Identification of Novel Pathways in the Pathogenesis of Diamond-Blackfan Anemia

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

by

Elena Bibikova

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ABSTRACT OF THE DISSERTATION

Identification of Novel Pathways in the Pathogenesis of Diamond-Blackfan Anemia

by

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Doctor of Philosophy in Molecular Biology
University of California, Los Angeles, 2014
Professor Hanna Mikkola, Chair

Diamond-Blackfan Anemia (DBA) is a genetic bone marrow failure syndrome, typically diagnosed in infants within the first year of life. It is characterized by macrocytic anemia, congenital abnormalities affecting the head, limbs, heart, and genitourinary system, and higher incidence of cancer, including leukemia and solid tumors. DBA is associated with mutations in ribosomal proteins, most commonly RPS19, and several groups have shown p53 pathway upregulation to play a significant role in DBA pathogenesis, though the overall mechanism of disease development remains unclear. Current treatments for DBA include corticosteroids, which have multiple side-effects in children, including short stature and delayed growth; blood transfusions, which carry a risk of organ failure from iron overload; and bone marrow transplants, which require immunosuppression and can result in mortality. Given the undesirable side effects of current DBA treatments, and the tumor suppressor function of p53, which makes it a
poor target for therapy, more research is needed to uncover pathways involved in DBA pathogenesis and to identify new drug targets that may be useful for treating DBA.

In this dissertation, we used primary human hematopoietic progenitor cells from fetal liver and cord blood that were transduced with lentivirus carrying shRNA against RPS19 as a model to identify novel pathways that play a role in DBA. In Chapter 2, we show that RPS19 deficient cells have reduced expression of the erythroid transcription factor GATA1, and that this reduction is p53-dependent and mediated by the inflammatory cytokine TNF-α. In Chapter 3, we describe the use of next-generation RNA sequencing to identify pathways dysregulated in RPS19 deficient cells, including inflammation mediated by chemokine and cytokine signaling, WNT signaling pathway, TGF-β signaling pathway, and the transcription factor c-MYC. Finally, in Chapter 4, we explore the role of microRNAs in DBA pathogenesis identified by next-generation microRNA-sequencing, and describe the role of the microRNA miR-34a in RPS19 deficient cells and in erythropoiesis. Taken together, our results suggest a novel role for inflammatory pathways in the pathogenesis of DBA, which can potentially be targeted to develop new treatments for this disease.
The dissertation of Elena Bibikova is approved.

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Shuo Lin

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Dinesh Rao

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Lily Wu

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Kathleen Sakamoto

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Hanna Mikkola, Committee Chair

University of California, Los Angeles
2014
I dedicate this dissertation to my family: to my grandfather, who always pushed me to be at my best; to my grandmothers who helped raise me; to my parents, whose unwavering support from the very beginning made all this possible; to my aunt, cousin, and everyone back home; and to my partner who supported me throughout this journey. I couldn’t have asked for a better family.
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PUBLICATIONS AND PRESENTATIONS


Chapter 1
Introduction

Diamond-Blackfan Anemia

Diamond-Blackfan Anemia (DBA) is a rare congenital bone marrow failure disorder, first identified by Josephs in 1936 (1), and later described by Diamond and Blackfan in 1938 (2). DBA is typically diagnosed within the first year of life, and is characterized by macrocytic anemia, reticulocytopenia, elevated fetal hemoglobin, high levels of erythroid adenosine deaminase (ADA) activity, and a lack of erythroid precursors in the bone marrow (3).

DBA is often associated with short stature, growth delay, and failure to thrive (4). Approximately 30-47% of patients also present with various congenital abnormalities, including craniofacial malformations, cardiac defects, urogenital malformations, limb abnormalities, and cleft palate (5). Additionally, patients with DBA have a predisposition to cancer including acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and a variety of solid tumors (3).

Classical treatment for DBA is focused on augmenting red blood cell (RBC) production with corticosteroids, such as prednisone and dexamethasone, supplying exogenous RBCs through repeated blood transfusions for patients not responsive to reasonable steroid doses, or replacing the deficient bone marrow through transplantation (5). All of the above treatments have unfavorable side effects, such as growth delay from continuous use of corticosteroids, iron overload from repeated transfusions, or immunosuppression required to sustain a bone marrow transplant (6).
Recently, a dietary supplement of the amino acid L-Leucine has been shown to be beneficial in some DBA patients (7). However, new effective therapies are still necessary for patients who do not respond to classical DBA treatments. Understanding the mechanism of DBA pathogenesis will help us develop new treatments for this disease.

*Ribosomal Defects in DBA*

Multiple bone marrow failure syndromes, including DBA, are associated with defects in ribosome biogenesis, and therefore termed “ribosomopathies (8).” Classical DBA is an autosomal dominant genetic disorder, which stems from haploinsufficiency or loss-of-function of ribosomal genes (5). The first DBA mutation was identified in 1999, from a study of a 7 year-old female with a balanced translocation [t(X;19)(p21;q13)] (9). The mutation was mapped to *RPS19*, which encodes a ribosomal component of the 40S ribosomal subunit, and is mutated in approximately 25% of DBA patients (10). Since then, other ribosomal mutations have been identified, including *RPS24* (2% of patients), *RPS17* (1%), *RPL35A* (2-4%), *RPL5* (7%), *RPL11* (5-10%), *RPS7* (1%), *RPS10* (2-6%), *RPS26* (2-6%), and *RPL26* (<1%) (11). Like the disease itself, these mutations are heterogeneous in nature and include missense, nonsense, frame shift, and splice mutations, as well as insertions, deletions, and rearrangements (11). Overall, ribosomal mutations have been implicated in approximately 70% of DBA cases to date, and more are being uncovered with high-throughput DNA sequencing technology (11).

From the study of these mutations, it has been established that DBA pathogenesis is related to defective ribosome biogenesis (8). The ribosome, which is
responsible for all protein synthesis in the cell, is made up of four ribosomal RNAs (rRNAs) contained in two ribosomal subunits: the smaller 40S subunit, made up of the 18S rRNA and RP(S) proteins, and the larger 60S subunit, made up of the 5.8S, 28S, and 5S rRNA, as well as the RP(L) proteins (12). Ribosome biogenesis is tightly regulated in the cell, and involves the maturation of the large precursor 90S rRNA into 18S, 5.8S, and 28S rRNA within the nucleolus (12). In patients with DBA, ribosome assembly is disrupted in different ways, based on the underlying mutation. For example, mutations in RPS19 lead to a defect in 18S rRNA maturation resulting from improper cleavage of the 21S rRNA precursor, which disrupts 40S subunit formation. In contrast, mutations in RPL11, RPL5, and RPL35a affect maturation of the 28S rRNA and assembly of the 60S ribosomal subunit (13). Despite these mutations affecting different stages of ribosome assembly, they similarly activate the ribosomal stress pathway in cells and lead to erythroid defects in the patient (12).

Role of p53 in DBA Pathogenesis

The classic result of ribosomal stress in the cell is activation of the master tumor suppressor p53 (14). While the exact mechanism behind this activation is unknown, the current model involves the release of free ribosomal assembly intermediates from the nucleolus into the nucleus, where they bind to MDM2, an E3 ubiquitin-protein ligase that negatively regulates p53 (15). To date, three ribosomal proteins have been found to bind MDM2, including RPL5, RPL11, and RPL23 (14). Upon binding, the free ribosomal proteins disrupt the interaction between MDM2 and p53, thus stabilizing p53 protein (15). Activation of the p53 pathway in DBA has been confirmed with various cell and
animal models including TF-1 cells, CD34+ primary cells, mouse models, and zebrafish, and is believed to contribute to cell cycle arrest and apoptosis in RP deficient cells (16-19), (Figure 1.1).

**Figure 1.1. Classical model of DBA pathogenesis.** Ribosomal protein insufficiency results in an imbalance of ribosomal assembly intermediates in the nucleolus, which spill out into the nucleus and bind to MDM2, thereby stabilizing p53 protein. Stabilization of p53 subsequently leads to p53 pathway activation, cell cycle arrest and apoptosis.

*Erythroid Defects in DBA*

Erythropoiesis is the process by which mature red blood cells develop in the bone marrow from undifferentiated hematopoietic stem cells (HSCs). Various cell surface markers, as well as expression of key erythroid transcription factors, can be used to discern the different stages of erythropoiesis in culture. Erythropoiesis begins when HSCs, which are positive for the cell surface marker CD34, differentiate into CD71+
erythroid progenitor cells: BFU-Es (burst-forming-unit-erythroid) and CFU-Es (colony-forming-unit-erythroid) (6). BFU-Es are the earliest erythroid progenitor cells, which form large colonies (bursts) in methylcellulose, and still express the hematopoietic progenitor marker CD34 (20). CFU-Es form smaller colonies in methylcellulose, are more differentiated than BFU-Es, and are also dependent on the cytokine erythropoietin (EPO) (20). These cells mature into pro-erythroblasts, followed by basophilic, polychromatic, and orthochromatic erythroblasts, which express CD71 and Glycophorin A, and reticulocytes (6). In the final stage of erythropoiesis, reticulocytes become enucleated to create mature red blood cells that carry oxygen throughout the body (21).

Several transcription factors are essential for erythropoiesis, including MYB, GATA2, GATA1, and SCL (22-24). MYB plays a role in early erythropoiesis by regulating the transcription of KIT, which encodes the receptor for stem cell factor (SCF) and is required for maintenance and proliferation of early erythroid progenitors (22). GATA2 is also expressed in early erythroid progenitor cells, but is replaced by GATA1 around the CFU-E stage, in what is termed the “GATA switch (25).” GATA1, comprised of two zinc finger domains and a transactivation domain, activates transcription of downstream targets by binding its consensus motif WGATAR in their promoters (26). Targets of GATA1 include EPOR, which encodes the EPO receptor, adult globin genes, heme biosynthesis enzymes, and erythroid membrane proteins (23). GATA1 also interacts with multiple co-factors, including FOG1 (friend of Gata1), and a complex comprised of SCL (stem cell leukemia), E2A (a member of the E-protein family), LMO2 (LIM doman only 2), and LDB1 (LIM domain-binding protein 1), which can modify its transcriptional activity (24).
DBA patients have persistently low reticulocyte counts and erythroblastopenia (absence or less than 5% erythroid precursors) in their bone marrow (12). Multiple studies have shown that BFU-Es and CFU-Es are most affected in DBA, while terminal differentiation proceeds normally in RP-deficient cells (16, 27, 28). Several erythroid transcription factors have also been implicated in DBA pathogenesis. Mutations in GATA1 have been identified in three rare X-linked cases of DBA (29), while MYB was found to be downregulated in the bone marrow of DBA patients with ribosomal protein mutations (30).

The Present Work

In this dissertation, our aim is to identify novel pathways that contribute to the pathogenesis of DBA and can potentially be targeted for drug development. To achieve this goal, we used primary human CD34+ cells transduced with lentivirus carrying shRNA against RPS19 to model the effects of ribosomal protein haploinsufficiency during erythropoiesis. In Chapter 2, we investigated the role of GATA1 in DBA pathogenesis and found that it was downregulated in RPS19 deficient primary human cord blood and fetal liver cells through a mechanism involving p53 and TNF-α. In Chapter 3, we used next-generation RNA sequencing (RNA-seq) to explore novel pathways dysregulated in RPS19 deficient fetal liver cells and identified inflammatory cytokine/chemokine signaling, TGF-β, and WNT pathways as potential candidates for further study. Finally, in Chapter 4, we explored the role of microRNA in DBA pathogenesis through microRNA-sequencing of RPS19 deficient fetal liver cells. The sequencing revealed miR-34a to be upregulated in RPS19 deficient cells, and we went
on to study the role of this microRNA in erythropoiesis using miR-34a expression and knockdown vectors in primary CD34+ cells.

Taken together, the results of this dissertation suggest a novel role for inflammatory pathways in DBA and identify potential new targets for drug development, including TNF-α, TGF-β, and GATA1 signaling pathways.
References


Chapter 2

Interplay of GATA1, p53, and TNF-α in DBA Pathogenesis

Abstract
Diamond-Blackfan Anemia is a congenital disorder characterized by defects in erythropoiesis, congenital abnormalities, and predisposition to cancer. Approximately 25% of DBA patients have a mutation in RPS19, which encodes a component of the 40S ribosomal subunit. Upregulation of p53 contributes to the pathogenesis of DBA in humans and zebrafish, but the link between ribosomal protein mutations and erythropoietic defects is not well understood. We found that RPS19 deficiency in hematopoietic progenitor cells leads to decreased GATA1 expression in the erythroid progenitor population and p53-dependent upregulation of TNF-α in non-erythroid cells. The decrease in GATA1 expression was rescued by inhibition of TNF-α or p53. The anemia phenotype in RPS19 deficient zebrafish was reversed by treatment with the TNF-α inhibitor etanercept. Our data reveal that RPS19 deficiency leads to p53-mediated increase in TNF-α and decreased GATA1 expression, suggesting a novel mechanism for the erythroid defects observed in DBA.

Introduction
DBA is characterized by macrocytic anemia, congenital abnormalities affecting the head, limbs, heart, and genitourinary system, and predisposition to cancer (1). Approximately 70% of DBA patients have mutations in genes encoding ribosomal
proteins, most frequently in RPS19 (2). Previous studies in human CD34+ cells, zebrafish, and mice have shown that haploinsufficiency of RPS19 is associated with upregulation of the tumor suppressor p53 (3-5). However, the link between ribosomal protein insufficiency and defects in erythropoiesis is not well understood.

Recently, mutations in the canonical erythroid transcription factor GATA1 have been found in three cases of DBA (6), suggesting a possible association between ribosomal protein deficiency and GATA1 (7). We therefore investigated whether RPS19 deficiency affects GATA1 expression in human hematopoietic progenitor cells and in zebrafish. Our results demonstrated that GATA1 mRNA and protein levels are downregulated in human hematopoietic progenitor CD34+ cells transduced with RPS19 shRNA. Furthermore, we report that downregulation of GATA1 is mediated through activation of p53 with increase in the inflammatory cytokine TNF-α. Treatment of RPS19 deficient zebrafish with the TNF-α inhibitor etanercept rescued the anemia phenotype. Our studies suggest a novel link between GATA1, p53, and TNF-α in the pathogenesis of DBA.

Materials and Methods

Cell Culture. Primary human CD34+ hematopoietic stem/progenitor cells were purified from cord blood (New York Blood Center) or from human fetal liver tissue (Advanced Bioscience Resources), using MACS cell separation (Miltenyi Biotec) and cryopreserved. Upon thawing, cells were cultured in x-Vivo15 media (Lonza) containing 10% FBS, FLT-3 (50ng/mL), TPO (50ng/mL), IL-3 (20ng/mL), IL-6 (20ng/mL), and SCF (50ng/mL).
**Lentiviral Transduction.** Primary CD34+ cells were transduced with lentivirus expressing shRNA against RPS19 (RPS19-1, RPS19-2) or Luciferase shRNA (Luc) at an MOI of 10 after 24 hours in culture. Cells were sorted for GFP 72 hours after infection and harvested for downstream assays as indicated in results. For p53 knockdown experiments, cells were transduced with lentivirus expressing p53 (8) or luciferase shRNA with mCherry and sorted for GFP+mCherry+ cells 72 hours after the initial transduction.

**Compounds.** Nutlin-3 (N6287, Sigma-Aldrich) was diluted in DMSO to a 10mM stock and added to cells at a final concentration of 10µM or 25µM for 24 hours. Etanercept (Amgen) was diluted according to manufacturer instructions, and added to zebrafish at a concentration of 2ng per embryo.

**Colony Assays.** GFP+ sorted hematopoietic cells were seeded in methylcellulose medium containing IL-3, SCF, GM-CSF, and EPO (H4434, StemCell Technologies), in triplicate, with 1000 cells per plate. Erythroid (BFU-E) and myeloid (CFU-G/M) colonies were counted 14 days later.

**qRT-PCR.** RNA was extracted using TRIzol (Life Technologies). RNA was transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). The qRT-PCR reaction was run with iQ™ SYBR Green MasterMix (Bio-Rad) using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). 7SL scRNA (9) was used as an internal control. Fold change of mRNA was calculated using the ΔΔCt method (10).
Western Blot. Antibodies against RPS19 (AB40833; Abcam) and GATA1 (sc-266; Santa Cruz Biotechnology) were used at a 1:200 dilution. β-actin mouse monoclonal IgG2a (A5316; Sigma-Aldrich) was used as a control at a 1:5000 dilution. The target proteins were analyzed using WesternBright Sirius Chemiluminescent Substrate for horseradish peroxidase (Advansta). Densitometry was performed using Image J 1.44v software (http://rsb.info.nih.gov/ij/) to quantify the data.

Zebrafish. Zebrafish were reared at 28.5°C at a 14-hr light/10-hr dark cycle. Embryos were obtained by natural spawning. 30 embryos were injected with rps19-specific morpholino at the one-cell stage as previously described (4), and with 2ng of etanercept at 4-5 hours post fertilization (hpf). RNA was prepared from embryos at 18 hpf for qRT-PCR. At day 3, embryos were stained with o-dianizidine to detect hemoglobin. For Western blot, embryos were collected at 18 hpf, dechorionated with pronase, de-yolked by pipetting in cold Ca-free Ringer solution, washed three times with the same solution, and placed in cold RIPA buffer with proteinase inhibitors. Protein content was detected using BSA assay. 10 µg of protein was loaded per lane and stained with antibodies against gata1 (AnaSpec) and tubulin (Cell Signaling). All zebrafish experiments were performed by Dr. Nadia Danilova (Dr. Shuo Lin’s Laboratory, UCLA).

TNF-α Detection. Cells were sorted for GFP 72 hours post-transduction and culture media was harvested 5 days later. TNF-α was detected with a Human TNF-α High Sensitivity ELISA kit (BMS223HS, eBioscience) according to the manufacturer’s instructions.
Statistics. P values for statistical significance were obtained using an unpaired Student t-test. The data are representative of at least two independent experiments. Threshold for significance was set at p<0.05.

Results
To investigate the link between RPS19 deficiency and erythropoietic defects, we examined whether RPS19 knockdown affects GATA1 expression. Primary human CD34+ cord blood or fetal liver hematopoietic stem and progenitor cells were transduced with lentiviral vectors expressing RPS19 or luciferase control shRNA. On Day 5 after transduction, RPS19 expression decreased by approximately 60% with increased levels of the p53 target, p21, and decreased levels of GATA1 and EPOR (Figure 2.1A). The decrease in GATA1 protein was confirmed by Western blot analysis (Figure 2.1B). RPS19 downregulation resulted in a significant decrease in erythroid colony formation, but a milder defect in myeloid colony formation (Figure 2.1C).

To confirm that GATA1 downregulation in the RPS19 deficient cord blood cells was due to decreased GATA1 transcription and not an artifact of fewer erythroid progenitors in the population, we sorted the RPS19 deficient hematopoietic cells for CD71, a marker of erythroid progenitors. We observed a decrease in GATA1 and EPOR expression in this population of cells (Figure 2.1D), consistent with our hypothesis that GATA1 transcription is decreased in erythroid progenitor cells.
Figure 2.1. GATA1 expression and erythroid colony formation is decreased in RPS19 deficient human and zebrafish hematopoietic progenitor cells. (A) Human CD34+ hematopoietic progenitor cells were infected with lentivirus carrying shRNA against RPS19 or luciferase control, sorted for GFP+ cells after 3 days, and analyzed 3 or 5 days after transduction. mRNA levels of RPS19, GATA1, and EPOR decreased in RPS19 deficient cells, while p21 mRNA increased compared to control 5 days after transduction. (B) GATA1 protein levels decreased in RPS19 deficient fetal liver cells (Scr = scrambled control shRNA; RPS19 = RPS19-2 shRNA) 3 days after transduction. (C) Colony formation in methylcellulose is reduced in cells with RPS19 knockdown. (D) GATA1 and EPOR expression is decreased in CD71+ RPS19 deficient erythroid progenitor cells 5 days after transduction.

Data are representative of at least two independent transduction experiments. *p<0.05, **p<0.01, ***p<0.001.

Bone marrow failure has been reported to be associated with an increased inflammatory response (11). In addition, TNF-α is known to be associated with inhibition
of erythropoiesis (12). Therefore, we hypothesized that RPS19 deficient cells activate pro-inflammatory cytokines, such as TNF-α, which has been shown to inhibit GATA1 activity in TF-1 cells (13). In agreement with this hypothesis, we found that both TNF-α mRNA and protein were upregulated in RPS19 deficient cells (Figures 2.2A and 2.2B). In RPS19-deficient zebrafish, TNF-α mRNA expression was increased 4.2 fold (Figure 2.2C). To investigate whether blocking TNF-α would rescue GATA1 downregulation and the erythroid defects in vivo, we treated rps19 morpholino-injected zebrafish with the TNF-α inhibitor etanercept and observed a significant increase in the number of red blood cells (Figure 2.2D), decreased levels of il-6 and p21 (Figure 2.2E), and restoration of gata1 expression in the morphants (Figure 2.2F).

To determine whether GATA1 downregulation and TNF-α upregulation were p53-dependent, we treated CD34+ cord blood cells for 24 hours with Nutlin-3, a drug that leads to p53 stabilization by blocking MDM2 (14). Nutlin-3 treatment increased expression of the p53 target p21 and TNF-α, and inhibited GATA1 expression (Figure 2.2G). Next, we co-transduced RPS19 deficient CD34+ cord blood cells with lentivirus expressing p53 or luciferase control shRNA and a mCherry selection marker, and analyzed GFP+mCherry+ cells 5 days after infection. We observed a decrease in p21 and TNF-α mRNA levels in cells transduced with p53 shRNA, as well as an increase in GATA1 levels (Figure 2.2H). Additionally, p53 knockdown led to a 2-fold increase in erythroid colony formation from RPS19 deficient cord blood cells (Figure 2.2I). These data show that GATA1 expression is downregulated in RPS19 deficient erythroid progenitors, is at least partly mediated by TNF-α upregulation, and is p53-dependent (Figure 2.2J).
Figure 2.2. Downregulation of GATA1 is mediated through the activation of p53 and TNF-α in human and zebrafish models. (A) CD34+ hematopoietic progenitor cells were transduced with lentiviral vectors carrying shRNA against RPS19 or luciferase control, sorted for GFP+ cells at 72 hours, and analyzed 5 days after infection. TNF-α mRNA is upregulated in RPS19 deficient cells, compared with control. (B) TNF-α protein levels are increased in the media from RPS19 deficient cells, compared with control, as measured by ELISA. Gray and black squares represent two individual measurements for each sample (the measurements overlap, except for the RPS19-3 sample). (C) TNF-α mRNA is upregulated in rps19 MO zebrafish at 18 hpf. (D) Treatment of rps19 MO zebrafish with the TNF-α inhibitor etanercept rescued the erythropoietic (see arrows) and developmental defects. (E) rps19 morphant zebrafish treated with etanercept showed reduced expression of p53 targets p21 and il-6. (F) Treatment with etanercept restored gata1 expression in rps19 MO zebrafish. (G) Stabilization of p53 by Nutlin-3 in CD34+ hematopoietic progenitor cells leads to dose-dependent upregulation of p21 and TNF-α, and downregulation of GATA1. (H) Downregulation of p53 in RPS19 deficient CD34+ cord blood cells reduced p21 and TNF-α expression, while increasing expression of GATA1. (I) Erythroid colony formation is increased in RPS19 deficient CD34+ cord blood cells co-transduced with p53 shRNA, compared to luciferase control shRNA. (J) Model of GATA1 downregulation through the combined effects of p53 and TNF-α in RPS19 deficient cells.

Data are representative of at least two independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Discussion

Based on these data, we conclude that GATA1 expression is downregulated in RPS19 deficient erythroid progenitors, which may contribute to the erythroid-specific lineage defects characteristic of DBA, and that this downregulation can be alleviated by the knockdown of p53 or TNF-α. There are several potential mechanisms by which downregulation of GATA may occur. First, increased expression of p53 in RPS19 deficient cells could directly antagonize the transcriptional activity of GATA1.

Exogenously expressed p53 has been shown to bind to GATA1 directly in K562 cells and inhibit its function (15). Therefore, it is possible that p53 activated in RPS19 deficient cells binds to GATA1, inhibiting its ability to transactivate erythroid-specific genes. This could also lead to reduced GATA1 transcription, since there are GATA1
binding sites in its own promoter (16), forming a negative feedback loop between GATA1 and p53 in RPS19 deficient cells.

Alternatively, GATA1 downregulation could be associated with increased levels of TNF-α, which has been shown to inhibit erythroid differentiation in K562, HEL, and TF1 cells (13, 17). This mechanism would be consistent with dexamethasone, which reduces TNF-α levels, having a beneficial effect in DBA patients (18), as well as with our data showing the effect of etanercept on zebrafish treated with RPS19 morpholino.

Other possible mechanisms that may contribute to reduced GATA1 expression in RPS19 deficient cells include decreased translation of GATA1 mRNA by defective ribosomes, alterations in GATA1 splicing, or downregulation of other, unknown factors upstream of GATA1 (such as RUNX1). These mechanisms remain to be explored.

In summary, our results demonstrate a direct link between RPS19 deficiency and GATA1 downregulation, which could explain the erythroid defects observed in DBA patients. We show that GATA1 downregulation is p53-dependent, and is, at least in part, mediated by increased TNF-α production in RPS19 deficient cells. Additionally, our results suggest that etanercept, and other TNF-α antagonists, may provide a potential approach to treat erythroid defects in patients with DBA.
Supplemental Data

Supplemental Methods

*Flow Cytometry.* Cells were incubated with human FcR binding inhibitor (eBioscience, Inc. #14-9161-73) followed by primary antibodies CD71-APC (BD Biosciences #551373) and biotinylated CD120a (TNFR1) (BD Biosciences #552536). After washing, Streptavidin-PE-Cy7 (BD Biosciences #557598) was added; all incubation times were 20 minutes and on ice. Data were collected on a DxP10 flow cytometer (Cytek) and analyzed using FlowJo Software (v.9.7.2).

**Table 2.1. shRNA Sequences**

<table>
<thead>
<tr>
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**Table 2.2. Primer Sequences for qRT-PCR**

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<td>ATCGGGTGTCCGCACTAAGTT</td>
<td>CAGCACGGGAGTTTGACCT</td>
</tr>
<tr>
<td>RPS19</td>
<td>Human</td>
<td>GCTCTAGAGTTACTGAAAAAGCA</td>
<td>CCCATAGCTTTGGCTATGGAGC</td>
</tr>
<tr>
<td>GATA1</td>
<td>Human</td>
<td>CCTCACCCGCGCCAAGAAG</td>
<td>CCATCTTCCGCATGGTCAG</td>
</tr>
<tr>
<td>EPOR</td>
<td>Human</td>
<td>ATCTGAGGCTCTCCTCAT</td>
<td>AGGCCCTCAAAGTCTGCTTC</td>
</tr>
<tr>
<td>p21</td>
<td>Human</td>
<td>ATCCCCTGTCTCTCCTTT</td>
<td>GCTGGCATGAAGCC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Human</td>
<td>CCACCGAGACCTCTCTCAATC</td>
<td>AGCTGCCCCTCAGCTTGAG</td>
</tr>
<tr>
<td>actin</td>
<td>Zebrafish</td>
<td>TCTCTTCCAGGCTTTTTCTTCT</td>
<td>CTCATCGTACTCTGCTTGCT</td>
</tr>
<tr>
<td>Il-6</td>
<td>Zebrafish</td>
<td>TCTCGTGTAACGACATCAGA</td>
<td>TCATCACGCTGGAGAAGTTG</td>
</tr>
<tr>
<td>p21</td>
<td>Zebrafish</td>
<td>TGAGAACTTACTGCGAGCTTCA</td>
<td>AGCTGCACTTGCTCTGAGC</td>
</tr>
<tr>
<td>tnf-α</td>
<td>Zebrafish</td>
<td>GGTGTTTGGGATCTTTTGG</td>
<td>CAAGCCACGCTGAAGAAAGG</td>
</tr>
</tbody>
</table>
Supplemental Figures

Figure 2.3. Effects of TNF-α and etanercept on normal human CD34+ cord blood cells. Human CD34+ cord blood cells were cultured in media, and after 5 days, were treated with 100ng TNF-α, 10µg etanercept, or 100ng TNF-α and 10µg etanercept. 24 hours later, the cells were collected for RNA. qRT-PCR showed that addition of TNF-α resulted in decreased GATA1 expression and increased p21 expression in the cells. This effect was rescued by etanercept.

Figure 2.4. TNFR1 expression is increased on the surface of CD71+ RPS19 deficient cord blood cells. Human CD34+ cord blood cells were transduced with lentivirus carrying shRNA against RPS19 or luciferase shRNA control. After 6 days in culture, cells were analyzed for CD71 and TNFR1 expression by flow cytometry. TNFR1 expression increased on the surface of RPS19 deficient CD71+ cells compared with luciferase control, but did not change in the CD71- population.
References


Chapter 3
Identification of Novel Pathways in DBA through RNA-Seq

Abstract
Diamond-Blackfan Anemia (DBA) is a congenital bone marrow failure syndrome, generally caused by a mutation in one of many ribosomal proteins, most frequently RPS19. Multiple studies have linked DBA with upregulation of the p53 pathway, triggered by ribosomal stress. However, little is known about alternative pathways involved in DBA pathogenesis. To answer this question, we used next-generation sequencing on the Illumina HiSeq platform to identify differentially expressed genes between RPS19 deficient and control primary hematopoietic progenitor cells. Our study identified 569 differentially expressed genes in our samples, which can be explored as potential targets for DBA diagnosis and therapeutics. Pathway and gene ontology analysis revealed enrichment of differentially expressed transcripts in inflammation and signaling pathways including p53, WNT, TGF-β, PI3 kinase, and others. Moreover, our analysis revealed suppression of MYC in RPS19 deficient cells, which we confirmed by qRT-PCR and further explored in our biological system.

Introduction
DBA is a congenital bone marrow failure syndrome that is commonly associated with mutations in ribosomal proteins, such as RPS19 (1), and upregulation of the p53 pathway as a result of ribosomal stress (2). In the classical model of DBA pathogenesis, mutations in ribosomal protein genes lead to an imbalance of ribosomal assembly
intermediates in the nucleolus, triggering nucleolar stress (3, 4). The free ribosomal proteins spill out into the nucleus, binding to the p53 inhibitor MDM2 and stabilizing p53 expression, which leads to cell cycle arrest and apoptosis in the cell (5). Because p53 is not a good target for drug development due to its critical function as a tumor suppressor, a greater understanding of the pathways that lead to DBA is needed to develop new therapies for the disease.

In this study, we use next-generation RNA sequencing on the Illumina HiSeq platform to compare gene expression changes between RPS19 deficient hematopoietic progenitor cells and controls. Among the 569 differentially expressed genes detected in our analysis was MYC, a global regulator of a multitude of processes in the cell, including transcription, translation, regulation of chromatin structure, DNA replication, and ribosome biogenesis (6). It is the latter function of MYC that made it an interesting target to explore in DBA pathogenesis.

MYC controls several aspects of ribosome biogenesis. It directly regulates transcription of rRNA through RNA pol I and RNA pol III (7, 8), and can upregulate transcription of ribosomal proteins through RNA pol II (9). Additionally, it regulates several proteins required for rRNA processing and ribosomal subunit transport, including nucleolar protein 56 (NOP56), block of proliferation 1 (BOP1), fibrillarin (FBL), dyskerin (DKC1), nucleolin (NCL), and nucleophosmin (NPM1) (10). MYC also regulates mRNA translation though transcription of initiation factors, including eIF4E, eIF2α, eIF4A1, and eIF4G1 (11). Finally, MYC forms a negative feedback loop with the ribosomal protein L11, which can bind to MYC and repress its transcriptional function to suppress ribosome biogenesis in the cell (12). Therefore, upon discovering MYC to be
downregulated in RPS19 deficient cells by high-throughput RNA-seq, we decided to further explore its role in DBA pathogenesis. We hypothesized that MYC is downregulated in DBA by a similar mechanism as MDM2, via binding of RPL11, and that this downregulation contributes to the growth arrest and erythroid defects observed in DBA.

**Materials and Methods**

*Cell culture.* Frozen primary CD34+ human fetal liver cells were obtained from the UCLA Center for AIDS Research (CFAR) Gene and Cellular Therapy Core. After thawing, cells were cultured in x-Vivo15 media (Lonza, #04-744Q) containing 10% FBS (Life Technologies), 0.45mg/mL Zosyn (UCLA Pharmacy), 0.25µg/mL Fungizone (Life Technologies, #15290), 50ng/mL Flt-3/Flk-2 (Sigma, #F3422), 50ng/mL TPO (R&D Systems, #288-TP), 20ng/mL IL-3 (R&D Systems #203-IL), IL-6 (R&D Systems, #206-IL), and 50ng/mL SCF (R&D Systems, #255-SC).

*Lentiviral transduction.* Primary CD34+ human fetal liver cells were transduced after 24 hours in culture with lentivirus expressing shRNA against RPS19 (RPS19-A, RPS19-B) or scrambled control (Scr) shRNA (constructs kindly provided by Dr. Stefan Karlsson, Lund University, Sweden). Cells were sorted for GFP after 72 hours and harvested for downstream assays. The MYC overexpression vector was created by inserting the MYC coding region from the pLenti-cMyc vector (kindly provided by Dr. Dean Felsher, Stanford University) into the pCDU-PGK-x-CMV-mCherry lentiviral vector (created in
our lab by Dr. Hee-Don Chae), under the PGK promoter. Empty pCDU-PGK-x-CMV-
mCherry vector was used as control.

**RNA-Seq.** Primary CD34+ human fetal liver cells transduced with shRNA against
RPS19 or scrambled control were sorted for GFP+ cells and harvested for RNA 72
hours after transduction. Total RNA was extracted using TRIzol (Life Technologies,
#15596018) and treated with Turbo DNase (Life Technologies, AM1907) before being
submitted to the UCLA DNA Microarray Core. At the Microarray Core, RNA was
evaluated for quality with the 2100 Bioanalyzer (Agilent Technologies). The cDNA
library was generated using the TruSeq Total RNA Sample Preparation Kit (Illumina,
Inc.) and sequenced on the Illumina HiSeq 2000 (Illumina, Inc.)

**RNA-Seq Data Analysis.** Alignment of the RNA-seq reads was performed with TopHat
(v1.4.1.) to human genome (hg19) and Ensembl _homo sapiens_ GTF file
(Homo_sapiens.GRCh37.61.gtf). Cuffdiff from Cufflinks (v1.3.0) was used to find
differential expression between control and RPS19 deficient samples (13). A parameter
of <0.05 False Discovery Rate (FDR) was used to determine significance. Genes that
were significantly up- or down-regulated in both RPS19 shRNA samples compared with
scrambled control were selected for further analysis. Pathway analysis was carried out
using the PANTHER (protein annotation through evolutionary relationship) classification
system gene list analysis tool (http://www.pantherdb.org/) (14-16). Preprocessing,
alignment, and differential expression analysis were performed at the UCLA DNA
Microarray Core; pathway analysis was performed by the end user.
**qRT-PCR.** Total RNA was extracted using TRIzol (Life Technologies, #15596018) and transcribed into cDNA using the SuperScript® First-Strand Synthesis System (Life Technologies, #18080-051). The qRT-PCR reaction was run with SYBR® Green PCR Master Mix (Life Technologies, #4309155) using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). Actin was used as the endogenous control. Fold change of mRNA was calculated using the ΔΔCt method.

**Colony Assays.** Sorted hematopoietic cells were seeded in methylcellulose medium containing IL-3, SCF, GM-CSF, and EPO (H4434, StemCell Technologies, #04444), in duplicate, with 1000 cells per plate. Colonies were counted 14 days later, and distinguished by their color and morphology.

**Liquid Erythroid Culture.** To differentiate cells towards the erythroid lineage, sorted hematopoietic cells were cultured in media containing 3U/mL EPO for one week. To access erythroid differentiation, cells were stained with antibodies against CD71-PE (BD Pharmigen™, #555537) and Glycophorin A-APC (BD Pharmigen™, #551336). Cells were analyzed on a FACSCalibur cytometer (BD Biosciences), and data were analyzed using FlowJo software (v7.6).

**Statistics.** P values for statistical significance were obtained using an unpaired Student t-test. Threshold for significance was set at p<0.05.
Results

RNA-Seq

To identify novel pathways that may be involved in DBA pathogenesis, we measured RNA expression profiles in primary CD34+ fetal liver cells transduced with shRNA against RPS19 or scrambled control shRNA, using high-throughput RNA sequencing (RNA-seq) on the Illumina HiSeq platform. Using a parameter of FDR < 0.05, we identified 569 differentially expressed genes in our samples, with 210 genes upregulated and 359 genes downregulated in the RPS19 deficient cells compared with control cells. Hierarchical clustering using this data set discriminated the cells transduced with two different shRNA sequences against RPS19 (RPS19-A, RPS19-B) from cells transduced with the scrambled shRNA control (Figure 3.1).
Figure 3.1. Heat map showing clustering of differentially expressed genes in RPS19 deficient hematopoietic progenitor cells. Human CD34+ fetal liver cells were transduced with shRNA against RPS19 (RPS19-A, RPS19-B) or scrambled shRNA control (Control). Change in expression is reported as log fold change of fragments per kilobase of exon per million (FPKM), represented by a color gradient, as shown on the right. Red represents genes with higher read counts (higher expression), while light yellow represents genes with lower read counts (lower expression).

Gene ontology (GO) analysis using the PANTHER classification tool revealed enrichment of differentially expressed genes in metabolic, cellular, cell communication, cell cycle, transport, developmental, immune system, and other biological processes.
Figure 3.2. Gene ontology classification of biological processes dysregulated in RPS19 deficient cells. 328 of 569 differentially expressed genes were classified based on their role in various biological processes.

Pathway analysis of the differentially expressed genes in RPS19 deficient cells identifies inflammation mediated by chemokine and cytokine signaling, p53, WNT signaling, TGF-β signaling, PI3 kinase, interleukin signaling, Huntington disease, insulin/IGF, p53 feedback, apoptosis, PDGF, integrin, heterotrimeric G-protein, RAS, and other signaling pathways to be dysregulated in RPS19 deficient cells (Table 3.1).
Table 3.1. Top pathway hits for differentially expressed genes between RPS19 deficient and control CD34+ fetal liver cells.

<table>
<thead>
<tr>
<th>Pathway</th>
<th># Genes</th>
<th>% Total Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation mediated by chemokine and cytokine signaling pathway (P00031)</td>
<td>17</td>
<td>3.20%</td>
</tr>
<tr>
<td>p53 pathway (P00059)</td>
<td>15</td>
<td>2.80%</td>
</tr>
<tr>
<td>Wnt signaling pathway (P00057)</td>
<td>11</td>
<td>2.10%</td>
</tr>
<tr>
<td>TGF-beta signaling pathway (P00052)</td>
<td>9</td>
<td>1.70%</td>
</tr>
<tr>
<td>PI3 kinase pathway (P00048)</td>
<td>9</td>
<td>1.70%</td>
</tr>
<tr>
<td>Interleukin signaling pathway (P00036)</td>
<td>9</td>
<td>1.70%</td>
</tr>
<tr>
<td>Huntington disease (P00029)</td>
<td>9</td>
<td>1.70%</td>
</tr>
<tr>
<td>Insulin/IGF pathway-protein kinase B signaling cascade (P00033)</td>
<td>8</td>
<td>1.50%</td>
</tr>
<tr>
<td>p53 pathway feedback loops 2 (P04398)</td>
<td>8</td>
<td>1.50%</td>
</tr>
<tr>
<td>Apoptosis signaling pathway (P00006)</td>
<td>7</td>
<td>1.30%</td>
</tr>
<tr>
<td>PDGF signaling pathway (P00047)</td>
<td>7</td>
<td>1.30%</td>
</tr>
<tr>
<td>Integrin signaling pathway (P00034)</td>
<td>7</td>
<td>1.30%</td>
</tr>
<tr>
<td>Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)</td>
<td>7</td>
<td>1.30%</td>
</tr>
<tr>
<td>Ras Pathway (P04393)</td>
<td>5</td>
<td>0.90%</td>
</tr>
</tbody>
</table>

Role of MYC in DBA Pathogenesis

Among the genes differentially expressed in our RNA-seq were the transcription factor MYC and several of its targets and interacting partners (MAX, TRRAP, MYCBP2, MGA, EP300, EP400, EIF4G1). Because MYC, like the p53 regulator MDM2, has been shown to bind directly to RPL11 (12), we hypothesized that the MYC network would be disrupted in RPS19 deficient cells by a mechanism similar to p53 activation, contributing to defective growth and erythropoiesis (Figure 3.3).
Figure 3.3. Model of MYC pathway dysregulation following ribosomal stress. Ribosomal protein insufficiency causes an imbalance of ribosomal assembly intermediates in the nucleolus, which spill out into the nucleus and bind to MYC, inhibiting its function. This contributes to further disruption of ribosome biogenesis, cell cycle arrest, apoptosis, and differentiation defects.

To test this hypothesis, we first set out to validate the expression of MYC pathway genes differentially expressed in the RNA-seq by qRT-PCR. The average expression change of select genes by qRT-PCR showed the same trend as the RNA-seq, but only changes in RPS19, MYC and EIF4G1 expression were significant, with p<0.05 (Table 3.2).
Table 3.2. Validation results of select gene expression by qRT-PCR. Fold change represents fold change between expression in RPS19 deficient cells and cells transduced with scrambled shRNA control. *p<0.05, **p<0.01, ***p<0.001, ns = not significant.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Average Fold Change by RNA-Seq</th>
<th>Average Fold Change by qRT-PCR</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPS19</td>
<td>0.25</td>
<td>0.20</td>
<td>***</td>
</tr>
<tr>
<td>MYC</td>
<td>0.77</td>
<td>0.55</td>
<td>**</td>
</tr>
<tr>
<td>EIF4G1</td>
<td>0.65</td>
<td>0.65</td>
<td>*</td>
</tr>
<tr>
<td>MAX</td>
<td>1.4 (up)</td>
<td>1.1 (up)</td>
<td>ns</td>
</tr>
<tr>
<td>TRRAP</td>
<td>0.60</td>
<td>0.85</td>
<td>ns</td>
</tr>
<tr>
<td>MYCBP2</td>
<td>0.62</td>
<td>0.83</td>
<td>ns</td>
</tr>
<tr>
<td>MGA</td>
<td>0.63</td>
<td>0.81</td>
<td>ns</td>
</tr>
<tr>
<td>EP300</td>
<td>0.55</td>
<td>0.88</td>
<td>ns</td>
</tr>
<tr>
<td>EP400</td>
<td>0.54</td>
<td>0.73</td>
<td>ns</td>
</tr>
</tbody>
</table>

Because MYC downregulation in RPS19 deficient cells was validated with qRT-PCR, we decided to further evaluate its role in DBA pathogenesis. To that end, we created a lentiviral vector carrying an mCherry marker to restore MYC expression in RPS19 deficient cells. We transduced primary CD34+ fetal liver cells with lentivirus carrying control GFP and mCherry vectors (Control), MYC expression vector with GFP control (Myc), RPS19 shRNA vector with mCherry control (RPS19), or MYC expression vector together with RPS19 shRNA (Myc+RPS19) and sorted the cells for the GFP+mCherry+ population 72 hours after transduction. RPS19 levels were decreased in both cells transduced with RPS19 shRNA, and cells co-transduced with RPS19 shRNA and MYC vector (Figure 3.4A). Interestingly, while MYC expression was fully restored in RPS19 deficient cells transduced with the MYC expression construct (Figure 3.4B), p21 expression remained high (Figure 3.4C), despite MYC’s noted ability to repress p21 expression (17). Additionally, restoration of MYC expression in RPS19 deficient cells
was not sufficient to rescue their erythropoietic defects, as measured by CD71 and Glycophorin A expression in erythroid liquid culture (Figure 3.4D), or their growth in methylcellulose colony assays (Figure 3.4E).

Figure 3.4. Overexpression of MYC is not sufficient to rescue the erythroid defects observed in RPS19 deficient cells. CD34+ fetal liver cells were transduced with lentivirus carrying control GFP and mCherry vectors (Control), a GFP control vector with a MYC mCherry vector (Myc), GFP RPS19 shRNA vector with an mCherry control vector (RPS19), or GFP RPS19 shRNA vector with a MYC mCherry vector (Myc+RPS19). GFP+mCherry+ cells were isolated by FACS 72 hours after transduction. (A) Cells transduced with RPS19 shRNA lentivirus showed reduced expression of RPS19. (B) Transduction of RPS19 deficient cells with the MYC expression construct restores MYC expression to levels seen in the control cells. (C) p21 overexpression in RPS19 cells is not mitigated by the addition of MYC. (D) MYC overexpression is not sufficient to rescue the erythroid defects seen in RPS19 deficient cells, as measured by flow cytometry for erythroid markers CD71 and Glycophorin A.
Overexpression of MYC in RPS19 deficient CD34+ fetal liver cells did not rescue their colony formation in methylcellulose. *p<0.05, **p<0.01, ***p<0.001.

Discussion
In this study, we used high-throughput RNA sequencing to identify novel pathways dysregulated in RPS19 deficient cells. RNA-seq revealed 569 differentially expressed genes between RPS19 deficient and control hematopoietic progenitor cells, which play a role in multiple biological processes including metabolism, cellular processes, cell communication, cell cycle regulation, transport, developmental processes, immune system function, and others. We identified the top five pathways dysregulated in RPS19 deficient cells as inflammation mediated by chemokine and cytokine signaling, p53, WNT signaling, TGF-β signaling, and PI3 kinase.

While the upregulation of the p53 pathway has been thoroughly described in DBA and served to validate our experimental system, the identification of an inflammatory pathway as the top hit on our RNA-seq was less expected, given that DBA is not classically considered an immune-mediated disease (1). However, this result is consistent with our previous findings of inflammation contributing to the erythroid defects seen in RPS19 deficient cells (Chapter 2). Therefore, this pathway is a good target for further exploration and lends evidence to the novel concept that inflammation plays a role in the pathogenesis of DBA.

Another target gene identified in the RNA-seq was MYC. Given MYC’s role in ribosome biogenesis (6), we decided to further explore its function in RPS19 deficient cells. We hypothesized that nucleolar stress in the cell leads to the release of ribosomal assembly intermediates, including RPL11, into the nucleus, binding MYC and inhibiting
its transcriptional function. We also hypothesized that restoring MYC expression to normal levels in RPS19 deficient cells would alleviate their erythroid phenotype, reduce p21 expression, and improve their growth in colony assays.

Contrary to our hypothesis, restoration of MYC expression in RPS19 deficient cells did not downregulate p21 or rescue their erythroid defects. There may be a number of reasons why this could be the case. First, MYC may be regulated post-translationally in RPS19 deficient cells, so restoration of mRNA expression may not be sufficient to have an effect on their phenotype. In that case, another approach should be used to activate MYC protein function in the cells. Alternatively, MYC downregulation may be a symptom of reduced growth and protein synthesis in RPS19 cells, rather than a cause. Also, MYC downregulation is required for terminal erythroid differentiation to occur (18), which may explain why constitutive expression of MYC failed to rescue erythroid defects in RPS19 deficient cells. In this case, transient overexpression of MYC may be more appropriate to explore its role in DBA.

In conclusion, our study identified several novel pathways dysregulated in RPS19 deficient cells, including inflammation mediated by cytokine and chemokine signaling, WNT signaling, TGF-β signaling, and PI3 kinase signaling. The role of these pathways in DBA pathogenesis should be explored further to find potential drug targets for the treatment of DBA.
References


transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks, *Nat Protoc* 7, 562-578.


Chapter 4
Role of MicroRNA in DBA Pathogenesis

Abstract
MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs that regulate gene expression by binding sequences on their target mRNAs. Recent studies have identified numerous miRNAs that function in normal erythropoiesis, as well as erythropoietic disorders such as sickle cell disease and MDS. However, the role of miRNAs in the pathogenesis of Diamond-Blackfan anemia (DBA) remains unknown. In this study, we used high-throughput microRNA sequencing (miRNA-seq) to analyze miRNA expression in RPS19 deficient human CD34+ hematopoietic progenitor cells. We identified 30 differentially expressed miRNAs in our samples compared with control cells, including members of the miR-34 family (miR-34a, miR-34b, and miR-34c). We went on to further characterize the role of miR-34a in RPS19 deficient cells and in normal erythropoiesis. Our results demonstrate that multiple miRNAs are dysregulated in RPS19 deficient cells and can potentially be important in the pathogenesis and treatment of DBA.

Introduction
MiRNAs are small, non-coding, 20-23 nucleotide fragments of RNA, typically transcribed from non-protein-coding genes or intronic regions of DNA. After transcription by RNA polymerase II or RNA polymerase III, the primary miRNA transcripts (pri-miRNA) are cleaved by the microprocessor complex Drosha-DGCR8 in the nucleus to
form precursor hairpin RNA (pre-miRNA), which is exported out of the nucleus by an Exportin-5-Ran-GTP complex and further cleaved by Dicer-TRBP to form the miRNA duplex. The 3’ strand of the cleaved miRNA duplex is usually degraded, while the 5’ strand is incorporated into the RISC complex, where it can repress target genes through mRNA cleavage, translation repression, or mRNA deadenylation (1).

Several miRNAs have been shown to function in erythropoiesis and erythropoietic disorders (summarized in Table 4.1). The first miRNA specifically identified in erythrocytes was miR-451 (2), which shares a locus with miR-144 and is regulated by GATA1 (3). Silencing of miR-451 in zebrafish leads to delayed erythroid maturation due to persistent expression of the miR-451 target gata2 (4), while silencing of miR-144 leads to persistent embryonic α-globin expression with increased levels of the miR-144 target klf4 (5). In CD34+ cord blood cells, downregulation of miR-221, miR-222, miR-223, and miR-24 is essential for terminal erythroid differentiation to occur (6-8). MiR-221 and miR-222 target KIT, which codes for the receptor of stem cell factor (SCF) ligand, and is normally repressed in terminal erythropoiesis (6). MiR-223 targets LMO2, and its upregulation leads to an increase in immature erythroid cells (8). Ectopic expression of miR-24, which targets activin type I receptor ACVR1B (ALK4), in CD34+ cells led to a 30% decrease in both BFU-E and CFU-E colony formation, while downregulation of miR-24 had the opposite effect (7).

A number of miRNAs affect erythropoiesis by targeting MYB, an essential erythroid transcription factor, which was also found to be expressed at lower levels in the bone marrow of DBA patients (9). These miRNAs include miR-15a, miR-16-1, miR-150, and miR-34a (10-12). Finally, miRNAs also play a role in final step of erythropoiesis:
chromatin condensation and enucleation. In mouse fetal liver progenitors, overexpression of miR-191 blocked erythroid enucleation, possibly through downregulation of its targets *Riok3* and *Mxi1*, which are required for chromatin condensation and enucleation to occur (13).

MiRNAs are also involved in numerous diseases including cancer (14), autoimmune disorders (15), and erythroid dysfunctions such as sickle cell disease (SSD) (16), and myelodysplastic syndrome (MDS) (17). Patients with SSD show downregulation of miR-320, which targets the receptor CD71 (*TFRC*) (16), while miR-145, which targets *FLI1*, is one of the genes deleted in 5q- syndrome, a type of MDS associated with a deleted 5q- chromosomal region that also contains the gene encoding ribosomal protein RPS14 (17). Recently, a study by Votavova et. al. (18), has also identified miR-34a to be upregulated in 5q- patients. However, the role of miRNAs in DBA pathogenesis remains unknown.
Table 4.1. MicroRNA in normal and defective erythropoiesis.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Experimental System</th>
<th>Function/Significance</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-15a</td>
<td>Human cord blood CD34+ cells</td>
<td>Identified as potential candidates causing elevated fetal hemoglobin expression</td>
<td>MYB</td>
<td>(10)</td>
</tr>
<tr>
<td>miR-16-1</td>
<td>Human cord blood CD34+ Cells and K562 cell line</td>
<td>Down-regulation required for terminal erythroid differentiation</td>
<td>ALK4</td>
<td>(7)</td>
</tr>
<tr>
<td>miR-24</td>
<td>K562 cell line, CD34+ cells from 5q- patients</td>
<td>Contributes to megakaryocytic differentiation; overexpressed in 5q- patients</td>
<td>MYB</td>
<td>(12, 18)</td>
</tr>
<tr>
<td>miR-34a</td>
<td>Zebrafish embryo</td>
<td>Required for erythropoiesis and α-globin gene expression; regulated by Gata1</td>
<td>klfd, gata2</td>
<td>(4, 5)</td>
</tr>
<tr>
<td>miR-144</td>
<td>CD34+ cells from 5q- patients</td>
<td>Gene deleted in 5q- patients</td>
<td>FLI1</td>
<td>(17)</td>
</tr>
<tr>
<td>miR-145</td>
<td>Human cord blood megakaryocyte-erythrocyte and megakaryocyte progenitors</td>
<td>Induces differentiation toward the megakaryocytic lineage at expense of the erythroid lineage</td>
<td>MYB</td>
<td>(11)</td>
</tr>
<tr>
<td>miR-191</td>
<td>Mouse primary erythrocyte progenitors</td>
<td>Down-regulation required for erythroblast chromatin condensation and enucleation</td>
<td>Riok3, Mxi1</td>
<td>(13)</td>
</tr>
<tr>
<td>miR-221</td>
<td>Human cord blood CD34+ cells</td>
<td>Down-regulation required for terminal erythroid differentiation</td>
<td>KIT</td>
<td>(6)</td>
</tr>
<tr>
<td>miR-222</td>
<td>Human cord blood CD34+ cells</td>
<td>Down-regulation required for terminal erythroid differentiation</td>
<td>LMO2</td>
<td>(8)</td>
</tr>
<tr>
<td>miR-320</td>
<td>Erythrocytes from patients with homozygous sickle cell disease (SSD)</td>
<td>Required for terminal erythroid differentiation; downregulated in patients with SSD</td>
<td>TFRC</td>
<td>(16)</td>
</tr>
</tbody>
</table>

Adapted with modifications from Hattangadi, et. al., (19).

In his study, we used primary human CD34+ cells transduced with RPS19 shRNA to identify and characterize miRNAs that may play a role in DBA pathogenesis. We identified miR-34 and miR-30 families to be upregulated in RPS19 deficient cells compared with controls, and went on to further characterize the role of miR-34a in erythropoiesis and the pathogenesis of DBA.
Materials and Methods

Cell culture. Frozen primary CD34+ human fetal liver cells were obtained from the UCLA Center for AIDS Research (CFAR) Gene and Cellular Therapy Core. After thawing, cells were cultured in x-Vivo15 media (Lonza, #04-744Q) containing 10% FBS (Life Technologies), 0.45mg/mL Zosyn (UCLA Pharmacy), 0.25µg/mL Fungizone (Life Technologies, #15290), 50ng/mL Flt-3/Flk-2 (Sigma, #F3422), 50ng/mL TPO (R&D Systems, #288-TP), 20ng/mL IL-3 (R&D Systems #203-IL), IL-6 (R&D Systems, #206-IL), and 50ng/mL SCF (R&D Systems, #255-SC).

Lentiviral transduction. Primary CD34+ human fetal liver cells were transduced after 24 hours in culture with lentivirus expressing shRNA against RPS19 (RPS19-1, RPS19-2) or Luciferase shRNA (Luc) at an MOI of 10. Cells were sorted for GFP after 72 hours and harvested for downstream assays. The miR-34a overexpression vector, pLenti-III-mir-GFP, was obtained from Applied Biological Materials Inc. (#mh10481). The pLenti-III-GFP-Blank vector (Applied Biological Materials Inc., #m001) was used as control. For miR-34a downregulation experiments, a lentiviral plasmid expressing a miR-34a “sponge” sequence (kindly provided by Dr. Dinesh Rao, UCLA) was modified to express mCherry instead of GFP, with empty mCherry vector used as control.

MicroRNA-Seq. Primary CD34+ human fetal liver cells were sorted for GFP+ cells and harvested for RNA 72 hours after transduction. Total RNA was extracted using TRIzol (Life Technologies, #15596018) and treated with Turbo DNAse (Life Technologies, AM1907) before being submitted to the UCLA DNA Microarray Core. At the Microarray
Core, RNA was evaluated for quality with the 2100 Bioanalyzer (Agilent Technologies). The cDNA library was generated using the TruSeq Small RNA Sample Preparation Kit (Illumina, Inc.) and sequenced on the Illumina HiSeq 2000 (Illumina, Inc.)

**MicroRNA-Seq Data Analysis.** Preprocessing, alignment, and miRNA quantification were carried out using miRDeep2 software (20). Differential expression analysis was performed with DESeq (v1.12.0), which tests for differential expression based on a model using negative binomial distribution for count data from high-throughput sequencing assays. Preprocessing, alignment, miRNA quantification, and differential expression analysis were performed at the UCLA DNA Microarray Core.

**qRT-PCR.** Total RNA was extracted using TRIzol (Life Technologies, #15596018). For mRNA analysis, RNA was transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, #170-8891). The qRT-PCR reaction was run with iQ™ SYBR Green MasterMix (Bio-Rad, #170-8887) using the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). Actin and 7SL scRNA were used as endogenous controls. Fold change of mRNA was calculated using the ΔΔC_t method. For miRNA analysis, 50ng of total RNA were reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies, #4366596) and individual microRNA primers provided as part of the TaqMan® MicroRNA Assays (Life Technologies, #4427975). The qRT-PCR reaction was run with TaqMan® Universal PCR Master Mix II (Life Technologies, #4324018) on the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). RNU6B was used as an endogenous control for miRNA experiments.
Colony Assays. GFP+ sorted hematopoietic cells were seeded in methylcellulose medium containing IL-3, SCF, GM-CSF, and EPO (H4434, StemCell Technologies, #04444), in duplicate, with 1000 cells per plate. Erythroid (BFU-E) and myeloid (CFU-G/M) colonies were counted 14 days later.

Western Blot. Protein was isolated from DBA patient lymphocyte cell lines (LCLs) by resuspending the cells in RIPA buffer with proteinase and phosphatase inhibitors, flash-freezing in liquid nitrogen, and spinning down 10,000g for 15 minutes in a tabletop centrifuge. Supernatant was collected and the protein was quantified using a BCA Protein Assay Kit (Thermo Scientific, #23227). 40µg of protein was loaded in a 12% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was blocked with 5% milk for one hour and probed with antibodies against p53 (Santa Cruz, #sc-126) at a dilution of 1:500 in 5% milk, and c-Myc (Cell Signaling, #9402) at a dilution of 1:1000 in 5% milk overnight at 4°C. For endogenous control, β-actin (Sigma-Aldrich, #A5316) was used at a 1:5000 dilution. The membrane was probed with goat anti-rabbit (Santa Cruz, #sc-2004) or goat anti-mouse (Santa Cruz, #sc-2005) secondary at a dilution of 1:5000 for one hour at room temperature, and target proteins were analyzed using enhanced chemiluminescence (ECL) substrate.

Statistics. P values for statistical significance were obtained using an unpaired Student t-test. Threshold for significance was set at p<0.05.
Results

MicroRNA-Seq

To identify novel miRNAs that may play a role in DBA pathogenesis, we measured miRNA expression profiles in primary CD34+ fetal liver cells transduced with shRNA against RPS19 (RPS19-1, RPS19-2), or luciferase shRNA controls (Luc-1, Luc-2) using high-throughput microRNA sequencing (miRNA-seq). After sorting the data based on unadjusted p-values calculated by DESeq, we identified 30 differentially expressed miRNAs in RPS19 knockdown fetal liver cells compared with controls (Figures 4.1 and 4.2). Among the miRNAs upregulated in RPS19 deficient cells were several members of the pro-apoptotic miR-34 family (miR-34a, miR-34b, and miR-34c), the miR-30 family (miR-30a, miR-30c, miR-30d, and miR-30e), miR-186, miR-320b, miR-196b, miR-148b, miR-221, miR-145, miR-4521, miR-154, miR-190b, miR-5189, miR-5091, miR-513b, and miR-769 (Figure 4.1). Downregulated miRNAs included miR-486, miR-99b, miR-411, miR-98, miR-144, miR-548I, miR-6718, miR-122, miR-548an, and miR-409 (Figure 4.2).
Figure 4.1. miRNAs enriched in RPS19 deficient fetal liver cells. Heat map from the miRNA-seq showing miRNAs enriched in RPS19 deficient CD34+ fetal liver cells compared with Luciferase shRNA control 72 hours after transduction.
**Figure 4.2. miRNAs downregulated in RPS19 deficient fetal liver cells.** Heat map from the miRNA-seq showing miRNAs downregulated in RPS19 deficient CD34+ fetal liver cells compared with Luciferase shRNA control 72 hours after transduction.

**Role of miR-34a in DBA**

Based on the data from the miRNA-seq, we decided to further explore the role of the miR-34 family in DBA pathogenesis. The miR-34 family genes are transcribed from two loci: one containing miR-34a, and the other containing miR-34b and miR-34c (21). Both genes are direct targets of p53 (21), and overexpression of miR-34a in K562 cells has been shown to inhibit cell proliferation, arrest cells in the G1 phase, and promote megakaryocytic differentiation (12). Targets of miR-34a include MYC and MYB (12, 21), which are both essential for normal erythropoiesis and have been found to be expressed at lower levels in bone marrow cells of DBA patients (9). We therefore hypothesized that upregulation of miR-34a in RPS19 deficient hematopoietic progenitors contributes to their inability to properly undergo erythropoiesis.

We first validated the results of the miRNA-seq by qRT-PCR and confirmed upregulation of miR-34a and miR-34b in RPS19 deficient fetal liver cells (Figure 4.3). Because the baseline expression of miR-34a was much higher in the cells than that of miR-34b, we focused on miR-34a for further studies.
Figure 4.3. miR-34a and miR-34b are overexpressed in RPS19 deficient primary human fetal liver cells. CD34+ fetal liver cells were transduced with lentivirus carrying two different shRNAs against RPS19, or against Luciferase as control. Total RNA was collected 72 hours after transduction. (A) RPS19 mRNA levels are decreased in primary human fetal liver cells transduced with shRNA against RPS19, compared with Luciferase shRNA controls. (B) miR-34a and (C) miR-34b are upregulated in RPS19 deficient fetal liver cells compared with Luciferase shRNA controls. qRT-PCR. *p<0.05, **p<0.01, ***p<0.001.

Next, we analyzed the expression of known miR-34a targets in our cells, c-MYB and c-MYC, and confirmed that they are down-regulated in primary fetal liver cells transduced with shRNA against RPS19, compared with control (Figure 4.4).

Figure 4.4. RPS19 knockdown leads to decreased expression of miR-34a targets c-MYB and c-MYC in CD34+ fetal liver cells. CD34+ fetal liver cells were transduced with lentiviral vectors carrying shRNA against RPS19 (RPS19-1, RPS19-2) or scrambled vector (Control). Total RNA was collected 72 hours after transduction.
Targets of miR-34a, (A) c-MYB and (B) c-MYC were downregulated in RPS19 deficient cells compared with control. qRT-PCR. *p<0.05, **p<0.01, ***p<0.001.

To test whether miR-34a upregulation was relevant in DBA, we tested five different lymphoblastoid cell lines (LCLs) derived from DBA patients (Figure 4.5A) for miR-34a expression. MiR-34a was significantly overexpressed in three out of five patient cell lines (287, 288, 320), with a milder, but not statistically significant increase in the other two cell lines (279, 316) compared with controls (Figure 4.5B). All five patients showed increased levels of the miR-34a transcriptional activator p53, and decreased levels of the miR-34a target c-MYC (Figure 4.5C).

<table>
<thead>
<tr>
<th>DBA Patients</th>
<th>Mutation</th>
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<tbody>
<tr>
<td>279</td>
<td>RPS19, intr. 5, donor splice site mutation, IVS5 +1g &gt; t</td>
</tr>
<tr>
<td>287</td>
<td>RPS19, intr. 2, acceptor splice site mutation, IVS2 -2a &gt; c</td>
</tr>
<tr>
<td>288</td>
<td>RPS19, intr. 5, donor splice site mutation, IVS5 +1t &gt; c</td>
</tr>
<tr>
<td>316</td>
<td>RPS19, ex.2, c.1A &gt; G p. Met1Val</td>
</tr>
<tr>
<td>320</td>
<td>RPS19, intr. 3, donor splice site mutation, IVS3 +1g &gt; t</td>
</tr>
</tbody>
</table>

**Figure 4.5. miR-34a is upregulated in LCLs from DBA patients.** LCLs from DBA patients and control individuals were analyzed for miR-34a expression by qRT-PCR, and for p53 and c-MYC expression by Western blot. (A) Table showing RPS19 mutations found in the DBA LCLs. (B) Significant miR-34a upregulation (p<0.05) was seen in 3/5 patients, with actin as the endogenous control. Data are represented as fold
change relative to the normal individual N2. (C) Western blot showing upregulation of p53 protein and downregulation of c-MYC protein in DBA LCL cells. *p<0.05, **p<0.01, ***p<0.001.

To determine whether miR-34a overexpression can inhibit erythropoiesis, we transduced normal CD34+ fetal liver and cord blood cells with a construct overexpressing miR-34a, or GFP control. Both fetal liver and cord blood cells showed increased levels of miR-34a and decreased expression of the miR-34a target BCL-2 after transduction (Figures 4.6A and 4.6C). However, erythroid colony formation was not adversely affected in either cell type (Figure 4.6B and 4.6D), leading us to conclude that miR-34a overexpression alone is not sufficient to inhibit erythropoiesis in primary CD34+ cells.

Figure 4.6. miR-34a overexpression does not significantly impair erythroid differentiation of human CD34+ cells. CD34+ fetal liver (A and B) and cord blood (C
and D) cells were transduced with lentivirus carrying miR-34a or GFP control. Cells were sorted for GFP at 72 hours and seeded for colony assays at 1000 cells per plate, or collected for RNA. (A and C) CD34+ fetal liver and cord blood cells transduced with miR-34a showed increased levels of miR-34a, and decreased mRNA levels of the miR-34a target BCL-2. qRT-PCR. (B and D) Overexpression of miR-34a in CD34+ fetal liver cells and cord blood cells did not significantly affect CFU-GM or BFU-E colony formation in methylcellulose. *p<0.05, **p<0.01, ***p<0.001.

Next, we wanted to check whether downregulation of miR-34a in RPS19 deficient fetal liver cells could rescue their growth defect, as observed through colony assays. To achieve this, we transduced primary CD34+ fetal liver cells with a scrambled shRNA control, miR-34a sponge sequence, RPS19 shRNA, or a combination of RPS19 shRNA with the miR-34a sponge sequence. Decreased expression of miR-34a was observed in cells carrying the sponge sequence (Figure 4.5A). However, downregulation of miR-34a was not sufficient to rescue the growth defect of RPS19 deficient cells observed in methylcellulose colony assays (Figure 4.5B).

Figure 4.7. Downregulation of miR-34a is not sufficient to rescue the erythroid defects observed in RPS19 deficient CD34+ cells. CD34+ fetal liver cells were transduced with lentivirus carrying scrambled control shRNA (SCR), a miR-34a sponge construct (34a), shRNA against RPS19 (RPS19), or a combination of both RPS19 shRNA and miR-34a sponge sequence (RPS19+34a). (A) Cells transduced with the miR-34a sponge construct with or without RPS19 shRNA showed reduced miR-34a expression. (B) Downregulation of miR-34a in RPS19 deficient CD34+ fetal liver cells...
Discussion

While miRNAs were shown to function in a myriad of diseases including MDS (22), little is known about the function of miRNA in DBA pathogenesis. To explore the role of miRNAs in DBA, we transduced primary human CD34+ cells with RPS19 shRNA and ran a miRNA-seq to identify differentially expressed miRNAs in RPS19 deficient samples compared with control cells. Our experiment identified 20 upregulated miRNAs and 10 downregulated miRNAs in RPS19 deficient cells.

Among the upregulated miRNAs were two notable families: miR-34 and miR-30, as well as miR-186, miR-320b, miR-196b, miR-148b, miR-221, miR-145, miR-4521, miR-154, miR-190b, miR-5189, miR-5091, miR-513b, and miR-769. The two genes of the miR-34 family are downstream of p53, and since p53 has been shown to be stabilized in DBA (23-25), this likely underlies the higher expression of these genes in RPS19 deficient cells. We became especially interested in the function of miR-34a in DBA, as this miRNA was also found to be heavily upregulated in 5q- syndrome by Votavova et. al. (18).

Contrary to our hypothesis that miR-34a would contribute to apoptosis of erythroid progenitors and defects in erythroid colony formation, overexpression of miR-34a showed no effect on hematopoietic colony formation in CD34+ fetal liver or cord blood cells, despite visible downregulation of its target BCL-2. This may mean that miR-34a upregulation is a symptom of DBA, rather than a contributing factor, or that it requires other factors to promote apoptosis in the cell. Likewise, knocking down miR-34a in
RPS19 deficient cells was not sufficient to rescue their defects in colony formation. It may be that knockdown of all three miR-34 family members (miR-34a, miR-34b, and miR-34c) is required to have an effect, or that other factors (e.g. p53, p21) trump the effect of miRNAs in the cells.

Aside from the miR-34 family, the miR-30 family of miRNAs (miR-30a, miR-30c, miR-30d, and miR-30e) also showed higher expression in RPS19 cells. This is interesting, because unlike miR-34, the miR-30 family has been shown to have anti-apoptotic effects in cardiomyocytes (26). However, the miR-30 family is considered to be tumor-suppressing, and is one of the miRNA families inhibited by c-MYC during tumorigenesis (27), leading to speculation that it may be regulated by p53 (28). Little is known about miR-30 function in erythropoiesis, and this area remains to be explored.

Several other upregulated miRNAs identified by our RNA-seq are involved in inflammation, consistent with our previous data that inflammation plays a role in DBA pathogenesis (Chapter 2, Chapter 3). MiR-186 and miR-148b target genes involved in autoimmune disease and innate immunity, respectively, and may be upregulated to modulate inflammation in the cells (29, 30). On the other hand, miR-221, which is downstream of TNF-α, and was found upregulated in a mouse model of rheumatoid arthritis (31), may contribute to inflammation in the cells. One of the targets of miR-221 is KIT (6), which has previously been shown to be downregulated in a DBA model (32), and miR-221 is required for early expansion of erythroid progenitor cells (6). It would be interesting to further investigate the role of this miRNA in DBA pathogenesis.

Finally, miR-145 was also among targets upregulated in the RNA-seq, which is counter-intuitive because this miRNA is deleted in the 5q- syndrome and contributes to
erythropoiesis (17). Its upregulation could be the result of a compensation mechanism in the cells, or a function of the difference between the 5q- syndrome and DBA.

Among miRNAs that were downregulated in RPS19 deficient cells are miR-486, miR-99b, miR-411, miR-98, miR-144, miR-548I, miR-6718, miR-122, miR-548an, and miR-409. Of these, miR-122 plays a role in iron homeostasis (33), miR-144 is a target of GATA1 and essential for erythropoiesis (3), and miR-98, which can be upregulated by glucocorticoids (34), is a negative regulator of p53 (35). The downregulation of miR-144 is consistent with our previous data showing GATA1 levels decreased in RPS19 deficient hematopoietic progenitor cells (Chapter 2). The role of miR-98 in DBA should be explored further, as its ability to downregulate p53 in response to glucocorticoids may be part of a mechanism by which corticosteroids are beneficial in the treatment of DBA.

In conclusion, our data show that multiple miRNAs are differentially expressed in RPS19 deficient hematopoietic progenitor cells, and while miR-34a does not appear to play a significant role in contributing to erythroid defects, the role of other miRNAs dysregulated in our DBA model remains to be explored.
References


Chapter 5

Summary

Conclusions and Future Directions

Several studies have previously shown the link between DBA pathogenesis and p53 pathway upregulation in multiple model systems including zebrafish, CD34+ cells, mice, and cells from DBA patients (1-4). From a clinical perspective, p53 is not an attractive target for drug development because of its critical role as a tumor suppressor (5). Therefore, there is a need to find alternative pathways to develop new therapies for DBA. In this study, we used three approaches to identify novel pathways involved in DBA pathogenesis.

In Chapter 2, we investigated the erythroid transcription factor GATA1 in RPS19 deficient primary hematopoietic progenitors, following a study by Sankaran et. al. (6) showing GATA1 mutations in three rare X-linked cases of DBA. We found both GATA1 mRNA and protein expression to be reduced in RPS19 deficient cells. To confirm that GATA1 reduction was not just a symptom of fewer erythroid progenitors being present in the cell population, we sorted the cells for CD71, an erythroid progenitor marker, and observed similar downregulation in GATA1 expression.

One possible mechanism by which GATA1 could be regulated in erythroid progenitors was through direct binding of p53, which has been shown to reduce GATA1-mediated transcription in K562 cells (7). Our studies confirmed that indeed, GATA1 downregulation was p53-dependent and could be rescued by shRNA-mediated knockdown of p53 in RPS19 deficient cells.
However, we also investigated an alternative mechanism, involving the inflammatory cytokine TNF-α. TNF-α has long been known to inhibit erythropoiesis (8), and was also associated with reduced GATA1 expression in TF-1 cells (9). Our results showed that TNF-α was overexpressed in RPS19 deficient hematopoietic progenitors, that this expression was p53-dependent, and that treatment of normal hematopoietic progenitors with TNF-α reduced expression of GATA1. To further investigate the role of TNF-α in DBA pathogenesis, we turned to a zebrafish model of DBA, which could better recapitulate the in vivo effects of inflammation, and also showed TNF-α upregulation. Treatment of rps19 morpholino zebrafish with the TNF-α inhibitor etanercept rescued their defects in red cell development, reduced levels of p21 and il-6 expression, and restored normal expression of GATA1.

Based on these findings, we concluded that GATA1 is downregulated in RPS19 deficient hematopoietic progenitors, and that this effect can be rescued by down-regulation of p53 or TNF-α. Previous work supports the relationship between p53 and TNF-α, and each may contribute to DBA pathogenesis, as p53 can activate TNF-α expression by binding to its promoter (10), and TNF-α can activate the p53 pathway through NF-kB (11). Thus, p53 and TNF-α could form a positive feedback loop, repressing erythropoiesis in response to ribosomal stress. This could help explain why the anti-inflammatory properties of corticosteroids may help alleviate symptoms of DBA (12), and opens up the possibility that TNF-α inhibitors, such as etanercept, may prove beneficial in treating patients with DBA.

In Chapter 3, we employed next-generation RNA sequencing on the Illumina HiSeq platform to identify genes differentially expressed between RPS19 deficient
hematopoietic progenitor cells and controls. Using this method, we identified several novel pathways affected in RPS19 deficient cells, including inflammation mediated by chemokine/cytokine signaling, WNT, and TGF-β signaling, which we are further exploring in our lab.

Additionally, RNA sequencing revealed downregulation of a major transcription factor, MYC, in RPS19 deficient cells. We went on to further characterize the role of MYC in DBA pathogenesis, but found that MYC upregulation alone did not rescue the erythroid defects observed in RPS19 deficient cells. One explanation for this is that MYC may be regulated post-transcriptionally in RPS19 deficient cells, and upregulation of MYC mRNA may not be sufficient to rescue the effects of reduced MYC protein expression. Another possibility is that MYC downregulation is just a symptom of cell cycle arrest as a result of p53 pathway upregulation, and not a primary cause of erythroid defects. Also, because MYC expression must be suppressed for terminal erythroid differentiation to occur (13), constant MYC expression may repress erythropoiesis, requiring an experimental system where MYC expression is transient to check its effect on erythropoiesis in RPS19 deficient cells.

Finally, in Chapter 4, we explored the role of microRNAs in DBA pathogenesis through high-throughput miRNA-seq. We identified two miRNA families, miR-30 and miR-34 that were upregulated in RPS19 deficient cells compared with controls. We went on to further study the role of miR-34a in DBA pathogenesis, and found that overexpression or downregulation of miR-34a alone was not sufficient to affect erythroid differentiation in normal or RPS19 deficient hematopoietic progenitor cells. It may be that the effect of miR-34a is compensated by changes in the other miR-34 family
members, miR-34b and miR-34c. In that case, a knockdown or overexpression of all three miR-34 family members would be required to see an effect. It may also be that miR-34a is dispensable for normal hematopoietic development, as has recently been suggested in a paper by Jain and Barton (14).

Aside from the miR-34 family, other important miRNAs have also been found to be dysregulated in our system, including the miR-30 family, miR-221, which is a TNF-α target shown to repress KIT (15, 16), and the GATA1 target miR-144 (17). It would be interesting to further study the role of these miRNAs in DBA pathogenesis, and to compare miRNA dysregulation among different bone marrow failure disorders. Additionally, it is important to repeat this study with cells from DBA patients, as RPS19 knockdown in normal cells may not fully recapitulate long-term changes that happen in the bone marrow of patients with DBA. miRNA profiles from DBA patients may not only be useful in finding novel pathways regulating the disease, but also as potential diagnostic markers to identify new cases of DBA.

Overall, the one consistent pattern that has come up in our study of novel DBA pathways is the role of inflammation in DBA pathogenesis. While DBA is not classically considered an immune-mediated disease (18), our studies show that inflammation plays a significant role in its pathogenesis. First, we observed upregulation of TNF-α in both RPS19 deficient hematopoietic progenitor cells and zebrafish. Second, the top pathway dysregulated in RPS19 deficient cells by RNA-seq was inflammation mediated by chemokine and cytokine signaling. Third, several inflammatory miRNAs came up on our miRNA-seq analysis, including the TNF-α target miR-221. Finally, treatment of rps19 morphant zebrafish with the TNF-α inhibitor etanercept was sufficient to rescue their
erythroid defects, reduce levels of p21 and il-6, and restore GATA1 expression. Future studies are needed to explore the role of inflammation in DBA pathogenesis, and to determine whether anti-inflammatory drugs, other than corticosteroids, may represent a new approach to treat patients with DBA.

References


