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TITLE: Signaling Pathways in Pathogenesis of Diamond Blackfan Anemia

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14. ABSTRACT

Diamond Blackfan Anemia (DBA) is a disorder that results in pure red cell aplasia, congenital abnormalities, and predisposition to cancer. The current treatment of steroids and chronic transfusions leads to significant morbidity. Approximately 25% of patients with DBA have mutations in RPS19. We previously generated a zebrafish model with RPS19 insufficiency that the phenotype is similar to that observed in patients with DBA. We also first described that p53 is upregulated in these fish injected with RPS19 morpholinos. To understand the mechanism by which RPS19 insufficiency leads to defects in erythropoiesis, we identified a p53 target, microRNA34a (miR34a), as being upregulated in human CD34+ fetal liver cells transduced with RPS19shRNA lentivirus. This not only led to decreased erythroid colony formation, but also aberrant erythroid differentiation. We hypothesize that RPS19 insufficiency mediates defects in erythropoiesis through upregulation of p53 and miR34a. To more rigorously test this hypothesis and identify new downstream targets and microRNAs, we propose three specific aims. In Aim 1, we will characterize the role of miR34a in RPS19 insufficient primary human hematopoietic stem cells in vitro. In Aim 2, we will study the role of miR34a in RPS19 insufficient primary human hematopoietic stem cells in vivo. In Aim 3, RNA-seq will be performed to identify novel transcripts and microRNAs that are aberrantly regulated downstream of RPS19 insufficiency in primary human hematopoietic stem cells. These studies will provide new insights into the molecular pathways downstream of ribosomal protein insufficiency in hematopoietic stem cells and potentially new targets for therapy.

15. SUBJECT TERMS- none provided

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The goal of this proposal is to understand the signaling pathways that lead to the pathogenesis of DBA. In the first aim, the role of miR34a will be investigated. In Aim 2, we proposed to perform RNA-seq and microRNA-seq to identify novel pathways. We will knock down RPS19 in human CD34+ fetal liver and cord blood cells and study genes identified by RNA-seq that are up- or down-regulated. In this manner, we hope to identify novel pathways and approaches to treat DBA.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

RPS19, DBA, signaling, pathways, RNA-seq, microRNA-seq, CD34+ cells

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

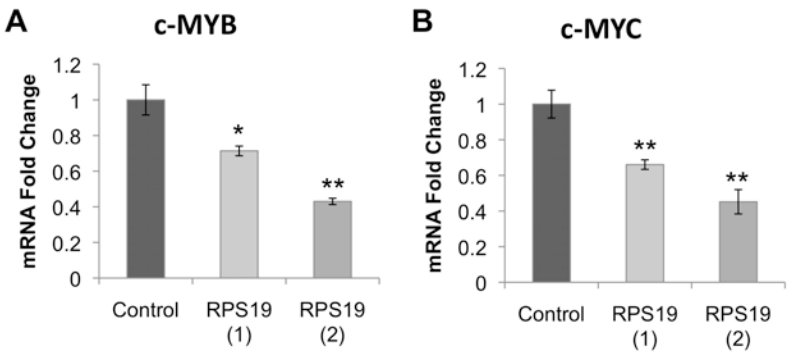
Task 1. To characterize the role of miR34a in RPS19 insufficient primary human hematopoietic stem cells *in vitro* (Months 1-12).

Major goal: To understand the mechanism of how miR34a upregulation could lead to defects in erythroid differentiation and proliferation.

Milestones:

a. We will transduce human CD34+ fetal liver hematopoietic stem cells with RPS19 shRNA lentiviral constructs and examine levels of miR34a and target genes c-Myb, c-Myc, Sirt1, and Notch1 at different stages of erythroid development (Months 1-3).

We have transduced human CD34+ fetal liver HSCs with RPS19 shRNA and showed that RPS19 deficiency leads to decreased expression of the miR34a targets c-myb and c-myc. Sirt1 and Notch1 expression remains unchanged in RPS19 deficient cells.

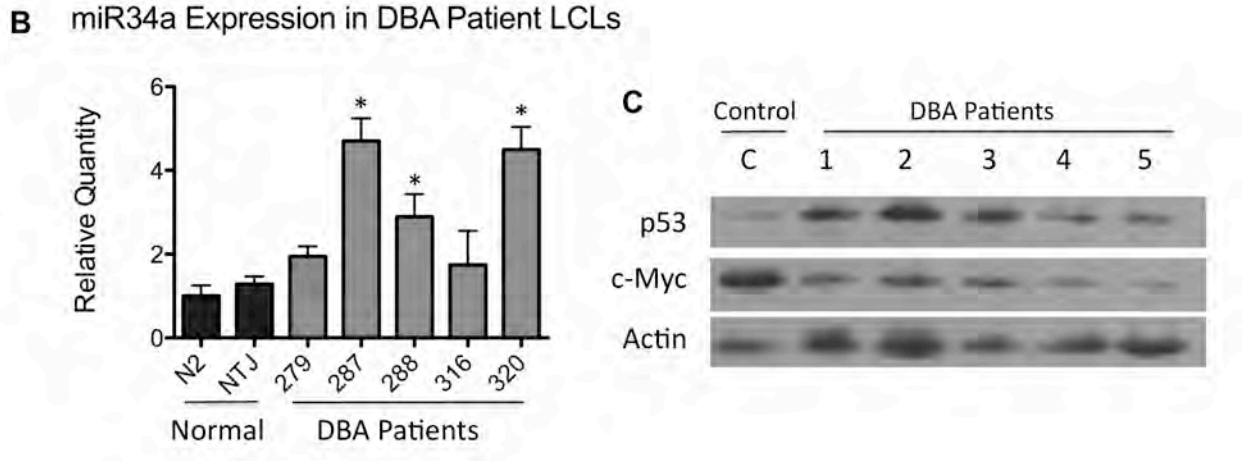


b. Study miR34a target gene expression (c-Myb, c-Myc, Sirt1, and Notch1) in lymphoblastoid cell lines (LCL) and CD34+ bone marrow progenitor cells from DBA patients with RPS19 mutations (Months 1-3).

These experiments have been performed and show there is significant variability in expression of c-Myb, c-Myc, Sirt1, and Notch1. The reason for this is most likely because of the fact that the LCL cells are immortalized by EBV and have different characteristics than primary normal HSCs.

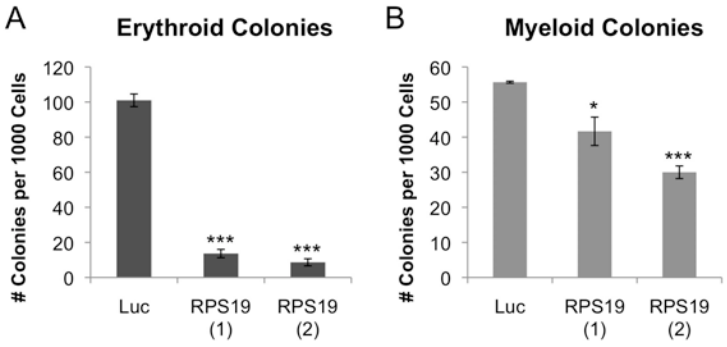
A

DBA Patients	Mutation
279	RPS19, intr. 5, donor splice site mutation, IVS5 +1g > t
287	RPS19, intr. 2, acceptor splice site mutation, IVS2 -2a > c
288	RPS19, intr. 5, donor splice site mutation, IVS5 +1t > c
316	RPS19, ex.2, c.1A > G p. Met1VAl
320	RPS19, intr. 3, donor splice site mutation, IVS3 +1g > t



c. We will infect CD34+ fetal liver cells with both RPS19 and miR34a shRNA lentivirus and examine effects on erythroid differentiation and proliferation by methylcellulose colony assays and FACS analysis (Months 3-6).

We first needed to demonstrate that CD34+ fetal liver and cord blood HSCs transduced with RPS19 resulted in defects in erythroid differentiation and to a lesser degree, myeloid differentiation. We showed this in methylcellulose colony assays but are currently performing experiments in a liquid culture system, which enables us to characterize specific stages of erythroid differentiation.



d. We will infect CD34+ fetal liver cells with RPS19 shRNA and miR34a lentivirus and examine effects on erythroid differentiation and proliferation by methylcellulose colony assays and FACs analysis (Months 3-6).

See above.

e. Examine miR34a target gene expression in cells infected with RPS19 and miR34a shRNA or RPS19 and miR34a lentivirus during erythroid differentiation (Months 6-9).

We will examine miR34a target gene expression once we have optimized liquid culture system of erythroid differentiation.

f. Study molecules involved in apoptotic pathways (Caspases-3, -7, and -9, PARP cleavage) in RPS19 + miR34a shRNA or RPS19 + miR34a lentivirus transduced CD34+ fetal liver cells during erythropoiesis (Months 9-12).

We will perform these experiments once we have optimized the liquid culture system of erythroid differentiation.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

- RPS19 deficiency leads to upregulation of miR34a and decreased expression of c-myb and c-myc through a p53-dependent pathway

- We have also performed RNA-seq experiments and submitted mRNA for microRNA-

seq. We have identified two interesting pathways involved downstream of RPS19 deficiency in human hematopoietic progenitor cells. These pathways involve FoxM1, which appears to be deregulated specifically in RPS19 deficient cells. The other protein that is deregulated is GDF15, which is expressed in response to stress erythropoiesis. These two new mechanisms are novel and have not been previously studied.

- We also identified that that RSP19 deficient hematopoietic progenitors have decreased GATA1 expression through a TNFalpha and p53-dependent pathway.

5. **CONCLUSION:** Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

Our results could lead to new insights in to the pathogenesis and treatment of DBA. The implications are far reaching. Some of the deregulated genes that we identified in DBA, have also been found to be deregulated in myelodysplastic syndromes (MDS). We plan to further investigate signaling pathways, in particular, those that might lead to new therapies for DBA and other bone marrow failure syndromes.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

   a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

      (1) Lay Press:

      International Innovations – featured my research globally http://www.research-europe.com/magazine/HEALTHCARE2/EX12/index.html

      (2) Peer-Reviewed Scientific Journals:

      N/A

      (3) Invited Articles:

      N/A

      (4) Abstracts:

Defective Nucleotide Metabolism Contributes to p53 Activation in Diamond-Blackfan Anemia.
Elena Bibikova, Nadia Danilova, Todd M. Covey, David Nathanson, Elizabeth Dimitrova, Anne Lindgren, Bertil Glader, Caius G. Radu, Kathleen M. Sakamoto*, and Shuo Lin*. Accepted for poster presentation at the American Society of Hematology meeting, New Orleans, LA. December 2013.

Transcriptional Profiling and Cytokine Signaling in the Pathogenesis of Diamond-Blackfan Anemia
Yoan Konto-Ghiorghi, Ph.D., Elena Bibikova, Anupama Narla, M.D., and Kathleen Sakamoto, M.D, Ph.D. Accepted for poster presentation at the American Society of Hematology meeting, New Orleans, LA. December 2013.

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

"Congenital Disorders of Bone Marrow Failure – Pathophysiology and Laboratory Diagnostic Advances. American Society for Clinical Laboratory Science, Los Angeles, CA. July 20, 2012.

"Molecular Characterization of Normal and Aberrant Hematopoiesis." Division of Pediatric Hematology/Oncology Monthly Research Seminar, August 10, 2012.

"Signaling pathways in normal and aberrant hematopoiesis" Jason Bennette Memorial Lectureship. Cohen Children's Hospital, September 16, 2013, Long Island, NY.

7. **INVENTIONS, PATENTS AND LICENSES:** List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

Nothing to report.

8. **REPORTABLE OUTCOMES:** Provide a list of reportable outcomes that have resulted from this research. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

Nothing to report.

9. **OTHER ACHIEVEMENTS:** This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for

based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

Degrees: Elena Bibikova will defend her Ph.D. thesis based on this work on December 19th, 2013.

Funding applied for this work: Hired a new postdoctoral fellow Minyoung Youn, PhD. She applied and was awarded a Child Health Research Institute Fellowship (Stanford University).

Hired a new postdoctoral fellow Yoan Konto, Ph.D. He applied for a Child Health Research Institute Fellowship (Stanford University), which is pending.

Applied for a Leukemia & Lymphoma Society Specialized Centers of Research (SCOR) – P.I. Beverly Mitchell, M.D. – not funded.

10. **REFERENCES:** List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).

Not applicable.

11. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

None attached. Will submit next year.

NOTE:

TRAINING OR FELLOWSHIP AWARDS: For training or fellowship awards, in addition to the elements outlined above, include a brief description of opportunities for training and professional development. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities may include workshops, conferences, seminars, and study groups.

We organized a grants workshop and professionalism workshop in 2012-2013. In addition, we have a developmental therapeutics seminar series and a bone marrow failure seminar series (through the Stanford Cancer Institute).

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

Not applicable.

QUAD CHARTS: If applicable, the Quad Chart (available on this eReceipt System https://cdmrp.org/Program_Announcements_and_Forms/ and under "Forms" on https://www.usamraa.army.mil) should be updated and submitted with attachments.

Not applicable.

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