Discoveries Journals
publishing the future

DISCOVERIES 2019, Jul-Sep, 7(3): e96
DOI: 10.15190/d.2019.9

Discoveries in IMAGES

Rpl5-Inducible Mouse Model for Studying Diamond-Blackfan Anemia

*Shideh Kazerounian[1, 3, *], Daniel Yuan[1], Matthew S. Alexander[2], Alan H. Beggs[1, 3, *], Hanna T. Gazda[1, 3]*

[1]Boston Children's Hospital, Division of Genetics and Genomics, The Manton Center for Orphan Disease Research, Boston, MA, USA
[2]University of Alabama at Birmingham and Children's of Alabama, Departments of Pediatrics and Genetics, Division of Neurology, Birmingham, AL, USA
[3]Harvard Medical School, Boston, MA, USA

**Corresponding authors*: Shideh Kazerounian, PhD, and Alan H. Beggs, PhD, Boston Children's Hospital, Division of Genetics and Genomics, The Manton Center for Orphan Disease Research, Boston, CLS 15th Floor, 300 Longwood Avenue; Boston, MA 02115; Phone: 617-919-217; Emails: shideh.kazerounian@childrens.harvard.edu and beggs@enders.tch.harvard.edu respectively.

Submitted: July 3rd, 2019; *Revised:* Sept. 9th, 2019; *Accepted:* Sept. 10th, 2019; *Published:* Sept. 30th, 2019;
Citation: Kazerounian S, Yuan D, Alexander MS, Beggs AH, Gazda HT. Rpl5-Inducible Mouse Model for Studying Diamond-Blackfan Anemia. *Discoveries* 2019, Jul-Sep; 7(3): e96. DOI: 10.15190/d.2019.9

ABSTRACT

Diamond-Blackfan anemia (DBA) is a rare congenital bone marrow disorder with mutations in ribosomal protein genes. Several animal models have been developed to study the pathological mechanism of DBA. Previously, we reported that the complete knock-out of both *Rpl5* and *Rps24* alleles were lethal, while heterozygous *Rpl5*$^{+/-}$ and *Rps24*$^{+/-}$ mice showed normal phenotype. To establish a more efficient mouse model for mimicking DBA symptoms, we have taken advantage of RNAi technology to generate an inducible mouse model utilizing tetracycline-induced down-regulation of *Rpl5*. After two weeks of treatment with doxycycline in drinking water, a subset of treated shRNA *Rpl5*$^{+/-}$ adult mice developed mild anemia while control mice had normal complete blood counts. Similarly, treated shRNA *Rpl5*$^{+/-}$ mice developed reticulocytopenia and bone marrow erythroblastopenia. Detection of DBA symptoms in these mice make them a valuable DBA model for studying the pathological mechanism underlying DBA and for further assessment of the disease and drug testing for novel therapies.

Keywords:

Diamond-Blackfan anemia, Ribosomal Protein L5, Rpl5-Inducible Mouse Model.

Abbreviations:

Diamond-Blackfan Anemia (DBA); Colony-forming Unit-Erythroid (CFU-E); Colony-forming Unit-Granulocyte and Monocyte (CFU-GM); Burst-forming Unit-Erythroid (BFU-E); Ribosomal Protein (RP);

Diamond-Blackfan anemia (DBA) is a rare congenital bone marrow disorder inherited in an autosomal dominant pattern and resulting from haploinsufficiency of ribosomal proteins. It is characterized by macrocytic anemia, reticulocytopenia, and bone marrow erythroblastopenia as well as congenital malformations in about 50% of patients[1].

The first major breakthrough in the molecular pathogenesis of DBA came from the discovery of the ribosomal protein S19 gene[2], followed by identification of mutations in 24 additional ribosomal protein (RP) genes including *RPL5*[3]. Additionally, mutations in two non-ribosomal

protein genes, *GATA1*, encoding a critical transcription factor for red blood cell maturation, and *TSR2*, encoding a pre-RNA processing protein, have been reported in a subset of patients[4-6]. Furthermore, we reported a significant decrease in the expression level of GATA1 protein but not mRNA in primary hematopoietic cells from patients with mutations in RP genes[6].

To address pathological mechanisms underlying DBA, several animal models have been generated. Homozygous *Rps19*$^{-/-}$ mice are embryonic lethal while heterozygotes had similar levels of RPS19 protein and mRNA as wild-type litermates[7]. We have reported that knocking-out *Rpl5* and *Rps24* alleles also leads to embryonic lethality, while heterozygous *Rpl5*$^{+/-}$ and *Rps24*$^{+/-}$ mice showed normal phenotypes at birth and throughout their development with no detectable differences between the expression levels of RPL5 and RPS24 mRNA and protein compared to those of wild-type mice[8]. Interestingly, a small number of these mice developed soft tissue sarcomas, also seen in some of patients with DBA[8]. Due to the severity of symptoms associated with *RPL5* mutations in patients, we decided to focus our studies on the molecular mechanism of RPL5 deficiency induced postnataly[3]. Here, we report the generation of a conditional *Rpl5* mouse line using an RNA interference (RNAi) approach. All animal studies were approved by Boston Children's Hospital's Institutional Animal Care and Use Committee.

A validated short-hairpin RNA (shRNA) construct targeting the mouse *Rpl5 mRNA transcript* was cloned into a TRE3G-based *Col1A1*-targeting vector (pColA1-TRE3G-GFP-miR30) and co-electroporated into pre-engineered KH2 embryonic stem cells, which contain a reverse tet-transactivator (rtTA) cassette integrated into the *Rosa26* locus[9]. In parallel, we obtained an shRNA-luciferase (shRNA *Renilla*, shRNA *Ren*) mouse line to use as a non-specific shRNA control line. Both mouse lines were generated by Mirimus Laboratories (Mirimus Inc., Brooklyn, NY) on the C57BL/6 background.

To assess the effects of *Rpl5* down-regulation, mice either heterozygous or homozygous for the shRNA at the *ColA1 and Rosa26* loci were generated. To increase the tetracycline effect for a stronger mRNA knockdown, we used mice that were heterozygous for *ColA1* and homozygous for *Rosa26*, which would increase the production of reverse tetracycline transactivator and therefore, the effect of tetracycline treatment (as described above). Henceforth, we will refer to shRNA *Rpl5*$^{+/-}$ mice as shRNA *Rpl5* and shRNA *Ren*$^{+/-}$ as control mice. Five to eight-week old female and male shRNA *Rpl5* mice were treated with 2mg/mL of doxycycline in drinking water[**10**]. After 2 weeks of treatment, a mild anemia was detected in about 20% of the treated shRNA *Rpl5* mice while control mice were normal (**Table 1**). Further hematological studies revealed marked reticulocytopenia in all treated shRNA *Rpl5* mice (n=3) (reticulocytes 1.77%, $0.1x10^6$/ul) versus control mice (n=3) (reticulocytes 3.8%, $0.24x10^6$/ul) and bone marrow erythroblastopenia (myeloid to erythroid linage 4.9 in all shRNA *Rpl5* mice and 3.4 in control mice). To further investigate erythropoiesis in shRNA *Rpl5* and control mice, methylcellulose colony assays[11] were performed on bone marrow cells isolated from these mice to quantify the number of burst-forming unit- erythroid BFU-E and colony forming unit-erythroid CFU-E colonies as well as colony forming-unit granulocyte macrophage (CFU-GM). Our results showed a decrease in the number of BFU-E and CFU-E colonies in shRNA *Rpl5* mice compared to control, which correlates to a decrease in the proliferation level of erythroid progenitor cells and a slight decrease of CFU-GM colonies (**Figure 1A**). We next performed flow cytometry on freshly isolated bone marrow cells from shRNA *Rpl5 and* control mice to compare the percentage of differentiated erythroid cells in each cell population. In our experiment, we examined two erythroid populations: less mature CD71^{high}Ter119^{med} and more mature CD71^{high}Ter119^{high12}. shRNA *Rpl5* mice had lower numbers of CD71^{high}Ter119^{med} (0.6% vs. 0.9%) and CD71^{high}Ter119^{high} (3.2% vs. 5.3%) cells as compared to control mice (**Figure 1B**). GATA-1 is a necessary factor for the survival and terminal differentiation of erythroid progenitors[**13**].

To assess expression levels of RPL5 and GATA1 proteins in shRNA *Rpl5* mice, western blots were performed on CD71^{high}Ter119^{high} cell population total cell lysates using anti-RPL5 (Novus Biologic, NBP1-31413) or anti-GATA-1 antibodies (Sc-1234)[8]. These results demonstrated a significant decrease in expression of both RPL5 (**Figure 1C**) and GATA1 (**Figure 1D**) in cells from the treated shRNA *Rpl5* mice compared to control

Table 1. Complete blood count in shRNA *Rpl5* and shRNA *Ren* mice treated with doxycycline

Parameter (Units)	shRNA *Rpl5* (Mean Result, n=3)	shRNA *Ren* (Mean Results, n=3)	Normal Range
RBC (M/µL)	**5.7 ±0.4**	**8.3±1.3**	**6.36-9.42**
Hb (g/dL)	**5.9±0.3**	**11.3±0.5**	**11.0-15.1**
HTC (%)	**26.8±1.9**	**41.8±7.3**	**35.1-45.4**
MCV (fl)	47.3±0.9	50.3±2.3	45.4-60.3
WBC (K/µL)	6.0±2.7	12.6±7.6	1.8-10.7
NE (K/µL)	1.0±0.2	4.2±3.0	0.1-2.4
LY (K/µL)	2.9±0.8	7.4±3.4	0.9-9.3
MO (K/µL)	0.07±0.005	0.54±0.66	0.0-0.4
EO (K/µL)	0.02±0.01	0.38±0.43	0.0-0.2
BA (K/µL)	0.01±0.0	0.17±0.09	0.0-0.2
PLT (K/µL)	674.3±292.3	626.3±349.7	592-2972

RBC: red blood cells (P=0.13); Hb: hemoglobin (P=0.0008); HTC: Hematocrit (P=0.117); MCV: corpuscular volume (P=0.3); WBC: white blood cell (P=0.46); NE: neutrophils (P=0.34); LY: lymphocytes (P=0.27); MO: monocytes (P=0.15); EO: eosinophils (P=0.45); BA: basophils (P=0.15); PLT: platelet count (P=0.92). Graphpad T test calculator website has been used to determine the P values based on standard division.

mice. On the other hand, quantitative PCR on two sorted erythroid populations showed similar levels of *Gata1* mRNA expression in shRNA *Rpl5* and control mice (data not shown), as was shown in human cells[6]. The reduction of GATA1 protein is likely due to the reduction of RPL5, impaired ribosomal erythropoiesis, lower numbers of ribosomes, and altered GATA1 translation, as reported in human cells[6,14].

In summary, we have generated and characterized a novel DBA mouse model, which allows an inducible and graded down-regulation of *RpL5* gene expression. These mice recapitulate the major features of DBA including anemia, reticulocytopenia, and bone marrow erythroblastopenia[1]. Therefore, these shRNA *Rpl5*$^{+/-}$ mice may provide an effective approach for studying DBA and testing novel therapies.

Acknowledgements

These studies were funded in part by generous support from the Mauch Family, a *Manton Center for Orphan Disease Research Junior Investigator Award*, and by *NIH R01HL107558* and *NIH K02HL111156* to HTG. SK and HTG performed the experiments, analyzed the data, and wrote the manuscript. DY performed the experiments and edited the manuscript. MSA designed the technical aspects of the mouse line and edited the manuscript. AHB and HTG edited the manuscript and advised with experiments. We also thank Vasilis Toxavidis and John Tigges of the Beth Israel Deaconess Medical Center Flow Cytometry Core Facility for their help in sorting bone marrow cells.

Conflict of Interest

The authors declare no competing financial interests.

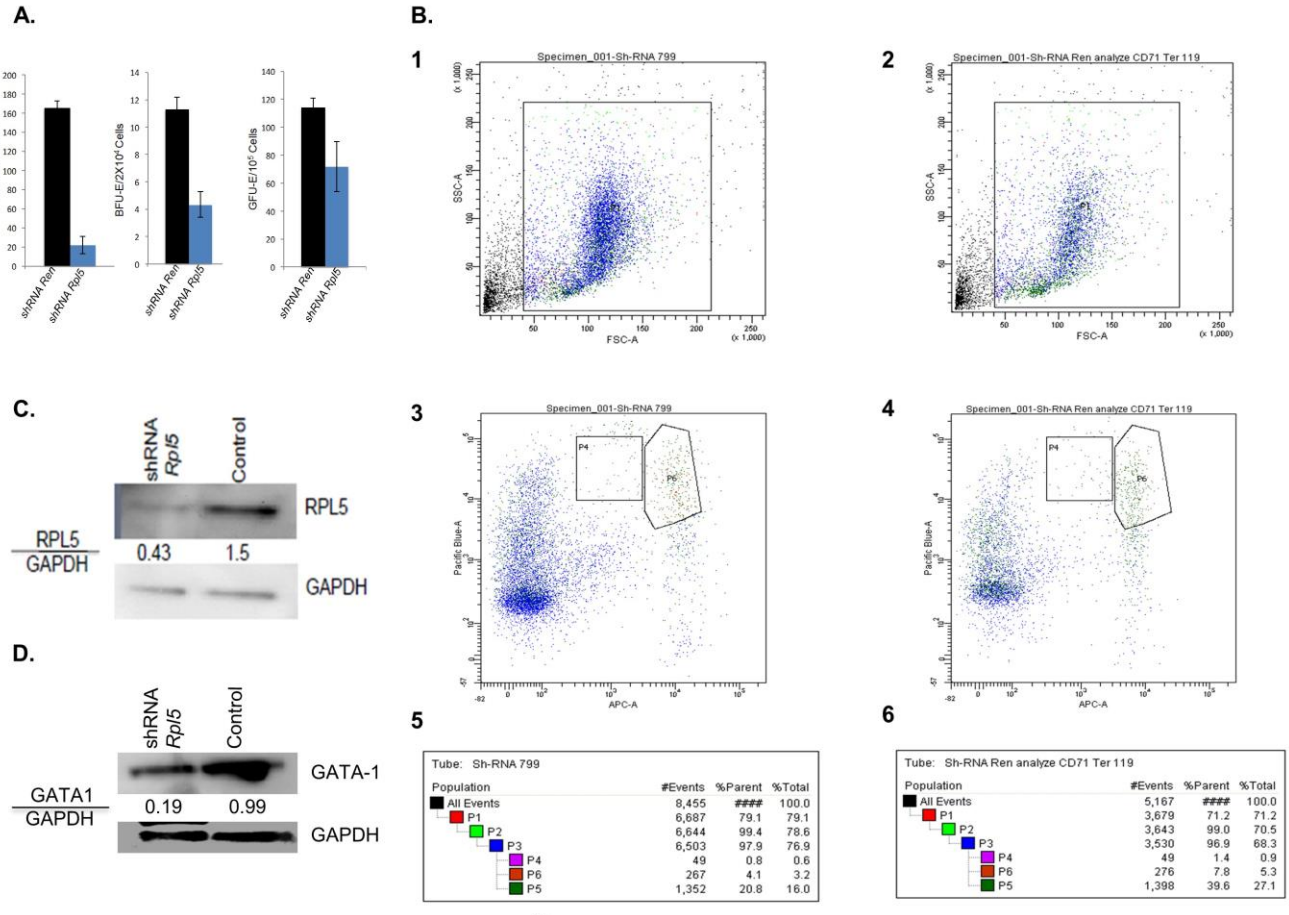

Figure 1. A. Methylcellulose colony assay on bone marrow cells from doxycycline treated shRNA Rpl5 and control mice (n=3), (CFU-E, P=0.0003; BFU-E, P=1; GFU-GM, P=0.3); **B.** FACS analysis of bone marrow cells from shRNA Rpl5 and control mice. Total bone marrow cells from shRNA Rpl5 (**B.1**) and control (**B.2**) mice; viable cells were gated for analysis and sorting. We used the cell-surface markers CD71 (Y-axis) and Ter119 (X-axis) to separate mature erythroids and erythroid precursors. The most mature erythroblasts are CD71lowTer119high, while the erythroid precursors are CD71highTer119intermediate cells. In our FACS analysis the gated population comprises CD71highTer119med cells (P4) and CD71highTer119high cells (P5) from shRNA Rpl5 (**B.3** and **B.5**) and control (**B4** and **B6**) mice; the results here are collected from one mouse per group and are representative of 6 independent experiments; in each experiment, cells were collected from 1-3 mice for each group and sorted independently; **C.** Western Blot analysis of RPL5 protein expressions in CD71highTer119high cells. There was a significant decrease in the expression level of RPL5 in the treated shRNA Rpl5 compared to the control mice; **D.** Western Blot analysis of GATA1 protein expressions in CD71highTer119high cells. There was a significant decrease in the expression of GATA1 in the shRNA Rpl5 group compared to the control mice. In these experiments, GAPDH was used as a loading control, and they are representative of 3 independent experiments.

References

1. Li H, Lodish HF and Sieff CA. Critical Issues in Diamond-Blackfan Anemia and Prospects for Novel Treatment. Hematol Oncol Clin North Am. 2018; 32: 701-12.
2. Draptchinskaia N, Gustavsson P, Andersson B, et al. The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. Nat Genet. 1999; 21: 169-75.
3. Ulirsch JC, Verboon JM, Kazerounian S, et al. The Genetic Landscape of Diamond-Blackfan Anemia. Am J Hum Genet. 2018.
4. Sankaran VG, Ghazvinian R, Do R, et al. Exome sequencing identifies GATA1 mutations resulting in Diamond-Blackfan anemia. J Clin Invest. 2012; 122: 2439-43.
5. Gripp KW, Curry C, Olney AH, et al. Diamond-Blackfan anemia with mandibulofacial dystostosis is heterogeneous, including the novel DBA genes

TSR2 and RPS28. Am J Med Genet A. 2014; 164A: 2240-9.
6. Ludwig LS, Gazda HT, Eng JC, et al. Altered translation of GATA1 in Diamond-Blackfan anemia. Nat Med. 2014; 20: 748-53.
7. Matsson H, Davey EJ, Draptchinskaia N, et al. Targeted disruption of the ribosomal protein S19 gene is lethal prior to implantation. Mol Cell Biol. 2004; 24: 4032-7.
8. Kazerounian S, Ciarlini PD, Yuan D, et al. Development of Soft Tissue Sarcomas in Ribosomal Proteins L5 and S24 Heterozygous Mice. Journal of Cancer. 2016; 7: 32-6.
9. Premsrirut PK, Dow LE, Kim SY, et al. A rapid and scalable system for studying gene function in mice using conditional RNA interference. Cell. 2011; 145: 145-58.
10. Jaako P, Flygare J, Olsson K, et al. Mice with ribosomal protein S19 deficiency develop bone marrow failure and symptoms like patients with Diamond-Blackfan anemia. Blood. 2011; 118: 6087-96.
11. Gazda HT, Kho AT, Sanoudou D, et al. Defective ribosomal protein gene expression alters transcription, translation, apoptosis, and oncogenic pathways in Diamond-Blackfan anemia. Stem Cells. 2006; 24: 2034-44.
12. Koulnis M, Pop R, Porpiglia E, Shearstone JR, Hidalgo D and Socolovsky M. Identification and analysis of mouse erythroid progenitors using the CD71/TER119 flow-cytometric assay. J Vis Exp. 2011 Aug 5;(54).
13. Pevny L, Simon MC, Robertson E, et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature. 1991; 349: 257-60.
14. Khajuria RK, Munschauer M, Ulirsch JC, et al. Ribosome Levels Selectively Regulate Translation and Lineage Commitment in Human Hematopoiesis. Cell. 2018; 173: 90-103 e19.

DISCOVERIES is a peer-reviewed, open access, online, multidisciplinary and integrative journal, *publishing high impact and innovative manuscripts* from all areas related to MEDICINE, BIOLOGY and CHEMISTRY.

This article is an Open Access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes;
2019, Applied Systems;