Deficiency of ribosomal protein S19 in CD34+ cells generated by siRNA blocks erythroid development and mimics defects seen in Diamond-Blackfan anemia


Diamond-Blackfan anemia (DBA) is a congenital red cell aplasia in which 25% of the patients have a mutation in the ribosomal protein S19 (RPS19) gene. To study effects of RPS19 deficiency in hematopoiesis we transduced CD34+ umbilical cord blood (CB) and bone marrow (BM) cells with 3 lentiviral vectors expressing small interfering RNA (siRNA) against RPS19 and 1 scrambled control vector. All vectors also express green fluorescent protein (GFP). Transduction with the siRNA vectors reduced RPS19 mRNA levels to various degrees, which resulted in erythroid defects, correlating to the degree of RPS19 down-regulation, and was rescued by expression of an siRNA-resistant RPS19 transcript. Erythroid colony formation capacity conjointly decreased with RPS19 levels in CD34+ BM and CB cells. In liquid culture supporting erythroid differentiation, RPS19-silenced as well as DBA patient CD34+ cells exhibited reduced proliferative capacity and impaired erythroid differentiation resulting in fewer erythroid colony-forming units (CFU-Es). When assaying myeloid development, a less pronounced influence on proliferation was seen. This study shows for the first time that RPS19 silencing decreases the proliferative capacity of hematopoietic progenitors and leads to a defect in erythroid development. (Blood. 2005;105:4627-4634)

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Introduction

Diamond-Blackfan anemia (DBA) is a hypoplastic congenital anemia that presents within the first 2 years of life. In addition to symptoms of anemia, patients display various other symptoms, including growth retardation, skeletal deformities, congenital heart defects, mental retardation, or other abnormalities. Patients show symptoms of anemia, patients display various other symptoms, including growth retardation, skeletal deformities, congenital heart defects, mental retardation, or other abnormalities. Twenty-five percent of the patients have a positive family history. Most cases of DBA are sporadic, but 10% to 25% have a sibling. The homozygous RPS19−/− mice exhibit an embryonic lethal phenotype prior to placental implantation in mice with C57BL/6J background. Unlike human RPS19-deficient DBA patients, the haploinsufficient RPS19+/− heterozygote mice have normal levels of RPS19 mRNA and display no hematologic abnormalities. Collectively, it is clear that novel murine and human model systems are necessary to develop a suitable model of RPS19 deficiency that allows extensive molecular studies. Recently, Matsson et al developed a mouse model with a targeted disruption of the RPS19 gene. The homozygous RPS19−/− mice exhibit an embryonic lethal phenotype prior to placental implantation in mice with C57BL/6J background. Unlike human RPS19-deficient DBA patients, the haploinsufficient RPS19+/− heterozygote mice have normal levels of RPS19 mRNA and display no hematologic abnormalities. Collectively, it is clear that novel murine and human model systems are necessary to develop a suitable model of RPS19 deficiency that allows extensive molecular studies. Recently, Matsson et al developed a mouse model with a targeted disruption of the RPS19 gene.
Materials and methods

Design and cloning of lentiviral siRNA vectors

The human RPS19 cDNA sequence (NM_001022) was searched for suitable siRNA target sequences starting with aag followed by 18 nucleotides. The 21st sense and antisense sequences were subjected to BLAST searches, eliminating sequences with more than 16 base pair (bp) homologies in the human genome. A control vector was designed by scrambling the B sequence so that there was no significant homology to any sequence in the human genome.

The RPS19 siRNA A oligonucleotide included gatgccggcagcaagactgtggctcccttttaagctgagctctttggagttaagagga and agtctttacagaaagcttttcagcttcgccgg. Sense and antisense siRNA sequences are shown in bold, and loop sequences are underlined. Hybridized oligonucleotides were phosphorylated by T4 DNA kinase and ligated into the pSpcar vector (OligoEngine, Seattle WA) into the BglII–HindIII site, downstream of the H1 promoter. The H1-hairpin-precursor cassette was excised from pSpcar with EcoRI and ClaI and further cloned into the EcoRI-ClaI site of the pLV-TH plasmid.

Design and cloning of lentiviral RPS19 rescue vector

The lentiviral construct pLV-mRIY containing an internal spleen focusing virus promoter that expresses high levels of a transcript coding for yellow fluorescent protein (YFP) together with a modified RPS19 cDNA sequence was used to silence RPS19 by RNA interference. A control vector was constructed by scrambling the B sequence so that there was no significant homology to any sequence in the human genome.

The RPS19 siRNA B oligonucleotide included gatgccggcagcaagactgtggctcccttttaagctgagctctttggagttaagagga and agtctttacagaaagcttttcagcttcgccgg. Sense and antisense siRNA sequences are shown in bold, and loop sequences are underlined. Hybridized oligonucleotides were phosphorylated by T4 DNA kinase and ligated into the pSpcar vector (OligoEngine, Seattle WA) into the BglII–HindIII site, downstream of the H1 promoter. The H1-hairpin-precursor cassette was excised from pSpcar with EcoRI and ClaI and further cloned into the EcoRI-ClaI site of the pLV-TH plasmid.

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The RPS19 siRNA C oligonucleotide included gatgccggcagcaagactgtggctcccttttaagctgagctctttggagttaagagga and agtctttacagaaagcttttcagcttcgccgg. Sense and antisense siRNA sequences are shown in bold, and loop sequences are underlined. Hybridized oligonucleotides were phosphorylated by T4 DNA kinase and ligated into the pSpcar vector (OligoEngine, Seattle WA) into the BglII–HindIII site, downstream of the H1 promoter. The H1-hairpin-precursor cassette was excised from pSpcar with EcoRI and ClaI and further cloned into the EcoRI-ClaI site of the pLV-TH plasmid.

Quantitative RT-PCR analysis

Total RNA was isolated from 1 × 10^6 G4P’CD34+ cells using the RNeasy micro kit as described by the manufacturer (Qiagen GmbH, Hilden, Germany), and cDNA was reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA). The expression level of RPS19 was analyzed by quantitative reverse transcriptase–polymerase chain reaction (Q-RT-PCR) using a LightCycler instrument (Roche, Idaho Falls, ID). The cDNA was used for quantitative PCR using Sybr Green (Sigma) for detection of PCR products. cDNA (2 μL) was used in a 15-μL final volume reaction containing 1 U Platinum Taq DNA Polymerase, 1 × buffer (provided with the enzyme), 0.8 mM dNTP (deoxyribonucleoside triphosphate), 3 mM MgCl2, 0.5 μg/mL BSA, 5% DMSO, 0.5 μM RPS19 forward (fw; 5’-GCC TGG AGT TAC TGT AAA AGA CG-3’), 0.5 μM RPS19 reverse (rev;
Northern blot analysis
Total RNA was isolated from GFP+ CB cells using TRIzol reagent, as described by the manufacturer (Invitrogen, Carlsbad, CA). RNA from each sample was loaded on a 1% agarose gel, transferred onto a Hybond membrane (Amersham Pharmacia, Buckinghamshire, United Kingdom), and hybridized using a full-length human RPS19 probe obtained by digesting the pSKR1Gw plasmid with AgeI and EcoRI. The probe was labeled with [32P] deoxycytidine triphosphate using a random priming kit (Amersham Pharmacia). The membrane was washed twice with 2× standard saline citrate (SSC) at 42°C, twice with 2× SSC + 1× sodium dodecyl sulfate (SDS) at 65°C and twice with 0.1× SSC + 1× SDS at 65°C and subsequently exposed to Kodak (Rochester, NY) x-ray film.

Antibodies and Western blot analysis
The chicken polyclonal anti-RPS19 antibody was derived from eggs immunized with 2 RPS19-derived peptides (C)GVMPHSFRSGKSIVA and (C)VEKDQDDGRKLTQPG. Immunoglobulin Y was extracted and loaded on a HiTrap normal human serum (NHS)–activated column (Amersham Biosciences, Buckinghamshire, England) containing covalent coupled Escherichia coli His-tagged RPS19 for affinity purification. Total cellular proteins were extracted by boiling in sample buffer containing 60 mM Tris (tris(hydroxymethyl)aminomethane) HCl, pH 6.8, 2% SDS, 5% vol/vol glycerol, 2% β-mercaptoethanol, 20 mM dithiothreitol. Proteins were separated by 15% SDS–polyacrylamide gel electrophoresis and blotted to polyvinyliden fluoride membrane (Hybond-P; Amersham Bioscience, Uppsala, Sweden) and analyzed with anti-RPS19 and anti-Actin antibodies (BD Biosciences, Lexington, KY). Proteins were visualized using chemiluminescence reagents (Western Lightning; Perkin Elmer Life Science, Boston, MA) according to the manufacturer’s protocol. Densitometry was performed on developed film by using Image J 1.30v software (http://rsb.info.nih.gov/ij/) to quantify the data.

 Colony assays
After each sorting, 2000 GFP+ BM or CB cells transduced with each vector were plated in quadruplicate 35-mm dishes in 1.1 mL H4230 methylcellulose (Stem Cell Technologies) with erythropoietin (Epo; 5 U/mL; Janssen-Cilag, Solland, Sweden), interleukin 3 (IL-3; 100 ng/mL; a gift from Novartis), and granulocyte-macrophage colony-stimulating factor (GM-CSF; 200 ng/mL; a gift from Novartis). The cells were cultured at 37°C in a humidified atmosphere with 5% CO2, 5% CO2, 5% CO2, and 5% CO2. Colony assays were performed on developed film by using Image J 1.30v software (http://rsb.info.nih.gov/ij/) to quantify the data.

 Results
To study the effects of RPS19 mRNA deficiency in hematopoiesis, we asked whether a DBA phenotype could be induced in healthy cells by permanent knockdown of RPS19 mRNA expression using siRNA-expressing lentiviral vectors. Three human siRNA sequences against RPS19 mRNA (RPS19-A, RPS19-B, RPS19-C) and 1 scrambled (Scr) control sequence were inserted into a lentiviral construct (pLV-THERPS19) to generate 3 separate siRNA vectors and 1 control vector (Figure 1A). CD34+ CB cells were transduced with the siRNA-expressing vectors, and GFP+ cells were sorted. Northern blot analysis of GFP+ cells shows that vectors LV-THERPS19-A, B, and C knock down RPS19 mRNA expression to various levels, while the LV-THERPS19-C vector did not affect the level of RPS19 mRNA, compared with mock-transduced cells (Figure 1B). RPS19 mRNA levels were further quantified by Q-RT-PCR, and, as indicated in the Northern blot, the silencing effect was mild in LV-THERPS19-A–transduced cells and most pronounced in LV-THERPS19-C–transduced cells (Figure 1C). Repeated Western blots of sorted cells, 3 (data not shown) and 5 days (Figure 1D) after transduction, show a reduction in RPS19 protein levels whereby the RPS19 protein is gradually decreasing over time because the reduction in protein levels was less pronounced and took longer time to develop. At 5 days following transduction, the siRNA vectors have generated haploinsufficient levels (40%-60% of normal) in the transduced cells (Figure 1E).
transduced BM cells from a previously studied DBA patient. The defect in RPS19-silenced cells was compared with Scr-generated cells, which showed the lowest levels of RPS19. The colony-forming defect was similar to that in CB cells (Figure 2C). CFU-G/GM colony formation capacity was also decreased, although the difference was not statistically significant (Figure 2A). The colony formation experiments were repeated with BM-derived CD34+ cells that were sorted out from gate R1 (CD71loGlyAlo) and R2 (CD71GlyAint) cells, as shown in Figure 3C. The stages of erythroid development by flow cytometry analysis were monitored for 5 independent experiments (mean of 2 independent experiments). The CFU-E and no BFU-E or CFU-G/GM colonies, showing that cells in gate R2 represent mostly erythroid progenitors (Table 2). Comparisons of the 3 fractions in RPS19-silenced BM cells lacking 1 allele of the RPS19 gene. Normal CD34+ BM cells transduced with the LV-TH-RPS19-B vector exhibited a similar decreased level in colony-forming capacity as with the LV-TH-Scr–transduced DBA cells.

Impaired erythroid differentiation and proliferation of erythroid progenitors in RPS19-deficient CD34+ BM cells mimics the erythroid phenotype in DBA

To investigate different stages of erythroid development, we cultured CD34+ GFP+ BM cells in liquid culture under conditions that are known to produce unilineage erythroid differentiation as previously described. In RPS19-deficient cells, erythroid proliferation decreased (Figure 3A). At days 7 and 14 cells were taken from the erythroid culture to determine the stage of erythroid maturation. At day 7, DAF staining showed a reduced fraction of hemoglobin-containing cells in cultures transduced with vectors LV-TH-RPS19-B and LV-TH-RPS19-C (Figure 3B). To monitor the stages of erythroid development by flow cytometry analysis, cells were stained with Glycophorin A and CD71 (Figure 3C), as described by Rogers et al, and sorted from gates R1, R2, and R3. The 3 distinct cell populations morphologically agree with Rogers et al; R1 (CD71GlyAlo/lo) cells contained 25% blasts and 75% myeloid cells, R2 (CD71GlyAlo) cells mostly contained immature erythroid cells, while the R3 (GlyAhi) cells were almost entirely composed of mature erythroid cells (Figure 3; Tables 1 and 2). Untransduced cells sorted out from gate R1 (CD71GlyAlo) at day 7 formed 115 CFU-G/GM and 3 BFU-E colonies per 1000 plated cells, while cells from gate R2 (CD71GlyAlo) formed 20 CFU-E and no BFU-E or CFU-G/GM colonies, showing that cells in gate R2 represent mostly erythroid progenitors (Table 2). Comparisons of the 3 fractions in RPS19-silenced BM cells...
showed a relative increase of the non–erythroid-committed cell fraction (R1) and a decreased fraction of erythroid blasts and mature erythroid cells (R2 and R3) day 7 (data not shown) and day 14 (Figure 3E). This indicates a failure in erythroid maturation at a differentiation stage earlier than the cells that were gated in R2. Q-RT-PCR from cells in gates R1 and R2 at day 7 showed that RPS19-silenced cells maintained RPS19 expression at levels within 30% to 60% of mock– and LV-TH-Scr–transduced cells in 2 separate experiments (data not shown).

Defects in erythroid development and proliferation can be rescued by RPS19 transgene expression.

To confirm that the erythroid defect was specifically caused by RPS19 deficiency we designed a lentiviral vector expressing the normal amino acid sequence but is no longer silenced by the coding sequence of RPS19. To rescue the RPS19 deficiency, the YFP together with RPS19. To rescue the RPS19 deficiency, the coding sequence of RPS19 was changed so that it codes for the normal amino acid sequence but is no longer silenced by the coding sequence of RPS19 was changed so that it codes for the normal amino acid sequence but is no longer silenced by the coding sequence of RPS19. The YFP expression was stable at 50% in YFP-control–transduced and LV-TH-Scr–transduced cells. Although YFP expression was stable at 50% in YFP-control–transduced and LV-TH-Scr–transduced cells. As expected YFP-control–transduced and YFP–LV-TH-RPS19-B–transduced cells had a reduced fraction of GlyA+ cells. Although YFP expression was stable at 50% in the other groups, the rescued YFP+ fraction of LV-TH-RPS19-B–transduced cells increased from 50% on day 2 to 70% on day 7 (data not shown), indicating a rescue of the proliferative defect.

Table 1. CD71lo GlyAint cells are erythroid blasts, while GlyAhi cells are more mature erythroid cells

<table>
<thead>
<tr>
<th></th>
<th>R1*, %</th>
<th>R2†, %</th>
<th>R3‡, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythroblast</td>
<td>0</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>Basophil</td>
<td>0</td>
<td>31</td>
<td>17</td>
</tr>
<tr>
<td>Polychromatic</td>
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<td>24</td>
</tr>
<tr>
<td>Orthochromatic</td>
<td>0</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>Blasts</td>
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<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Promyelocytes</td>
<td>50</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>10</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Macrophages/monocytes</td>
<td>15</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

*Differential cell counts of 2 to 300 cells from gates R1, R2, and R3 after 14 days of culture in erythroid expansion medium.

†R1 contains 25% blasts and 75% mature myeloid cells.
‡R2 contains 8% blasts and 71% immature erythroid cells.
§R3 contains almost only mature erythroid cells.

Table 2. CD71lo GlyAint cells only give rise to erythroid colonies

<table>
<thead>
<tr>
<th></th>
<th>R1, no.</th>
<th>R2, no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-G/GM/1000 plated cells</td>
<td>115.4</td>
<td>0.0</td>
</tr>
<tr>
<td>BFU-E/1000 plated cells</td>
<td>3.3</td>
<td>0.3</td>
</tr>
<tr>
<td>CFU-E/1000 plated cells</td>
<td>0.1</td>
<td>20.8</td>
</tr>
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</table>

Colonies formed by 1000 untransduced CD71lo GlyAint (R1) and CD71lo GlyAhi (R2) cells separated by FACS after 6 days in erythroid expansion culture. Cells were plated in semisolid medium containing erythropoietin, IL-3, SCF, and GM-CSF. The cell population in R1 mostly has a CFU-G/GM-forming potential, while R2 contains only cells with an erythroid colony-forming potential. The colony numbers represent 2 separate experiments. Cells in R3 were mature erythroid cells, so colony assays were not performed.
Reduced myeloid proliferation but maintained myeloid differentiation of CD34+ cells expressing siRNA against RPS19

Since some patients develop neutropenia, thrombocytopenia, or both during the course of DBA, we asked whether the myeloid proliferation and differentiation is altered in cells transduced with siRNA against RPS19. Transduced GFP BM cells were cultured in medium containing IL-3, SCF, and G-CSF for 21 days. Decreased proliferation capacity in cells transduced with vectors LV-TH-RPS19-B and -C is shown in Figure 5A. Transduction with vector LV-TH-RPS19-A did not affect the proliferation in myeloid culture conditions. RPS19 expression in silenced cells was 20% to 60% of normal in cells from gates 1 to 3 at day 14 as determined by Q-RT-PCR (data not shown). To study the effects of RPS19 silencing on granulocytic development, cells from day 14 were stained with CD33 and CD13, and 3 distinct CD33+ cell populations were studied as sorted: CD13+ (gate 1), CD13+ (gate 2), and CD13++ (gate 3) (Figure 5B). Differential counts of the different populations are shown in Table 3. In myeloid culture conditions RPS19-silenced cells contained a higher fraction of CD13++ cells (Figure 5C), showing that even though proliferation is reduced, the myeloid maturation is not arrested.

Myeloid differentiation is less sensitive than erythroid differentiation to the RPS19 deficiency

To compare the effects of RPS19 silencing on proliferation and maturation of myeloid and erythroid cells, sorted GFP+CD34+ BM cells were cultured under conditions that enable differentiation into both myeloid and erythroid lineages (IL-3, SCF, G-CSF, and erythropoietin). Figure 6A shows the number of GFP+ cells at different time points during the liquid culture expansion. The efficient RPS19 knockdown by vectors LV-TH-RPS19-B and -C clearly reduced the proliferation capacity, while cells transduced with the least effective vector LV-TH-RPS19-A show no significant difference compared with the cells transduced with the Scr control vector. The erythroid versus myeloid lineage distribution of cultured cells changes in RPS19-silenced cells. The ratio of cells positive for CD41 or CD13 divided by cells positive for Glycophorin A increased in RPS19-silenced cells (Figure 6B), indicating that erythroid differentiation is more sensitive to RPS19 silencing than the myeloid differentiation.

Table 3. Phenotypic maturation as determined by CD13 and CD33 correlates to morphological differentiation

<table>
<thead>
<tr>
<th>Gate</th>
<th>Blast</th>
<th>Promyelocytes</th>
<th>Myelocytes</th>
<th>Neutrophils</th>
<th>Macrophages/monocytes</th>
<th>Erythropoiesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>41</td>
<td>6</td>
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<tr>
<td>2</td>
<td>4</td>
<td>29</td>
<td>8</td>
<td>58</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>86</td>
<td>0</td>
</tr>
</tbody>
</table>

Differential counts of 2 to 300 cells on day 14 in myeloid expansion medium within gates 1 (CD33+CD13+), 2 (CD33+CD13+), and 3 (CD33+CD13+).
Giri et al. followed 28 steroid-refractory DBA patients, of which 11 patients, pointing toward a bilineage progenitor defect. Similarly, an impairment along the granulocyte-macrophage pathway in G/GM colony-forming potential. Gate R2 (CD71 negative, myeloid cell population with some BFU-E but mostly CFU-E) and 2 different stages of erythroid differentiation. After 14 days, 3 populations, 1 fraction of hemoglobin-containing cells. After 7 days, RPS19-silenced cell cultures contained a reduced number of myeloid colony formation. Reduced in RPS19 silenced CD34 cells. RPS19-silenced erythroid cell cultures contain a higher fraction of cells in gate R1 and a lower fraction of cells in gates R2 and R3, displaying similar ratios as CD34+ BM cells from a DBA patient lacking 1 RPS19 allele cultured under the same conditions. Since CD34+ BM cells from RPS19-deficient DBA patients and from healthy RPS19-silenced BM cells fail to form normal ratios of erythroid progenitor cells, we suggest a significant block in erythroid development prior to the formation of CFU-Es as a major cause of erythroblastopenia and anemia in DBA patients. Our findings do not rule out additional reduction in erythroid differentiation at the CFU-E stage and beyond. This failure of early erythroid development can be caused by a requirement of relatively high RPS19 expression during a critical stage of erythroid development. Previous studies show that RPS19 expression is higher in early hematopoietic progenitor cells than in more differentiated erythroid cells. In a careful in vitro study using peripheral blood cells from DBA patients, Ohene-Abuakwa et al. found that the main defect in DBA erythropoiesis is somewhat later (after the Epo-triggered onset of erythroid differentiation). No erythropoietin was used during the first 7 days of culture, and this difference may account for the differences in our and their findings.

RPS19 silencing decreased myeloid proliferation in myeloid liquid culture. However, the cells that succeeded to proliferate were mature myeloid cells, even to a larger extent than the control cells. In addition, culture of transduced cells in liquid medium that enables both erythroid and myeloid differentiation increased the fraction of cells belonging to the myeloid (CD13+) or megakaryocyte (CD41+) lineage compared with the erythroid lineage (GlyA+). Cell proliferation was always lower in RPS19-silenced cells, pointing toward a general progenitor proliferation defect in RPS19-deficient DBA, that agrees with the proliferation deficiency of early (CD34+/CD38+) progenitor cells from DBA patients in liquid culture.

The hematopoietic system has a great proliferative potential, capable of increasing cell production 5- to 10-fold with a 2.5-fold higher relative turnover of erythroid cells compared with myeloid cells. Reduced proliferation capacity is a possible explanation to the hematopoietic and developmental defects in DBA. The increased sensitivity to RPS19 silencing during erythroid compared with myeloid differentiation could be due to an impairment of the translational machinery or a disruption of an unknown extraribosomal function of RPS19. For example, ribosomes lacking RPS19 or containing mutated forms may alter the translation rate constants for transcripts important for erythroid development. An example of this mechanism of translational regulation is the interferon-γ-induced phosphorylation and release of ribosomal protein L13a that specifically inhibits ceruloplasmin translation. Another possibility is that RPS19 has an extraribosomal function interacting with factors involved in erythropoiesis. RPS19 has been shown to interact with fibroblast growth factor 2 in NIH3T3 cells, and it is possible that RPS19 also interacts with factors in erythroid development.

To understand the pathogenesis in DBA it is important to have access to cells in which the pathologic process can be induced and studied. Our model system presented in this paper significantly simplifies future studies on ribosomal and extraribosomal mechanisms involved in RPS19 deficient DBA.

**Acknowledgments**

We thank Zhi Ma for assistance in cell sorting; Ingridh Astrand-Grundström and Dr Per-Gunnar Nilsson for expert evaluation of differential counts; Dr Sarah Ball for advise on DAF staining; and Karin Olsson, Eva Nilsson, and Dr Ann Brun for help with the Northern blot and Q-RT-PCR analyses.
References


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