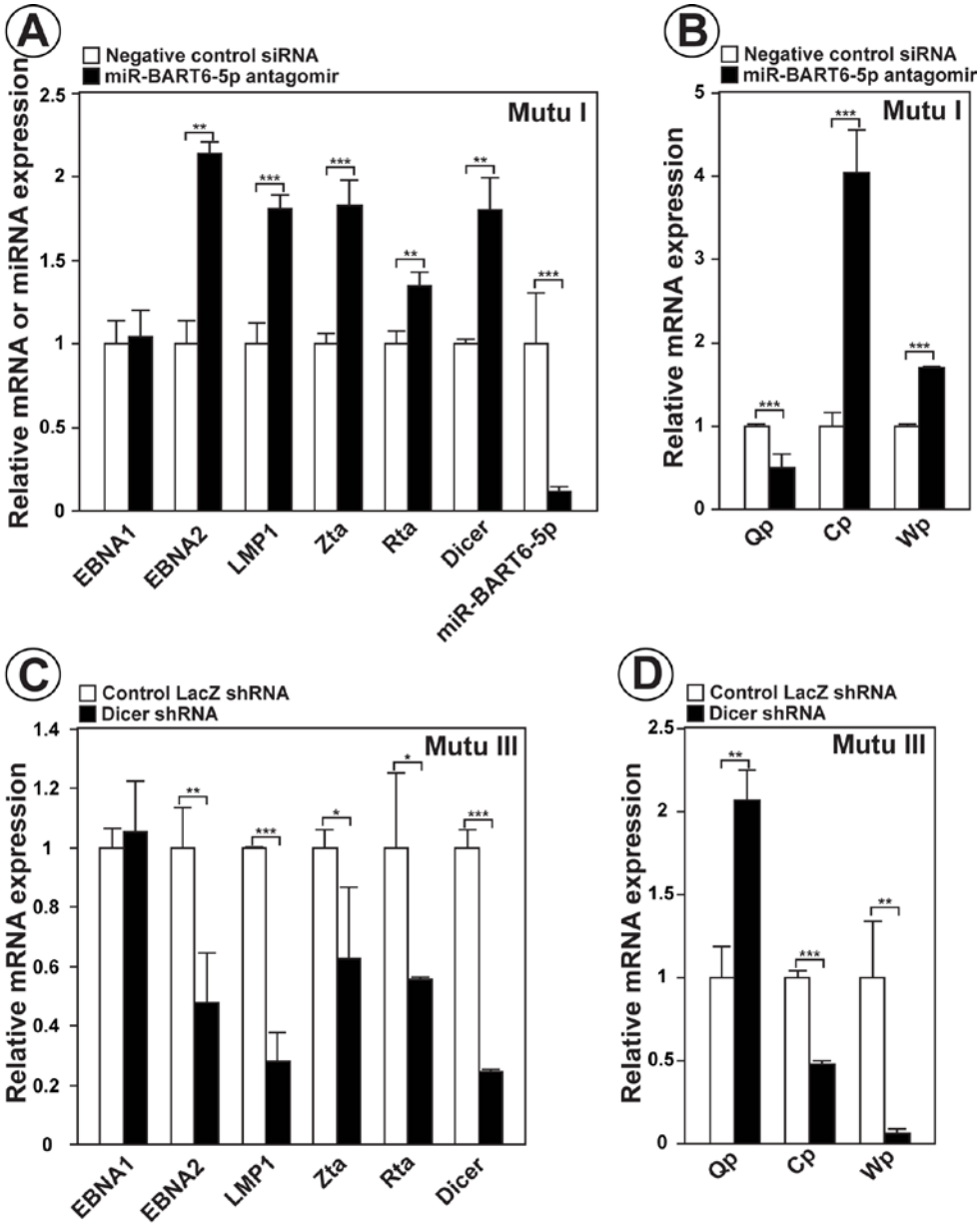

Fig. 7. The effects of miR-BART6-5p and Dicer silencing in B lymphoma cells. (**A**) Upregulation of EBV genes critical for latency III and lytic replication in Mutu I cells transfected with the miR-BART6-5p antagomir or control siRNA. Dicer and miR-BART6-5p levels were also monitored. Three independent qRT-PCR assays were performed. Mann-Whitney *U*-test, **p<0.005, ***p<0.001. Error bars, SEM (n=3). (**B**) Changes in viral promoter activities were induced in Mutu I cells by the miR-BART6-5p antagomir. Three independent qRT-PCR assays were done. Mann-Whitney *U*-test, ***p<0.001. Error bars, SEM (n=3). (**C**) Down regulation of EBV genes critical for latency III and lytic replication was detected in Mutu III cells transfected with the Dicer targeting shRNA expression plasmid or control vector containing shRNA against LacZ. Three independent qRT-PCR assays were done. Mann-Whitney *U*-test, *p<0.05, **<0.005, ***p<0.001. Error bars, SEM (n=3). (**D**) Changes in viral promoter activities were induced in Mutu III cells by Dicer knock-down. Three independent qRT-PCR assays were performed. Mann-Whitney *U*-test, **p<0.005, ***p<0.001. Error bars, SEM (n=3).

Conclusions:

Significance of Dicer repression by miR-BART6 for the viral life cycle

Most significantly, we provided evidence that miR-BART6-5p suppresses Dicer expression through binding to four target sites present within the 3'UTR of the human Dicer mRNA. Interestingly, these four target sites are not conserved in the mouse or even chimpanzee Dicer mRNA, revealing that miR-BART6-5p RNAs target only human Dicer. In view of the EBV host specificity, i.e., EBV infections occur only in human, silencing of Dicer by miR-BART6-5p might have been established during the course of EBV evolution into a human-specific virus. It may be prudent to discount species conservation, usually used as one of the important parameters for target prediction programs, when target genes of a miRNA from a virus with narrow host range are screened.

Our miRNA array analysis revealed that Dicer suppression by miR-BART6-5p RNAs leads to suppression of many miRNAs. It has been reported that EBV infection of primary B cells results in a dramatic downregulation of host cell miRNA expression, implying the presence of a suppressor of miRNA expression encoded by the virus (8). It was proposed that EBV may manipulate the expression of miRNAs as a major regulatory step in the viral life cycle, while host cells may potentially use miRNAs in response to EBV (8,9). It appears that miR-BART6-5p likely is this viral miRNA suppressor and plays a critical role in the EBV virus life cycle by silencing Dicer and regulating the expression of miRNAs.

Suppression of Dicer by miR-103/107 and the consequent global reduction of miRNA synthesis have been reported (10-13). It appears that EBV has acquired miR-BART6 to mimic the powerful strategy of miR-103/107 to downregulate host cell miRNA production, which may be necessary to respond to host immune response and help EBV to stay in a specific state of latency and not initiate lytic viral replication. Suppression of miR-BART6-5p by antagomir indeed resulted in activation of EBNA2, LMP1, Zta, and Rta genes, critical for transition to type III latency or lytic replication. In addition, the type III latency-specific Cp and Wp promoter activities were dramatically activated by miR-BART6-5p antagomir, whereas the type I and type II latency-specific Qp promoter activities were suppressed by the antagomir. Reduction of Dicer and consequent suppression of specific miRNAs that control these factors may be one possible mechanism to affect different viral promoter activities.

In conclusion, our results suggest the important roles played by EBV miR-BART6 RNAs in the regulation of viral replication and latency. Naturally occurring pri-miR-BART6 mutation and editing may be an adaptive selection to counteract the miR-BART6 function.

References:

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10. Forman, J. J., Legesse-Miller, A., and Coller, H. A. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105**(39), 14879-14884
11. Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008) *Nature* **455**(7209), 58-63
12. Tokumaru, S., Suzuki, M., Yamada, H., Nagino, M., and Takahashi, T. (2008) *Carcinogenesis* **29**(11), 2073-2077
13. Martello, G., Rosato, A., Ferrari, F., Manfrin, A., Cordenonsi, M., Dupont, S., Enzo, E., Guzzardo, V., Rondina, M., Spruce, T., Parenti, A. R., Daidone, M. G., Bicciato, S., and Piccolo, S. (2010) *Cell* **141**(7), 1195-1207

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

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

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 or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) 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):
1. Post-transcriptional gene regulation by an Editor: ADAR and its role in RNA Editing.	Louis Valente, Yukio Kawahara, Boris Zinshteyn, Hisashi Iizasa, & Kazuko Nishikura	Post-transcriptional gene regulation: RNA processing in eukaryotes. (Ed. Jane Wu) Wiley-VCH book	June 2012	☐Submitted X 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 J. Biol. Chem. sometime this 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_____

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

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

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

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:

Dr. Nishikura pioneered in the field of A→I RNA editing mediated by ADAR. Dr. Nishikura's group discovered that ADAR is in fact a dsRNA-specific adenosine deaminase. Her group cloned the first ADAR gene family member, ADAR1, leading to cloning of ADAR2 and ADAR3 genes. Dr. Nishikura's group conducted extensive biochemical studies on the molecular mechanism of ADAR action, 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 Dr. Nishikura's group has shown that primary transcripts of miRNA are subject to A→I RNA editing and also that editing of pri-miRNA results in inhibition of miRNA expression and function.

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

1995-present Professor, The Wistar Institute, Philadelphia, PA

Honors:

2009-2013 Ellison Medical Foundation Senior Investigator Award

Editorial Board Member:

2011-2016 *J. Biol. Chem.*

Invited Speaker (2011-2013, limited to international meetings):

2011	Gordon Research Conferences on “RNA Editing,” Chair, Galveston, TX
2011	ASBMB (American Society for Biochemistry and Molecular Biology) Symposium on "RNA Biology", Washington, DC
2012	Frontiers in Biology, Stanford, CA
2013	Gordon Research Conferences on “RNA Editing,” Galveston, TX
2013	Gordon Research Seminars on “RNA Editing,” Keynote Speaker, Galveston, TX

C. Selected Peer-reviewed Publications (selected from 91):

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-independent dimerization of adenosine deaminases acting on RNA 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.
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.
8. Iizasa, H. and Nishikura, K. 2009. A new function for the RNA-editing enzyme ADAR1. Nat. Immunol. 10: 16-18.
9. Nishikura, K. 2010. Functions and regulation of RNA editing by ADAR deaminases. Annu. Rev. Biochem. 79: 321-349.
10. Wulff, B-E, Sakurai, M., and Nishikura, K. 2011. Elucidating the inosinome: global approaches to adenosine-to-inosine RNA editing. Nat. Rev. Genetic. 12: 81-85.
11. Wulff, B-E and Nishikura, K. 2012. Modulation of microRNA expression and function by ADARs. Curr. Top. Microbiol. Immunol. 353: 91-109.