Reduced ribosomal protein gene dosage and p53 activation in low-risk myelodysplastic syndrome

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Reduced gene dosage of ribosomal protein subunits has been implicated in 5q− myelodysplastic syndrome and Diamond Blackfan anemia, but the cellular and pathophysiologic defects associated with these conditions are enigmatic. Using conditional inactivation of the ribosomal protein S6 gene in laboratory mice, we found that reduced ribosomal protein gene dosage recapitulates cardinal features of the 5q− syndrome, including macrocytic anemia, erythroid hypoplasia, and megakaryocytic dysplasia with thrombocytosis, and that p53 plays a critical role in manifestation of these phenotypes. The blood cell abnormalities are accompanied by a reduction in the number of HSCs, a specific defect in late erythrocyte development, and suggest a disease-specific ontogenetic pathway for megakaryocyte development. Further studies of highly purified HSCs from healthy patients and from those with myelodysplastic syndrome link reduced expression of ribosomal protein genes to decreased RBC maturation and suggest an underlying and common pathophysiologic pathway for additional subtypes of myelodysplastic syndrome.

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Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous group of blood cell disorders characterized by defective hematopoiesis and increased susceptibility to leukemia; it is thought to involve abnormalities in HSCs. Approximately 50% of affected patients have blood cell cytogenetic abnormalities, of which deletions of chromosome 5q are the most common and portend a favorable prognosis.1 Identification of causal genes in 5q− and other MDS subtypes has been challenging, but recent advances in genetics and genomics have enhanced our understanding of how specific chromosomal alterations and their molecular consequences contribute to the pathogenesis of MDS.2,3

On the basis of a large-scale RNAi screen, Ebert et al identified RPS14 as a critical gene on 5q whose hemizygosity in BM cells recapitulates many of the features in 5q− MDS.2 Intriguingly, erythroid abnormalities in 5q− MDS are similar to those in Diamond Blackfan anemia (DBA), a dominantly inherited disorder in which germline mutations in one allele of either a 40S (encoded by RPS genes) or a 60S (encoded by the RPL genes) ribosomal protein gene have been identified.4,5 However, there are also important differences between the blood cell phenotypes of the 2 conditions; thrombocytosis and megakaryocytic dysplasia are cardinal features of 5q− MDS but not of DBA. Furthermore, recent work from Starczynowski et al suggests that 2 microRNAs are critical mediators of the 5q− phenotype because knockdown of these genes in immature hematopoietic cells leads to megakaryocytic dysplasia and thrombocytosis after transplantation in mice.6 Increased understanding of how ribosomal protein mutations cause disease might provide additional insight into the pathogenesis of MDS and DBA.

Spontaneous and induced ribosomal protein mutations have been studied in many model organisms, including yeast, flies, plants, fish, and mice.7−11 Studies designed on the basis of on these models indicate that reduced dosage of ribosomal protein genes compromises ribosome biogenesis, protein synthesis, cell proliferation, and cell survival.11,12,14 Although these processes are likely to account for impaired organismal and cellular growth, the mechanisms responsible for tissue-specific phenotypes, including anemia and thrombocytosis, remain unresolved.

Additional insight into the pathogenesis of ribosomal protein-mediated BM failure has come from recent work in our laboratory: mice with ribosomal protein mutations develop epidermal melanocytosis and dark skin in which activation of the transcription factor p53 is a critical event.15,16 Here, we ask whether these observations can be applied to understand the pathogenesis of MDS on the basis of conditional and BM-specific ablation of a ribosomal protein subunit, Rps6.12 We found that activation of p53 is necessary to elicit a BM failure phenotype in mice whose features recapitulate essential aspects of 5q− MDS and that suggests specific roles for p53 activation in both HSC dysfunction and defects in red cell maturation. In addition, we found that ribosomal protein dysregulation occurs in non-5q− MDS in human patients, suggesting a shared pathogenic mechanism among different MDS subtypes.
Methods

Generation of ribosomal protein mutant mice and animal experiments

We obtained mice carrying Tg.MxCre from The Jackson Laboratory, Rps6lox from S. Volarevic and G. Thomas (University of Cincinnati),15 Tg.K5Cre from S. Artandi (Standard University), and J. Jorcano (Epithelial Biomedicine Division, CIEMAT), and TgTrp53loxp from T. Jacks (Massachusetts Institute of Technology).18 Rps19Dsk3lox (C3HeB/FeJ), Tg.K5Cre (C3HeB/FeJ), Trp53lox (129/SvJ), and Tg.MxCre (C57BL/6J) were maintained on an inbred background. Mdm2puro21 and /-/+ controls were (129/SvJ x C57BL/6J)F1 littersmates. Rps6lox+/+;Tg.MxCre/+ were generated by crossing Rps6lox+/+ (129/SvJ x C57BL/6J F1) with Tg.MxCre/+ and observed an intermediate between nonmutant and Rps6loxloxp+;Tg.K5Cre+/- animals. Then, 7- to 9-week-old animals were given 250 μg of polyinosinic:polycytidylic acid (polyI:C) intraperitoneally (Invivogen) on days 1, 3, and 5. All experiments in which we used Rps6loxlox+;Tg.MxCre/+ animals were performed at least 16 weeks after polyI:C treatment.

Erythrocyte adenosine deaminase (eADA) was evaluated as previously described.20 All experiments were performed under a protocol approved by the Stanford Administrative Panel on Laboratory Animal Care.

Immunofluorescence, histology, and cytology

Adult skin or femurs were fixed in 4% paraformaldehyde. Femurs were decalcified in 500mM EDTA, pH7.2. Immunofluorescence was carried out with p53 (Novocastra) or von Willebrand factor (VWF; Dako) antisera after antigen retrieval with 0.01M citrate buffer, pH 6, in a pressure cooker. Skin sections were incubated with goat antirabbit Cy3 antisera (Jackson ImmunoResearch Laboratories) and ProLong antifade reagent with DAPI (Invitrogen). Femur sections were incubated with goat antirabbit biotinylated antisera (Jackson ImmunoResearch Laboratories), Vectastain Elite Avidin-Biotin complex reagent (Vector Labs), and Tyramide-Fluorescein amplification reagent (PerkinElmer). Ter119 antisera (BD Pharmingen), mouse antirabbit biotinylated antisera (BD Pharmingen), avidin-biotin complex reagent, and Tyramide-Fluorescein amplification reagent (PerkinElmer) were used for double immunofluorescence staining on femur sections after a second treatment with 0.01M citrate buffer, pH 6, in a pressure cooker. Histologic sections were stained with hematoxylin and eosin. BM cytopsins, and peripheral blood smears were stained with Wright Giemsa reagents.

Immunophenotyping, purification of, and assay for Rps6 protein from mouse BM

Total BM cells were c-kit enriched with the use of magnetic beads (Miltenyi Biotec) and stained with fluorochrome-conjugated antibodies (supplemental Table 3, available on the Blood Web site; see the Supplemental Materials link at the top of the online article) and propidium iodide (Molecular Probes). Cell populations were fractionated on the basis of Pronk et al21 or Socolovsky et al22 (supplemental Table 4). Quantitative measurements presented in Figure 5A, C, and D are based on 1 million cells from each of 2-3 animals per genotype, with values from Rps6loxlox+;Tg.MxCre+/ animals divided by a factor of 2.13 (or 1.18 for Rps6loxlox+;Tg.MxCre/+;Trp53T550a) to account for the reduction in BM cellularity (Table 2); comparisons between genotypes therefore correspond to absolute differences on a per-animal basis. Cells were sorted or immunophenotyped with FACS Aria II cell sorters (Becton Dickinson), and data were analyzed with FlowJo Version 7 software (TreeStar).

Rps6 protein levels were evaluated from BM cells after staining for surface antigens, fixation with 1.6% paraformaldehyde, permeabilization with cold methanol, and staining with fluorescently conjugated mouse Rps6 antisera (Cell Signaling Technology). Mean fluorescence intensity for a population of cells is a surrogate for Rps6 protein levels.

Results

Rps6 hemizygosity causes robust p53 activation in skin and BM

We previously reported mutations of Rps19 and Rps2016 as the cause of 2 dominantly inherited dark skin mutants, Dark skin 3 (Dsk3) and Dark skin 4 (Dsk4), respectively, that were identified during the course of a forward genetic screen for new pigmentation abnormalities.15,19 These animals exhibit increased accumulation of epidermal melanocytes in the footpads, ears, and tail that becomes apparent shortly after weaning and persists throughout adult life. We observed a similar phenotype in animals with keratinocyte-specific hemizygosity for a third ribosomal protein, Rps6 (Rps6loxlox+;Tg.K5Cre+').16 In additional studies, we showed that activation of p53 is both necessary and sufficient for the pigmentation phenotype. Thus, mutations in 3 different components of the 40S ribosome trigger a common pathophysiologic pathway that causes accumulation of p53, epidermal melanocytosis and dark skin.

Rps19Dsk3 animals develop a mild blood cell phenotype (a 5%-10% reduction in RBC counts), and Rps20Dsk4 animals have no blood cell abnormalities.16 However, we also noticed that Rps19Dsk3 animals exhibited skin color darkening that was intermediate between nonmutant and Rps6loxlox+;Tg.K5Cre+/- animals (Figure 1A), which suggested that the level of skin darkening might serve as a proxy for the extent of p53 activation. We investigated this observation more carefully using tail skin sections processed and quantified in parallel. Immunofluorescence for p53 is undetectable in the skin of nonmutant animals, whereas Rps19Dsk3 animals and Rps6loxlox+;Tg.K5Cre+/ animals have a 15-fold and 94-fold increase in staining, respectively, compared with nonmutant animals (Figure 1B-C). Thus, hemizygosity for Rps6 has a stronger effect on p53 activation than the Rps19Dsk3 mutation.

To investigate how the aforementioned difference would manifest itself in the BM, we crossed Rps6loxlox+ mice to animals carrying an interferon-inducible Cre driver that has been used previously to modify genes in the adult BM. Tg.MxCre23 At 7-9 weeks of age, nonmutant (+/-, Rps6loxlox+;Tg.MxCre+/) and mutant (Rps6loxlox+;Tg.MxCre+/) animals were injected with polyLC to activate the Mx promoter and were evaluated 16-75 weeks later.

The effect of Cre-mediated recombination on Rps6 mRNA levels was assessed by the use of quantitative RT-PCR on peripheral blood and sorted BM cells from nonmutant and Rps6loxlox+;Tg.MxCre+/- animals. Overall, and in all cell populations examined (peripheral blood, CD71+ erythrocytes [supplemental Table 4], and megakaryocyte-erythrocyte progenitors), Rps6 expression was significantly reduced (P = .001, .042, and .006, respectively) in Rps6loxlox+;Tg.MxCre+/- animals compared with nonmutant controls (supplemental Figure 1A-E). The levels of reduction varied, presumably because of differences in Cre-mediated excision (supplemental Figure 1A-E); yet, the overall decrement in Rps6 expression persisted over time (supplemental Figure 1D-E). Notably, the degree of Rps6 mRNA reduction correlated with alterations in peripheral blood cell counts (supplemental Figure 1C,E). We also measured Rps6 protein expression in mutant and nonmutant BM cells using a FACS-based approach and observed an ~35% reduction (supplemental Figure 1F-G).

To assess the effects of ribosomal protein mutations on p53 in the BM, we developed a protocol for immunohistochemistry
of femur sections. Although different transgenic strategies were used for Rps6 in the skin (Tg.K5Cre) and BM (Tg.MxCre followed by polyI:C injection), the level of p53 immunostaining in the BM still correlated with the extent of skin darkening (Figure 1B-E), consistent with previous reports that hemizygosity for Rps19 is generally more severe than hemizygosity for Rps6.10,12

**Hematologic characterization of Rps6 mutants**

We first examined peripheral blood and BM from nonmutant (+/+, Rps6lox+/+; and Tg.MxCre/+), and Rps6lox+/+;Tg.MxCre/+ mutant animals. The MxCre transgene by itself had no effect on blood cell counts (supplemental Table 2); however, Rps6lox+/+;Tg.MxCre/+ animals exhibited a robust macrocytic anemia that worsened over
time (Figure 2, Table 1, supplemental Figure 2 and Table 2). At 16 weeks after induction of Cre recombinase, mutant animals had significant alterations in RBC count (36% reduction, \( P < .001 \)) and mean corpuscular volume (25% increase, \( P < .001 \)), and a modest reduction in reticulocyte count (supplemental Table 2). Mutant mice also exhibited marked leukopenia (and associated lymphopenia) and a progressive thrombocytopenia (Figure 2, Table 1, supplemental Figure 2 and supplemental Table 2). In addition, a spectroscopic assay for eADA function in peripheral blood (a biomarker for disease activity in DBA patients) demonstrated a 2-fold increase in enzyme activity in mutant compared with nonmutant control animals (Table 1).

We next asked whether Trp53 was required for the hematologic changes caused by BM-specific reduction of Rps6 gene dosage. *Rps6lox;Tg.MxCre/+* animals were crossed with those carrying a Trp53 hemizygosity and observed a prominent megakaryocytosis (a 2.7-fold increase in megakaryocyte number) and marked hypocellularity in *Rps6lox/+*; *Tg.MxCre/+* compared with control animals (Figure 3A-B). Immunofluorescence for VWF, an immunohistochemical marker for megakaryocytes in normal BM and MDS, revealed a 2.3-fold increase in megakaryocytes in ribosomal protein mutant marrow (Figure 3C-D). To evaluate the degree of hypocellularity and to assess the role of p53 in this process, we flushed marrow from the femurs and tibias of nonmutant, *Rps6lox/++;Tg.MxCre/+* and *Rps6lox/++;Tg.MxCre/+;Trp53ko* animals and counted the number of cells. Mutant animals (*Rps6lox/++;Tg.MxCre/+*) exhibited a 50% reduction in cellularity that was abrogated completely by loss of p53 (*Rps6lox/++;Tg.MxCre/+;Trp53ko; Table 2).

We evaluated lineage specific blood cell precursors on the basis of BM cytology. From cytospin preparations of BM samples flushed from the lower extremities, we observed a marked reduction in the number of lymphoid cells in *Rps6* mutant animals, whereas immature and mature granulocytic and monocytic populations were unaffected (Table 2). The number of lymphocytes in *Rps6lox/+*; *Tg.MxCre/+* BM was significantly reduced (by 56%, \( P = .006 \)), and partially rescued in the absence of p53 (*Rps6lox/+;Tg.MxCre/+;Trp53ko*).

We examined the cytology of RBC precursors from *Tg.MxCre/+* and *Rps6lox/+;Tg.MxCre/+* and *Rps6lox/+;Tg.MxCre/+;Trp53ko*.
BM on the basis of recent work by Pronk21 and Socolovsky22 in which the morphology of specific, sorted cell populations from the mouse myeloerythroid hierarchy was carefully assessed. We scored cells as either immature (PreCFU-E and CFU-E; supplemental Table 4) or mature erythroid precursors (basophilic, polychromatophilic, and orthochromatic erythroblasts; supplemental Table 4) and observed a significant relative increase in the number of immature erythroid precursors \( (P < .001, \text{Figure } 3E, \text{Table } 2, \text{supplemental Table } 4) \) and a significant decrease in mature erythroid cells \( (P < .016, \text{Figure } 3E, \text{Table } 2, \text{supplemental Table } 4) \). Both of these changes were dependent on \( \text{Trp53} \) (Table 2).

As a complementary means of evaluating the erythroid lineage in \( \text{Rps6}^{\text{lox/+}}; \text{Tg.MxCre/+} \) mice using \( \text{Ter119} \) (a marker for mature RBCs)22 and \( \text{p53} \). Akin to our previous findings (Figure 1D-E), we observed a significant \( (P = .013) \) increase in \( \text{p53} \)-positive cells in the BM of mutant animals (supplemental Figure 3A-B). Ter119 staining of nucleated cells (dapi-positive), however, was significantly reduced \( (P < .016) \) in the \( \text{Rps6} \) mutant compared with nonmutant animal (supplemental Figure 3A,C). We also found that although a fraction of the nucleated, Ter119-positive cells also stained with \( \text{p53} \), most \( \text{p53} \)-positive cells were not mature RBCs (Ter119-positive), suggesting that \( \text{p53} \) acts at multiple stages during hematopoiesis.

Megakaryocytes were significantly increased and showed distinct cytologic changes, with frequent, small dysplastic, monolobated megakaryocytes in BM from \( \text{Rps6}^{\text{lox/+}}; \text{Tg.MxCre/+} \) mice (Figure 3F-G). Thus, from multiple perspectives, BM-specific...
hemizygosity for Rps6 causes defects that recapitulate key clinical features of 5q− MDS,26 including defects in red cell maturation and megakaryocyte dysplasia accompanied by thrombocytosis.

**p53 activation mimics ribosomal protein deficiency in the BM**

Except for the lymphoid abnormalities, the hematologic alterations we observed depended completely on the presence of Trp53. To ask whether activation of p53 was sufficient to trigger these abnormalities, we took 2 approaches to elicit moderate levels of p53 activation because robust activation of p53 in developing BM causes a failure to initiate primitive erythropoiesis and embryonic death.26

In one approach, we took advantage of Mdm2mutant7-9 mice in which expression of Mdm2, a negative regulator of p53 stability and activity, is reduced ~70% relative to wild-type mice.26 We measured p53 expression in the BM of Mdm2mutant/+, and nonmutant (+/+ ) controls. Although p53 expression in nonmutant marrow was negligible, immunofluorescent staining in Mdm2mutant/ mice was increased by 5.2-fold compared with control animals (Figure 4A-B). To extend the initial results on the lymphocyte and red cell lineages described by Mendrysa et al,26 we counted megakaryocytes in BM sections and observed a significant megakaryocytosis (P < .001) in Mdm2mutant/ compared with nonmutant (+/+ ) controls (Figure 4C-D). In addition, immunofluorescence for WVF was significantly increased in BM sections from Mdm2 mutant animals (Figure 4E).

As a complementary approach, we administered the chemotherapeutic agent, 5-fluorouracil, whose ability to cause apoptosis and growth arrest in cells has been attributed to its ability to induce p53,27 and which has been widely used in mice to investigate the biology of stress hematopoiesis. On the basis of a regimen from the work of Agosti et al,28 we administered a single dose of 150 mg/kg to wild-type control mice and observed an ~20% decrease in RBC count and an ~60% increase in platelet count 11 days later (Figure 4F-G). These values returned to near pretreatment levels by day 25. In contrast, the response to 5-fluorouracil in Trp53ok/kok animals was markedly attenuated; at day 11, both RBC and platelet counts were significantly different (P = .007 and P = .0075, respectively) from nonmutant animals. Thus, both genetic and pharmacologic approaches in vivo suggest that p53 activation induces erythrocyte and megakaryocyte abnormalities similar to those caused by Rps6 hemizygosity.

**Effects of Rps6 hemizygosity on hematopoiesis**

Recent work by Pronk et al demonstrated that specific cell populations in the mouse myeloid/erythroid hierarchy can be identified and prospectively separated via differential expression of the cell-surface markers endoglin (Eng/CD105), CD150 (Slamf1), and CD41 (Itga2b).21 Multipotent progenitors (MPPs) can be distinguished from HSCs, granulocyte-monocyte populations distinguished from megakaryocyte (McP) and erythrocyte precursors, and erythroid progenitors can be divided into a maturation hierarchy: PreMegE (megakaryocyte-erythroid precursor), PreCFU-E, and CFU-E/ProEry populations.

To explore the ontogeny of the erythroid and megakaryocytic defects caused by BM-specific Rps6 hemizygosity in the mouse, we analyzed specific precursor populations from the BM as described in Pronk et al21 (HSC and MPP populations were identified on the basis of differential expression of CD34, MPP−/−CD34+, and HSC−/−CD34−). We measured and compared 6 separate types of cells between Rps6ok/ok, Tg.MxCre/+ mutant and nonmutant (Tg.MxCre/+ ) controls and observed 2 salient features. First, mutant animals exhibited a significant paucity in the absolute number of HSCs (Figure 5D, supplemental Figure 4). To explore the ontogeny of the erythroid and megakaryocytic defects caused by BM-specific Rps6 hemizygosity in the mouse, we measured and compared 6 separate types of cells between Rps6ok/ok, Tg.MxCre/+ mutant and nonmutant (Tg.MxCre/+)

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### Table 2. Effect of Rps6 hemizygosity and Trp53 mutations on BM cell counts

<table>
<thead>
<tr>
<th></th>
<th>Tg.MxCre/+</th>
<th>Rps6ok/ok; Tg.MxCre/+</th>
<th>Rps6ok/ok; Tg.MxCre/+; Trp53ok/ok</th>
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</thead>
<tbody>
<tr>
<td>Immature erythroid*</td>
<td>13.50 ± 1.66</td>
<td>40.00 ± 3.24†</td>
<td>13.67 ± 3.71† NS§</td>
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<tr>
<td>Mature erythroid*</td>
<td>37.5 ± 1.26</td>
<td>21.0 ± 1.72†</td>
<td>39.0 ± 4.16† NS§</td>
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<td>Lymphocyte</td>
<td>70.75 ± 6.02</td>
<td>29.75 ± 5.02†</td>
<td>52.00 ± 1.73⁴§</td>
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<tr>
<td>Myeloblast</td>
<td>2.5 ± 0.65</td>
<td>3.25 ± 0.48</td>
<td>4.67 ± 1.67</td>
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<tr>
<td>Immature myeloid†</td>
<td>19.75 ± 0.75</td>
<td>21.00 ± 1.96</td>
<td>19.67 ± 3.53</td>
</tr>
<tr>
<td>Mature myeloid†</td>
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<td>121.75 ± 7.22</td>
<td>108.00 ± 6.93</td>
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<td>Monocyte/macrophage</td>
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<td>22.25 ± 3.25</td>
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<tr>
<td>Cellularity (× 10⁵)</td>
<td>7.59 ± 0.42</td>
<td>3.56 ± 0.28†</td>
<td>6.39 ± 0.61† NS§</td>
</tr>
</tbody>
</table>

NS indicates not significant.

*BM cytology was evaluated from 4 nonmutant, 4 Rps6ok/ok; Tg.MxCre/+; and 3 Rps6ok/ok; Tg.MxCre/+; Trp53ok/ok animals. A total of 260 cells were evaluated per animal. Immature erythroid cells are Pre CFU-E or CFU-E cells as defined by Pronk et al (supplemental Table 4); mature erythroid cells are either basophilic, polychromatophilic or orthochromatophilic erythroblasts (supplemental Table 4); immature myeloid cells are promyelocytes or myelocytes; and mature myeloid cells are band or segmented neutrophils. The number of cells within each population is given as mean ± SEM with P values based on 2-tailed Student t test, a P < .01, b P < .05, c P < .001.

†Nonmutant versus Rps6ok/ok; Tg.MxCre/+.
‡Nonmutant vs Rps6ok/ok; Tg.MxCre/+ vs Rps6ok/ok; Tg.MxCre/+; Trp53ok/ok.
§Nonmutant vs Rps6ok/ok; Tg.MxCre/+; Trp53ok/ok.

††Nonmutant versus Rps6ok/ok; Tg.MxCre/+.
‡‡Nonmutant vs Rps6ok/ok; Tg.MxCre/+ vs Rps6ok/ok; Tg.MxCre/+; Trp53ok/ok.
§§Nonmutant vs Rps6ok/ok; Tg.MxCre/+; Trp53ok/ok.

[BM cellularity determined by flushing 2 femurs and 2 tibias from 7 nonmutant, 7 Rps6ok/ok; Tg.MxCre/+; and 3 Rps6ok/ok; Tg.MxCre/+; Trp53ok/ok animals. Values are given as mean ± SEM with P values based on 2-tailed Student t test, a P < .01, b P < .05, c P < .001.]
megakaryocyte-like cells can be easily distinguished on the basis of their size and morphology, Figure 6A.) In nonmutant animals, the proportion of wells that give rise to megakaryocyte-only colonies was very low (generally 1-2 wells per 96-well plate). However, in mutant animals, there was a 20- to 30-fold increase in the proportion of wells that gave rise to megakaryocyte-only colonies (Figure 6A).

We performed a similar experiment sorting for MPP, PreMegE, or MkP populations, placing 10 cells per well in a 96-well plate, and then counting the number of megakaryocytes on each of the following 3 days. Similar to results observed with the single-cell assay for HSCs (Figure 6A), the number of megakaryocytes recovered from MPP cells (measured as mean number of megakaryocytes per well of a 96-well plate) was significantly greater (P = .01) in mutant compared with nonmutant animals (Figure 6B). However, no differences in megakaryocyte number were detected between mutant and control cultures established from PreMegE and MkP populations (Figure 6C-D), which is surprising given the conventional view of the megakaryocyte lineage in which there is a stepwise progression from HSC to MPP to PreMegE to MkP (Figure 7B).

We considered whether this apparent paradox—increased numbers of MPP-derived megakaryocytes without a concomitant increase in PreMegE- or MkP-derived megakaryocytes—might be explained by differences in plating efficiency or cell survival. We measured plating efficiency by counting total cell number (without regard to morphology) after 1 day of culture and observed no differences between mutant and nonmutant HSC, MPP, PreMegE, or MkP cultures (supplemental Figure 5). We did, however, observe that cell growth over the next several days was reduced in all mutant cultures, consistent with a cell-autonomous process that causes general growth impairment.

In summary, hemizygosity for Rps6 in the BM causes not only a profound reduction in overall cellularity but also affects the relative distribution of hematopoietic subpopulations, with a relative decrease in the number of HSCs, CFU-E/ProEry, CD71<sup>+</sup>, and CD71<sup>+</sup>-cells, accompanied by a relative increase in all other subpopulations. In culture, growth of each subpopulation is impaired, except for MPP-derived megakaryocytes, which are increased in mutant compared with nonmutant cultures.

**Effect of reduced ribosomal protein gene dosage in human BM cells**

To investigate how hemizygosity for RPS6 would affect human blood cell development, we studied the effects of RPS6 shRNAs in...
Cord blood samples were sorted (Lin−/H11002 CD34+/H11001 CD38−/H11002 CD90+/H11001 CD45RA−/H11002) to enrich for HSCs,29 infected with a lentivirus containing short hairpin RNA sequences targeting RPS6 (or RPS14 for control), and then cultured under conditions to allow myeloid and erythroid differentiation.2 After 10 days, cultures were analyzed for cell-surface markers of myeloid (CD11b+/H11001), mature erythroid (GPA+/H11001), and immature erythroid (CD71+/H11001 GPA−/H11002) differentiation.

In all cases, shRNA constructs targeting RPS6 (or RPS14) caused a decrease in the erythroid/myeloid ratio (Figure 5E), the extent which correlated with the efficiency of knockdown as assessed by quantitative RT-PCR (supplemental Figure 6A). In addition, RPS6 knockdown led to a block in erythroid differentiation as assessed by presence of the erythroid lineage cell surface marker GPA (Figure 5F-G), similar to observations in BM-specific Rps6 mutant mice (Figure 5B-D), and consistent with the 5q− MDS phenotype.

A role for reduced ribosomal protein gene dosage in both DBA and 5q− MDS suggested to us that other forms of MDS might also involve altered expression of ribosomal protein genes. To explore this idea, we interrogated the results of Affymetrix microarray measurements carried out on 8 MDS patient HSC samples (7 low-risk and 1 intermediate-risk, none of whom carried 5q−; supplemental Table 1),30 compared with 11 age-matched healthy control samples. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE30201 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30201).
Among 54,676 probes on the array, mRNA levels for 3,672 were decreased and 1,159 were increased in the MDS patient samples at a false discovery rate of 0.01%. However, ribosomal protein genes are disproportionately represented among the differentially expressed genes: 0.7% of the differentially expressed probes represented ribosomal protein genes, whereas 0.4% of all the probes on the array represented ribosomal protein genes ($P = 0.005$, 2-tailed z-statistic, 95% confidence interval, z = 2.821). More strikingly, of 74 ribosomal protein genes represented on the array, mRNA levels for 21 were decreased, and only 1 was increased (Figure 7A). Thus, ribosomal protein gene expression is dysregulated—and largely repressed—in HSCs from a group of non-5q MDS patients.

Discussion

5q- MDS is characterized by abnormal erythrocyte maturation with macrocytic anemia, thrombocytosis, and a predisposition to leukemia.25 Intriguingly, DBA, a congenital BM failure syndrome, exhibits erythroid defects similar to 5q- syndrome, and in recent studies researchers suggest that both conditions are caused by reduced dosage of one or more ribosomal protein genes.2,5 To date, the underlying cellular pathophysiology and potential approaches to treatment for these diseases have been difficult to unravel.
During earlier studies of pigmentary mutations in mice, we realized that reduced dosage of the ribosomal protein genes, Rps6, Rps19, and Rps20, led to activation of p53 in which the differential and specific response of individual cell types in the skin and blood gave rise to a pleiotropic syndrome that was likely relevant to 5q- MDS and DBA. We have now tested this hypothesis more directly and found that hemizygosity for Rps6 in mouse BM and reduced dosage of ribosomal protein genes in human cells recapitulate cardinal features of the human condition. In addition, studies designed on the basis of genome-wide expression analysis reveal that abnormal expression—and specifically down-regulation—of ribosomal protein genes characterizes BM stem cells from patients with low-risk, non-5q- MDS. These results connect genotype to phenotype for a relevant animal model of MDS and suggest that ribosomal protein gene dysregulation may play a role in the pathogenesis of a broad group of BM-failure syndromes.

Our work uses Rps6 as a tool to model 5q- MDS (and to inform our understanding of DBA), yet human mutations of RPS6 have not been identified in either condition. However, germline mutations for RPS6...
may be lethal (as they are in mice).32 Our results confirm that loss-of-function for Rps6 is more severe than loss-of-function for Rps19 or Rps20 because keratinocyte-specific hemizygosity for Rps6 has a more dramatic effect on p53 induction compared with germline mutations in Rps19 or Rps20 and because germline mutations in Rps19 or Rps20 have a very mild effect on BM.16 We note that Rps6 phosphorylation serves as an indicator of physiologic stress and is downstream of the mammalian target of rapamycin pathway, and from that perspective, Rps6 is not “just another ribosomal protein,” which may help to explain why lymphopenia develops in our BM-specific Rps6 mutants but not in other models of 5q− MDS (or DBA). However, our results also suggest that the effect of Rps6 mutation-induced BM disease in other BM lineages is similar to that caused by mutations in other ribosomal protein genes, at least with regard to dependence on p53.31,32 We also note that extensive ribosomal protein gene resequencing has not yet been reported for MDS; indeed, our results suggest that some patients with low-risk MDS and normal cytogenetics may have somatic mutations of ribosomal protein genes other than RPS14, including RPS6.

Similar to the work of Barlow et al,31 we find that many of the BM phenotypes in Rps6 mutant animals depend on the presence of p53. We find that activation of p53 is necessary for peripheral blood cell abnormalities (leukopenia, macrocytic anemia, thrombocytopenia, and elevated EADA activity), BM hypocellularity, and erythrocyte maturation in mutant BM. We note that parallel mechanisms are likely to exist in other contexts. Activation of p53 during ischemia contributes to cell death, tissue injury, and organ failure.34,35 and recent studies of animal models of Fanconi anemia and Treacher Collins syndrome suggest that stabilization of p53 during embryogenesis induces characteristic developmental abnormalities.36,37 These observations support an emerging paradigm in which activation of p53 function can give rise to a variety of developmental or disease-related phenotypes depending on the amount and tissue-specific context in which activation occurs.38

Our studies of hematopoietic subpopulations suggest that reduced ribosomal protein dosage and consequent p53 activation can perturb multiple and independent steps in blood cell development. Impairment of HSC growth is consistent with both a general role for ribosome function and/or a proapoptotic effect of p53 activation on HSCs.3,39,40 In this context, the relative increase of downstream progenitors (MPP, PreMegE, Pre CFU-E, MkP) observed is likely to reflect homeostatic regulation within the BM.41 However, the reduction of more mature erythroid progenitors (CFU-E/ProEry, CD71+, and CD71−) points to a specific block in erythroid maturation and may therefore account for the observation that 5q− MDS and DBA frequently present with a selective reduction in RBC development. Finally, akin to observations by Adolfsson et al42 our studies of megakaryocyte-like growth from cultured hematopoietic cell subpopulations suggest that reduced ribosomal protein gene dosage unmasks an alternative pathway for megakaryocyte development directly from HSC and/or MegE rather than via PreMegE and MkE subpopulations.

In previous studies of large-scale gene expression in MDS CD34+ cells, investigators have shown that reduced expression of ribosomal protein genes is a hallmark of 5q− MDS31,44; revealed that pathways related to interferon, thrombopoietin, and Wnt signaling are dysregulated in all MDS subtypes;5 and have also identified specific sets of genes that correlate with disease subtype and/or prognosis.3,44 At first glance, some of the results reported by Sridhar et al44 appear opposite to what we find, with elevated levels of ribosomal protein gene expression observed in CD34+ cells in non-5q− MDS. However, a key difference between the work reported here and previous studies31,43-45 is that our expression profiling studies use highly purified HSC (Lin−CD34+CD123−CD90−CD45RA−) from MDS patients. This approach both enriches for HSCs (which constitute approximately 1% of total CD34+ cells) and diminishes the presence of multilineage and lineage committed progenitor cells that are also CD34+. This is particularly important for patients with high-risk MDS, in which the CD34+ population is likely to contain a large proportion of actively dividing cells that are metabolically active. In fact, in the work from Sridhar et al, expression of ribosomal protein genes in all CD34+ cells is elevated in patients with high-risk (transformed) MDS compared with low-risk (stable) MDS,44 whereas the patients studied here all represent low-risk MDS. Nonetheless, additional studies, likely designed on the basis of large-scale sequencing, will be required to investigate whether the reduction of ribosomal protein gene expression we observe in HSCs from non 5q− MDS represent a critical pathophysiologic event or a secondary consequence of altered growth rate.

Taken together with genetic studies of Diamond-Blackfan anemia, our work suggests that the hematopoietic system is especially sensitive to alterations in ribosomal protein gene dosage. This intersection—of forward genetic approaches in human disease with reverse genetic approaches in mouse models—is reminiscent of an analogous connection between components of the primary cilium and a spectrum of human diseases represented by Bardet-Biedl syndrome, polycystic kidney disease, and heterotaxy.46 Additional studies in mouse models will illuminate the extent to which non 5q− MDS and other forms of BM failure represent ribosomopathies.

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Authorship

Contribution: K.A.M. generated the Rps6 mutant mice, analyzed the skin, peripheral blood, and BM phenotypes, performed experiments on Mdm2 mutant animals and analyzed the genome wide expression analysis from human bone marrow cells, with help from M.G.P. and C.Y.P.; W.W.P. carried out the siRNA knockdown and genome-wide expression analysis in human HSCs, with help from J.V.P. and C.Y.P.; R.B. evaluated Rps6 protein levels in the BM of mutant mice; B.E.G. measured eADA activity in the blood of mice. They all thank H. Manuel for technical support.

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References


5. Lipton JM, Ellis SR. Diamond Blackfan anemia.


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K5Cre transgenic line appropriate for tissue-spe-


24. Chuang SS, Jung YC, Li CY, von Willebrand factor is the most reliable immunohistochemical marker for megakaryocytes of myelodysplastic syndrome and chronic myeloproliferative disor-


26. Mendrysa SM, McElwee MK, Michalowski J, et al. O'Leary KA, Young KM, Perry ME. mdm2 Is criti-

27. Liu TX, Howlett NG, Deng M, et al. Knockdown of zebrafish Fancd2 causes developmental abnor-


30. Greenberg PL. Relationship of differential gene expres-


32. Jones NC, Lynn ML, Gaudenz K, et al. Prevention of the neurocysticercus Treherrera collinsi syn-


34. Fiskum G, Rosenthal RE. Verczki V, et al. Pro-

35. Matsusaka H, Ide T, Matsuho S, et al. Tar-

36. Jones NC, Lynn ML, Gaudenz K, et al. Prevention of the neurocysticercus Treherrera collinsi syn-

37. Liu TX, Howlett NG, Deng M, et al. Knockdown of zebrafish Fancd2 causes developmental abnor-

38. Vossen KH, Lane DP. p53 in health and dis-


40. Miyake K, Utsugisawa T, Flygare J, et al. Riboso-


42. Adolfsson J,mansson R, Buza-Vidas N, et al. Identification of Flt3+ hematopoietic stem cells lacking erythro-megakaryocytic potential a re-

drome is associated with deregulation of ribosomal-

44. RIBOSOMAL PROTEIN MUTATIONS IN MYELODYSPLASIA 3633
Reduced ribosomal protein gene dosage and p53 activation in low-risk myelodysplastic syndrome

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