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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. We have further characterized the role of miR-34a in RPS-19 deficient CD34+ human fetal liver cells. We have identified additional microRNAs that are deregulated in RPS19-deficient CD34+ human fetal liver cells. We have also characterized downstream signaling pathways that regulate erythropoiesis in RPS19-deficient hematopoietic progenitor cells, in particular those that involve inflammation and DNA damage. These studies will provide new insights into the molecular pathways downstream of ribosomal protein insufficiency in hematopoietic stem cells and potentially novel targets for therapy.

15. SUBJECT TERMS
RPS19, DBA, signaling, pathways, RNA-seq, microRNAs, CD34+ cells

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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. We have further characterized the role of miR-34a in RPS-19 deficient CD34+ human fetal liver cells. We have identified additional microRNAs that are deregulated in RPS19-deficient CD34+ human fetal liver cells. We have also characterized downstream signaling pathways that regulate erythropoiesis in RPS19-deficient hematopoietic progenitor cells, in particular those that involve inflammation and DNA damage. These studies will provide new insights into the molecular pathways downstream of ribosomal protein insufficiency in hematopoietic stem cells and potentially novel targets for therapy.

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1. INTRODUCTION:

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 and other relevant microRNAs 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:

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

3. ACCOMPLISHMENTS:

Statement of Work

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.

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 through a p53-dependent pathway. We are continuing to analyze the expression and role of Sirt1 and Notch1. This milestone is 100% completed.

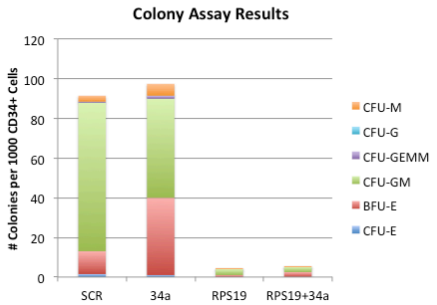
b. Study miR34a target gene expression (c-Myb, c-Myc, Sirt1, and Notch1) in lymphoblastoid cell lines (LCL) and CD34+ bone marrow progenitor cells from DBA patients with RPS19 mutations (Months 1-3).

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. We are in the process of obtaining and analyzing bone marrow samples from patients with DBA. This milestone is 75% completed.

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 demonstrated that downregulation of miR34a was not sufficient to rescue the defects observed in RPS19-deficient CD34+ cells. 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. This milestone is 75% completed.

Downregulation of miR34a is not Sufficient to Rescue the Defects Seen in RPS19 Deficient Cells



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.

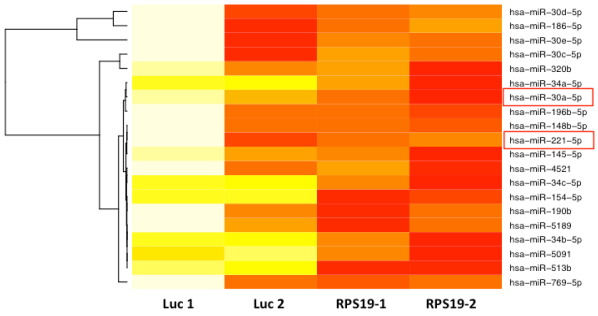
e. Examine miR34a target gene expression in cells infected with RPS19 and miR34a shRNA or RPS19 and miR34a lentivirus during erythroid differentiation (Months 6-9).

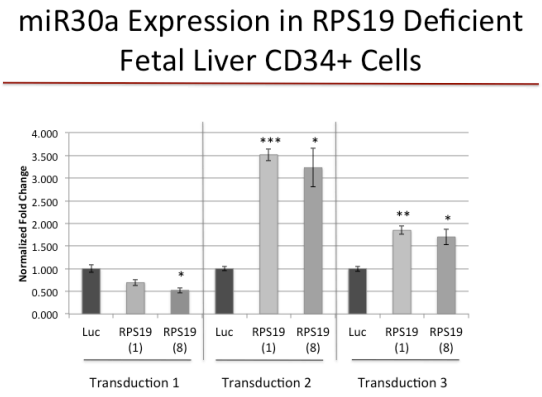
We are examining miR34a target gene expression. These experiments are ongoing. This milestone is 75% completed.

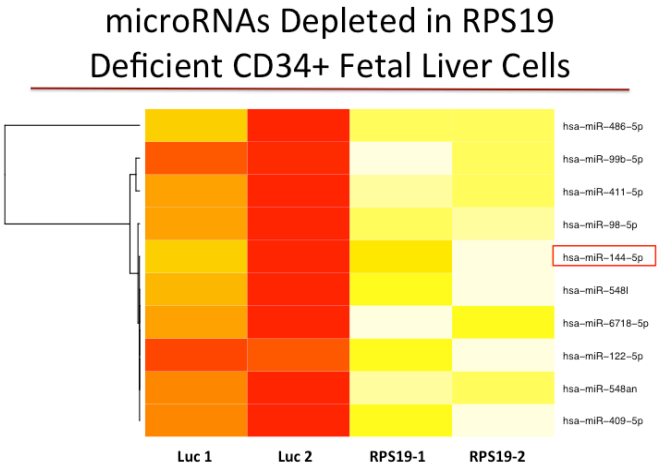
f. Study molecules involved in apoptotic pathways (Caspases-3, -7, and -9, PARP cleavage) in RPS19 + miR34a shRNA or RPS19 + miR34a lentivirus transduced CD34+ fetal liver cells during erythropoiesis (Months 9-12).

We are performing these experiments once we have optimized the liquid culture system of erythroid differentiation. This milestone is 75% completed.

We have also explored additional microRNAs identified in RPS19-deficient human fetal liver cells from microRNA-seq experiment, including miR30 and miR221.







Task 2. To characterize the role of miR34a in RPS19 insufficient primary human hematopoietic stem cells *in vivo* (Months 12-24).

Major goal: To understand how miR34a regulates hematopoietic stem cell engraftment and erythropoiesis in a mouse model of RPS19 insufficiency.

a. Human CD34+ fetal liver hematopoietic stem cells will be infected with either RPS19 shRNA alone or RPS19 shRNA + miR34a lentivirus then injected into NOD-SCID IL2R gamma (NSG) null mice; blood, bone marrow and spleen cells will be analyzed by FACs for short-term engraftment after 4-6 weeks. Colony assays will also be performed with bone marrow cells from mice (Months 12-14).

b. Human CD34+ fetal liver hematopoietic stem cells will be infected with either RPS19 shRNA alone or RPS19 shRNA + miR34a lentivirus then injected into NOD-SCID IL2R gamma (NSG) null mice; blood, bone marrow and spleen cells will be analyzed by FACs for long-term engraftment after 12-16 weeks. Colony assays will also be performed with bone marrow cells from mice (Months 14-18).

c. We will analyze cell cycle profile and apoptotic/survival of engrafted, transduced cells as described above (Months 18-24).

For a, b, and c, we were able to transplant human CD34+ cells transduced with RPS19 shRNA, but the mice did not engraft. Therefore, the remaining experiments under Task 2 were not possible.

Task 3. We will identify transcripts and microRNAs that are overexpressed or downregulated in RPS19 insufficient primary human hematopoietic stem cells (Months 18-36).

Major goal: To understand the role of novel genes downstream of RPS19 insufficiency that regulate erythropoiesis using a systems biology approach.

a. Characterize transcripts that have been identified as being significantly downregulated or upregulated RPS19 insufficient human CD34+ fetal liver stem cells and examine expression in LCLs and CD34+ bone marrow cells from DBA patients with RPS19 mutations (Months 18-21).

These experiments were completed and we are now preparing a manuscript on the role of FoxM1 in erythropoiesis and RPS19-deficient HSPCs.

b. Perform RNA-seq using microRNA primers to identify microRNAs that are aberrantly regulated in RPS19 shRNA insufficient human CD34+ fetal liver cells (Months 18-22).

We have completed the microRNA-seq experiments in collaboration with Dr. Stan Nelson at UCLA (subcontract). We are working with our bioinformaticians to analyze the data.

c. Examine the expression of specific microRNAs identified by RNA-seq in DBA patient LCLs and CD34+ bone marrow progenitors from DBA patients with RPS19 mutations (Months 22-24).

We plan to do this once the analysis of the micro-RNA-seq data is complete.

d. Characterize the role of the most significantly up- or downregulated microRNAs on erythropoiesis *in vitro* by overexpressing them in RPS19 insufficient CD34+ fetal liver cells using methylcellulose colony assays and FACs analysis (Months 24-30).

We plan to do this once the analysis of the micro-RNA-seq data is complete.

e. Characterize the most significantly up- or downregulated microRNAs on erythropoiesis in vivo by overexpressing them in RPS19 insufficient CD34+ fetal liver cells and transplanting into NSG mice to examine both short-term and long-term engraftment (Months 30-36).

This will most likely not be feasible given the inability of RPS19-deficient human CD34+ cells to engraft in NSG mice.

Summary of what was accomplished under these goals:

- RPS19 deficiency leads to upregulation of miR34a and decreased expression of c-myb and c-myc through a p53-dependent pathway. Furthermore, additional microRNAs are deregulated in RPS19-deficient CD34+ human fetal liver cells.

- We attempted experiments proposed in Aim 2 with primary human CD34+ fetal liver cells injected into NSG mice. We confronted difficulties in that these cells did not engraft. Therefore, these experiments could not be completed.

- We have also performed RNA-seq experiments and submitted mRNA from RPS19 knockdown human fetal liver cells. 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 and this paper was published in Blood.

- We completed the micro-RNA-seq screen with human fetal liver cell CD34+ cells and are waiting for the bioinformatics analysis. Our goals are to characterize significantly up- or downregulated microRNAs in RPS19-deficient cells in the future. Since the funding has been expended, we cannot request a no-cost extension to complete these studies. We plan to submit another grant to the DOD this fall, depending on our microRNA-seq results.

What opportunities for training and professional development have the project provided?

Graduate student: Former graduate student Elena Bibikova defended her Ph.D. thesis based on this work on December 19th, 2013. Dr. Bibikova now works as a research scientist at Acerta Pharma, Inc.

Postdoctoral fellows: Postdoctoral fellow Minyoung Youn, PhD. She received 2 years of funding from the Child Health Research Institute Fellowship (Stanford University) to study signaling pathways in RPS19-deficient hematopoietic stem and progenitor cells for two years 9/1/2013 to 8/30/2015.

Postdoctoral fellow Joseph Park, M.D., Ph.D. applied for and received a CHRI fellowship for two years to study the role of RPS14 deficiency in del(5q) myelodysplastic syndrome. Techniques learned from the CDMRP project will be used for Dr. Park's project.

NIH training grant: As a result of the work funded by the CDMRP, I was able to obtain an NIH T32 training grant to support postdoctoral fellows studying Pediatric Nonmalignant Hematology and Stem Cell Biology and an NIH R56 grant.

T32DK098132 4/1/2014-3/31/2019
National Institutes of Health (NIDDK)
Training in Pediatric Nonmalignant Hematology and Stem Cell Biology
Sakamoto, PI
- The goal is to train postdoctoral fellows in nonmalignant hematology and stem cell biology

R56DK107286-01
National Institutes of Health (NIDDK) 9/10/15-8/31/16
Signaling Pathways in MDS
Sakamoto, PI and Shuo Lin, co-PI
-The goal of this project is to characterize inflammatory signaling pathways that mediate RPS14-induced anemia in MDS.

How were the results disseminated to communities of interest?

The data were presented at UCLA during graduate student Elena Bibikova's thesis defense and at the American Society of Hematology meeting in 2014 and 2015 by Dr. Minyoung Youn.

What do I plan to do during the next reporting period to accomplish the goals?

I plan to continue to focus on microRNAs that are deregulated in RPS19-deficient CD34+ fetal liver cells as described in the SOW and milestones above and to submit another DOD New Idea grant in 2016.

4. IMPACT:

What was the impact on the development of the principal disciplines of the project?

Our results could lead to new insights into 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 RPS14 del(5q) myelodysplastic syndromes (MDS). We plan to further investigate downstream signaling pathways, in particular, those that might lead to new therapies for DBA

and other bone marrow failure syndromes. For FoxM1, there are inhibitors that are available, which could be studied as potential approaches to treat DBA.

What was the impact on other disciplines?

Since RPS14-deficiency is identified in 5q- MDS patients, the signaling pathways and deregulated proteins are common in both DBA and MDS. This has resulted in a funded NIH R56 grant on signaling pathways in 5q- MDS. Our findings are relevant to MDS and potentially other ribosomapathies.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

See above.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Could not complete the *in vivo* experiments due to lack of engraftment of human CD34+ RPS19-deficient cells into NSG mice.

Actual or anticipated problems or delays and actions or plans to resolve them

See above.

Changes that had a significant impact on expenditures

None.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None.

6. PRODUCTS:

Journal publications

Danilova N, Bibikova E, Covey TM, Nathanson D, Dimitrova E, Lindgren A, Glader B, Radu CG, **Sakamoto KM***, and S Lin*. The Role of DNA damage response in zebrafish and cellular models of Diamond Blackfan Anemia. Disease Models and Mechanisms, 7:895-905, 2014. (*co-senior authors)

Bibikova E, Youn MY, Danilova N, Konto-Ghiorghi Y, Ono-Uruga Y, Ochoa R, Narla A, Glader B, Lin S, and **KM Sakamoto**. TNF-α mediated inflammation in RPS19 deficient hematopoietic progenitors represses GATA1 through a p53-dependent mechanism. Blood, 7:895-905, 2014.

Rankin EB, Narla A, Park JK, Lin S, and **KM Sakamoto**. Biology of the bone marrow microenvironment and myelodysplastic syndromes. Mol Genet Metab. 116:24-28, 2015.

Books, conference papers, and presentations

Youn MY, Bibikova E, Danilova N, Ono-Uragi Y, Konto-Ghiorghi Y, Ochoa R, Narla A, Glader B, Lin S, and KM Sakamoto. RPS19 Deficiency Leads to GATA1 Downregulation through TNF-mediated p38 MAPK Activation. Accepted for a poster presentation. American Society of Hematology, San Francisco, CA 2014.

Youn MY, LaVasseur C, Narla A, and KM Sakamoto. Loss of FoxM1 promotes erythroid differentiation through increased proliferation of erythroid progenitors. Accepted for poster presentation. American Society of Hematology, Orlando, FL 2015.

Website or other internet sites

None.

Technologies or techniques

See publications.

Inventions, patent applications, and /or licenses

None.

Other products

None

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name: Kathleen M. Sakamoto, MD, PhD
Project Role: PI
No change in effort.

Name: Elena Bibikova, PhD

Project Role: Graduate Student
Nearest person month worked: 3 (over the past year)
Contribution to Project: Dr. Bibikova performed all the work proposed in the project.
Funding support: CDMRP grant

Has there been a change in the active other support of the PI or key personnel since the last reporting period.

R56DK107286-01
National Institutes of Health (NIDDK) 9/10/15-8/31/16
Signaling Pathways in MDS
Sakamoto, PI and Shuo Lin, co-PI
-The goal of this project is to characterize inflammatory signaling pathways that mediate RPS14-induced anemia in MDS.

Acerta, Inc. 1/1/15-12/31/15
Analysis of BTK and PI3Kd inhibitors in Normal and Neoplastic Myeloid cells
Sakamoto, PI
- The goal is to study the effects of BTK and PI3Kd inhibitors in normal hematopoietic stem and progenitor cells and acute myeloid leukemia cells.

Hyundai Hope on Wheels 1/1/15-12/31/16
The Role of CREB in the Pathogenesis of ALL and as a Target for Therapy.
Sakamoto, PI
- The goal is understand how CREB regulates growth of ALL cells and how it can be targeted for ALL patients.

Leukemia and Lymphoma Society of America 10/1/14-3/31/16
Screen to Lead Program
Targeting CREB in Acute Myeloid Leukemia
Sakamoto, PI
- The goal is to study CREB as a target for AML therapy.

T32DK098132 4/1/14-3/31/19
National Institutes of Health (NIDDK)
Training in Pediatric Nonmalignant Hematology and Stem Cell Biology
Sakamoto, PI
- The goal is to train postdoctoral fellows in nonmalignant hematology and stem cell biology.

Research grant 8/1/15-7/31/16
St. Baldrick's Foundation
Sakamoto PI
The Role of RSK in Acute Myeloid Leukemia and as a Target for Therapy
- The goal is to study RSK1 in the pathogenesis of AML and inhibitors for potential

therapy.

Research grant 1/1/16-12/31/16
USC Parker Institute for Childhood Cancer Research/William Lawrence & Blanche Hughes Foundation
Sakamoto, PI
- CREB inhibition in Pediatric Acute Lymphoblastic Leukemia

Since Dr. Bibikova has graduated, postdoctoral fellow, Dr. Minyoung Youn has taken over this project.

Name: Minyoung Youn, PhD
Project Role: Postdoctoral Fellow
Nearest person month worked: 6
Contribution to Project: Will perform experiments proposed in the project.
Funding support: Stanford Child Health Research Institute postdoctoral fellowship, and NIH R56 grant.

What other organizations were involved as partners?

We continue to collaborate with Dr. Stan Nelson for the microRNA-seq data (as proposed in the original proposal).

Organization Name: UCLA
Location of Organization: Los Angeles, CA.
Partner's contribution to the project: collaboration

8. SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES:

None.