Thrombocytopenia and erythrocytosis in mice with a mutation in the gene encoding the hemoglobin β minor chain

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Diverse mutations in the genes encoding hemoglobin (Hb) have been characterized in human disease. We describe here a mutation in the mouse Hbb-b2 gene, denoted Plt12, that precisely mimics the human hemoglobin Hotel Dieu variant. The mutation results in increased affinity of Hb for oxygen and Plt12 mutant mice exhibited reduced partial pressure of O2 in the blood, accompanied by erythrocytosis characterized by elevated erythropoietin in increased affinity of Hb for oxygen and Plt12 mutant mice. Survivors displayed a marked thrombocytopenia without signifying deficiencies in the numbers of megakaryocytes or megakaryocyte progenitor cells. The lifespan of platelets in the circulation of Hbb-b2Plt12Plt12 mice was normal, and splenectomy did not correct the thrombocytopenia, suggesting that increased sequestration was unlikely to be a major contributor. These data, together with the observation that megakaryocytes in Hbb-b2Plt12Plt12 mice appeared smaller and deficient in cytoplasm, support a model in which hypoxia causes thrombocytopenia as a consequence of an inability of megakaryocytes, once formed, to properly mature and produce sufficient platelets. The Plt12 mouse is a model of high O2-affinity hemoglobinopathy and provides insights into hematopoiesis under conditions of chronic hypoxia.

Erythrocytosis, the presence of abnormally high red blood cell mass or hemoglobin, may be congenital or acquired. Examples of acquired erythrocytosis include polycythemia vera, a clonal hematopoietic stem cell disease associated with mutations in the JAK2 gene; conditions of pathological production of erythropoietin (EPO); and disease-associated hypoxias that drive reactive EPO-mediated red blood cell production (1, 2). Congenital causes of erythrocytosis include those with defects in oxygen sensing, including mutations in the von Hippel Lindau (VHL), prolyl hydroxylase 2 (PHD2), and hypoxia inducible factor 2α (HIF-2α) genes; mutations in the gene encoding EPO receptor, commonly relieving the receptor from negative regulatory mechanisms; mutations affecting 2,3-biphosphoglycerate (2,3-BPG) mutase activity, resulting in high oxygen-affinity hemoglobin (Hb); and high oxygen affinity-causing mutations in the globin chain-encoding genes themselves (1–3).

The adult Hb molecule contains two α and two β chains. The Hb tetramer is formed by association of two αβ halves (α1β1 and α2β2) and exists in two interchangeable conformations: the T, or “tense”, low oxygen affinity; and R, or “relaxed”, high oxygen affinity states (4). More than 100 Hb variants have been described in humans that display increased oxygen affinity. The altered affinity results in a low rate of oxygen release to the tissues and relative tissue hypoxia. In many patients, a compensatory erythrocytosis ensues, driven by hypoxia-induced enhancement of EPO production and increased erythropoiesis (1, 2). Although generally well tolerated in the young, high oxygen-affinity hemoglobinopathies are frequently associated with thrombotic complications in older patients (4). Hb variants with high oxygen affinity can be grouped into various molecular classifications (4). Destabilization of the low oxygen-affinity state can result in variants altering the normal αβ2 interface, α1ε2 contacts, or at the C terminus of the β chain. A second class of variants is proposed to result from reduced interaction of Hb with 2,3-BPG, which normally binds between the deoxy-Hb β chains to modulate Hb oxygen affinity. Variants causing structural alterations affecting the oxygen-binding heme pocket have also been described. The relative abundance of abnormal to normal Hb also influences hematological outcome and is influenced by several factors, most notably whether the variation occurs within the α or β chain. Although many Hb variants reported to have high oxygen affinity appear not to be linked with erythrocytosis, this may be attributable to relatively modest effects on affinity having a minimal effect in vivo, or where variation causes reduced expression or protein instability. In these last instances, anemia with a reduced Hb level may occur (4).

Rodent models have provided insights into the effects of sequence polymorphisms on hemoglobin oxygen binding affinity. Adaptive modifications to a high altitude environment in the deer mouse have provided insights into structural changes within the hemoglobin molecule that allow enhanced oxygen affinity (5). Mouse models of human disease alleles are uncommon; however, genome-wide or targeted mutagenesis approaches in the mouse have been used to study the erythrocytosis associated with the high oxygen-affinity Rainier mutation (6) and the hemolytic anemia caused by the destabilizing Presbyterian mutation (7) in β globin. We describe here a mutation in the mouse Hbb-b2 gene that mimics the human Hotel Dieu mutation (8, 9).

The mutation results in increased affinity of Hb for oxygen and causes thrombocytopenia in heterozygous mice and lethality accompanied by exacerbated thrombocytopenia and erythrocytosis when homozygous.

Results

Plt12, an N-nitroso-N-ethylurea-Induced Mutation Causing Thrombocytopenia and Erythrocytosis. In an N-nitroso-N-ethylurea (ENU) mutagenesis screen for genes controlling blood cell production, male BALB/c mice were treated with ENU and then mated with untreated BALB/c females. The Plt12 founder G1 mouse displayed thrombocytopenia with a platelet count of 40 × 109/L, significantly lower than the average platelet count for BALB/c mice (128 ± 413 × 109/L). Upon progeny testing, the founder Plt12 G1 mouse transmitted the thrombocytopenic trait (500–1000 × 109 platelets/L) to approximately half of its offspring (Fig. 1A). When affected Plt12 mice were intercrossed,
4 of 103 weaned offspring exhibited exacerbated thrombocytopenia (fewer than 360 × 10^9 platelets/L) and a marked erythrocytosis (over 15 × 10^12 red blood cells/L; Fig. 1B), which caused a distinctive erythema, particularly evident in the ears and feet (Fig. 1C), and became moribund by 5–6 wk of age. Histopathological examination of moribund mice revealed splenomegaly attributable to excessive erythropoiesis, erythroid foci, and fatty degeneration in the liver, excess mononuclear cells and dilution of the sinusoids in the bone marrow, and, in most mice, emphysema (Table 1 and Fig. 2). Together, these data suggest that Plt12 is a semidominantly acting mutation causing mild thrombocytopenia in heterozygous mice and preweaning or early adult lethality accompanied by exacerbated thrombocytopenia and erythrocytosis when homozygous.

Plt12 Encodes a Variant of the Hemoglobin β Minor Chain. To map the chromosomal location of the Plt12 mutation, affected BALB/c Plt12 mice were outcrossed to unaffected C57BL/6 mice. Linkage was observed for a 2.1-Mb region on chromosome 7 between the markers D7Mit218 and D7Mit220 (Fig. S1A). The region contained 87 olfactory receptors (OlfR), which were excluded from further analysis. The majority of exons from the 20 other annotated genes within this interval were sequenced (Table S1). A single base pair mutation in the gene coding Hb β minor chain (Hbb-b2) was found in affected Plt12 mice but not wild-type C57BL/6 or BALB/c animals nor unaffected mixed background (C57BL/6 × BALB/c) littermates. The identified mutation, a GAT to GGT codon change, is predicted to cause substitution of the aspartic acid at amino acid 100 of the Hb β minor chain by glycine (Fig. S1B).

Significant mortality of homozygous Hbb-b2Plt12/Plt12 mice was also evident on a C57BL/6 genetic background: in contrast to the expected 25%, only 3.5% of offspring weaned from heterozygous parents were homozygous for the Hbb-b2Plt12 allele (4 Hbb-b2Plt12/Plt12, 79 Hbb-b2Plt12/+ , and 36 Hbb-b2Plt12/+ from a cohort of 119 offspring genotyped). However, whereas BALB/c Hbb-b2Plt12/Plt12 animals subsequently succumbed within weeks of weaning as described above, most C57BL/6 Hbb-b2Plt12/Plt12 survivors remained ostensibly healthy for at least a year of life. To minimize complications caused by failing health, all analyses on adult Hbb-b2Plt12/Plt12 mice were subsequently performed on mice at 45–55 d of age, unless otherwise indicated, in which the Hbb-b2Plt12 mutation had been backcrossed 6–18 generations to C57BL/6.

Hemoglobin Binds Oxygen at Abnormally High Affinity in Hbb-b2Plt12/Plt12 Mice. The precise amino acid change observed in Hbb-b2 in Plt12 mice has been described in humans and is referred to as the Hotel Dieu mutation (8, 9). In humans, this mutation results in production of a high oxygen-affinity form of Hb and in both reported cases, heterozygosity of this allele caused a pronounced erythrocytosis (8, 9). Accordingly, the partial pressure of oxygen (pO2), partial pressure of carbon dioxide (pCO2), oxygen saturation (SaO2), and p50 (partial pressure of oxygen at which hemoglobin is 50% saturated) in arterial blood samples from Hbb-b2Plt12/Plt12 mice and their Hbb-b2Plt12/+ littermates were determined by using a blood gas analyzer. Hbb-b2Plt12/Plt12 mice had abnormally low p50 values, consistent with a higher affinity of hemoglobin for oxygen. pO2 levels were also lower in Hbb-b2Plt12/Plt12 mice, indicating that the presence of fewer free oxygen molecules in the blood compared with wild-type mice, and, consistent with these observations, the percentage of all of the available hemoglobin binding sites saturated with oxygen (SaO2) was elevated in Hbb-b2Plt12/Plt12 mice. pCO2 levels were unaltered (Fig. 3A).

In contrast to humans, mice have two β globin genes: Hbb-b1, which encodes the β major chain (βmaj); and Hbb-b2, which encodes the β minor chain (βmin) (Fig. S1C). The β-chain variants are normally expressed in unequal amounts resulting in approximately fourfold more βmaj hemoglobin over the βmin variant (10, 11). Chromatographic analysis of blood from Hbb-b2Plt12/Plt12 mice revealed that whereas the expected excess of βmin over βmaj was evident in wild-type mice, only the βmin variant was present in the mutants (Fig. 3B). Because previous studies have linked increased βmin expression with high serum EPO concentrations (12, 13), this observation is likely to reflect the excess EPO evident in Hbb-b2Plt12/Plt12 mice (see below).

Excessive Erythropoiesis in Hbb-b2Plt12/Plt12 Mice. Examination of an additional cohort of mice revealed that in addition to severe thrombocytopenia and erythrocytosis, Hbb-b2Plt12/Plt12 mice also displayed a slightly elevated white blood cell count (Table 2). In mice heterozygous for the Hbb-b2Plt12 mutation, modest thrombocytopenia was accompanied by a high red blood cell count and hematocrit, but this was significantly milder than observed in Hbb-b2Plt12 homozygotes (Table 2). The hematopoietic phenotype in Hbb-b2Plt12/Plt12 mice was intrinsic to bone marrow cells, with erythrocytosis and thrombocytopenia developing in wild-type recipients 3–5 mo after Hbb-b2Plt12/Plt12 bone marrow transplants (Table 2). The erythrocytosis was accompanied by an elevated number of nucleated erythroid cells in the bone marrow. Similarly, the spleens of Hbb-b2Plt12/Plt12 mice were enlarged due to selectively expanded erythropoiesis, with eightfold elevation in the number of nucleated erythroid cells evident in this organ (Table S2). The splenic architecture was disrupted, with grossly expanded red pulp and small lymphoid follicles (Fig. 2). These features were reproduced in wild-type recipients of Hbb-b2Plt12/Plt12 bone marrow transplants (Table 2 and Table S2).

Fig. 1. Plt12, an ENu-induced mutation that causes semidominant erythrocytosis and thrombocytopenia. (A) The low platelet count observed in the Plt12 founder G1 mouse was transferred to second generation (G2) offspring consistent with a heritable, dominantly acting mutation. (B) Red blood cell and platelet numbers in a cohort of offspring from affected Plt12 parents revealing a proportion of animals with both exacerbated thrombocytopenia and marked erythrocytosis. (C) Erythema, particularly evident in the ears, in Plt12 mice. The control mouse (Left) had a platelet count of 1060 × 10^9/L and a red cell count of 11.49 × 10^12/L. The affected mouse (Right) had a platelet count of 353 × 10^9/L and a red cell count of 16.44 × 10^12/L.
sections of these organs was not significantly different from that in wild-type mice (Table S2). However, Hbb-b2₉declare/Plt12 mice were more actively cycling, with significantly more LSK cells in the G₁ phase of the cell cycle than wild-type controls (Fig. 6A). The number and identity of hematopoietic cells within the peritoneal cavity were also unaltered in Hbb-b2₉declare/Plt12 mutants (Table S2).

Oxygen concentration influences hematopoietic stem cell (HSC) activity, particularly cell cycling, with low O₂ tension associated with maintenance of quiescence (15). In Hbb-b2₉declare/Plt12 mice, the number of cells in the lineage-negative Sca1⁺ c-Kit⁺ (LSK) fraction, in which all hematopoietic stem cells reside, was no different to that in wild-type controls, nor were the numbers of long-term (LT) HSCs, short-term (ST) HSCs, or multipotent progenitor cells (Fig. 6A). However, stem cells in Hbb-b2₉declare/Plt12 mice were more actively cycling, with significantly more LSK cells in the G₁ phase of the cell cycle than wild-type mice, at the expense of cells in G₀, and this was also observed in hematopoietic progenitor cells (Fig. 6B).

**Table 1. Pathological lesions in Plt12 mice**

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Frequency (%)</th>
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<tr>
<td></td>
<td>Healthy Plt12/Plt12</td>
</tr>
<tr>
<td>Liver</td>
<td>CS7BL/6 (n = 3–11)</td>
</tr>
<tr>
<td>Fatty degeneration</td>
<td>27</td>
</tr>
<tr>
<td>Erythroid foci</td>
<td>100</td>
</tr>
<tr>
<td>Erythropoiesis</td>
<td>100</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>Gross enlargement</td>
<td>100</td>
</tr>
<tr>
<td>Excess erythropoiesis</td>
<td>100</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
</tr>
<tr>
<td>Emphysema</td>
<td>64</td>
</tr>
<tr>
<td>Pigmented macrophages in alveoli</td>
<td>36</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
</tr>
<tr>
<td>Vacuolation of ventricle walls</td>
<td>11</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
</tr>
<tr>
<td>Excess mononuclear cells</td>
<td>100</td>
</tr>
<tr>
<td>Bone trabeculae overgrowth</td>
<td>9</td>
</tr>
<tr>
<td>Dilatation of marrow sinusoids</td>
<td>50</td>
</tr>
<tr>
<td>Loss of abdominal fat</td>
<td>100</td>
</tr>
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</table>

The frequency of lesions was determined by post mortem examination and examination of hematoxylin and eosin-stained tissue sections from mice of the indicated genotypes.

The altered erythropoiesis and thrombopoiesis in Hbb-b2₉declare/Plt12 mice was not accompanied by defects in other myeloid cell lineages; numbers of granulocytes and monocytes in the blood were within the normal range, as were the numbers of morphologically recognizable granulocytic cells, monocytes, and eosinophils, and the numbers of their clonogenic progenitors, in the bone marrow and spleen (Table 2 and Tables S2 and S3). Flow-cytometric analysis of myeloid progenitor cells, defined by expression of specific combinations of cell surface markers, confirmed similar numbers of progenitors in Hbb-b2₉declare/Plt12 mice compared with wild-type controls (Fig. 6D). The circulating concentration of thrombopoietin (TPO), the major cytokine regulator of steady-state platelet number, is normally inversely correlated with platelet number, a feedback regulatory mechanism controlled by platelet-associated TPO receptor-mediated cytokine clearance (14). Unexpectedly, relative to wild-type controls, an abnormally low circulating concentration of TPO was observed in Hbb-b2₉declare/Plt12 mice (Fig. S2A). However, reduced TPO did not appear to be the cause of the thrombocytopenia, because administration of TPO at doses sufficient to elevate platelet counts in wild-type mice did not significantly increase platelet counts in Hbb-b2₉declare/Plt12 mutants (Fig. S2B).

Other Hematological Parameters in Hbb-b2₉declare/Plt12 Mice. The altered erythropoiesis and thrombopoiesis in Hbb-b2₉declare/Plt12 mice was not accompanied by defects in other myeloid cell lineages: numbers of granulocytes and monocytes in the blood were within the normal range, as were the numbers of morphologically recognizable granulocytic cells, monocytes, and eosinophils, and the numbers of their clonogenic progenitors, in the bone marrow and spleen (Table 2 and Tables S2 and S3). Flow-cytometric analysis of myeloid progenitor cells, defined by expression of specific combinations of cell surface markers, confirmed similar numbers of progenitors in Hbb-b2₉declare/Plt12 mice compared with wild-type controls (Fig. 6D). The number and identity of hematopoietic cells within the peritoneal cavity were also unaltered in Hbb-b2₉declare/Plt12 mutants (Table S2).

Thrombocytopenia in Hbb-b2₉Declare/Plt12 Mice Is Attributable to Defective Platelet Production. The frequency of megakaryocyte progenitors, measured as cells capable of production of mega-karyocyte colonies in clonogenic assays, was normal in the bone marrow and spleens of Hbb-b2₉declare/Plt12 mice (Table S3). Similarly, the number of megakaryocytes observed in histological sections from bone marrow and spleens of Hbb-b2₉declare/Plt12 mice was not significantly different from that in wild-type mice (Table S2). However, Hbb-b2₉declare/Plt12 megakaryocytes appeared smaller than normal with reduced cytoplasm (Fig. 5A-C). The lifespan of circulating platelets in Hbb-b2₉declare/Plt12 mice was normal (Fig. S5A), and splenectomy did not correct the thrombocytopenia (Fig. 5C), suggesting that altered half-life or increased sequestration associated with splenomegaly did not account for the low numbers of platelets in these mice. The circulating concentration of thrombopoietin (TPO), the major cytokine regulator of steady-state platelet number, is normally inversely correlated with platelet number, a feedback regulatory mechanism controlled by platelet-associated TPO receptor-mediated cytokine clearance (14). Unexpectedly, relative to wild-type controls, an abnormally low circulating concentration of TPO was observed in Hbb-b2₉declare/Plt12 mice (Fig. S2A). However, reduced TPO did not appear to be the cause of the thrombocytopenia, because administration of TPO at doses sufficient to elevate platelet counts in wild-type mice did not significantly increase platelet counts in Hbb-b2₉declare/Plt12 mutants (Fig. S2B).

**Fig. 2.** Disrupted tissue morphology in Hbb-b2₉Declare/Plt12 mice. Micrographs of hematoxylin/eosin-stained histological sections of representative tissue sections from Hbb-b2₉Declare/Plt12 (right) and wild-type Hbb-b2⁰ mice (left). Fatty degeneration in liver (A), lung emphysema (B), and expanded red pulp in spleen (C) can be observed in Hbb-b2₉Declare/Plt12 mice compared with their healthy littermate controls.

**Fig. 3.** Hbb-b2₉Declare leads to production of a high O₂-affinity variant of Hb. (A) Comparison of the partial pressure of oxygen (pO₂), partial pressure of carbon dioxide (pCO₂), oxygen saturation (SaO₂), and p50 (partial pressure of oxygen at which hemoglobin is 50% saturated) in arterial blood samples from Hbb-b2₉Declare mice and Hbb-b2⁰ littermates. (B) Alkaline cellulose acetate and acid citrate agar electrophoretic analysis of blood indicating the expected excess of αβmaj over αβmin in wild-type mice, with dominance of the αβmin variant in Hbb-b2₉Declare/Plt12 mice. Mean values are indicated with error bars denoting 1 SD. *P < 0.05 for comparison of data from Hbb-b2₉Declare/Plt12 mice with that of Hbb-b2⁰ controls.

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Table 2. Erythrocytosis and thrombocytopenia in Plt12 mutant mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Peripheral blood</th>
<th>Transplants</th>
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<tr>
<td></td>
<td>+/+ (n = 19)</td>
<td>+/+ (n = 6)</td>
</tr>
<tr>
<td>Platelet count (×10⁶/mL)</td>
<td>1205 ± 244</td>
<td>839 ± 3</td>
</tr>
<tr>
<td>Red cell count (×10⁶/mL)</td>
<td>10.8 ± 0.5</td>
<td>11.7 ± 0.6</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>52.3 ± 2.3</td>
<td>50.5 ± 2.0</td>
</tr>
<tr>
<td>White cell count (×10⁶/mL)</td>
<td>10.7 ± 1.6</td>
<td>9.7 ± 2.2</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>9.2 ± 1.6</td>
<td>8.0 ± 2.0</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.2</td>
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</table>

Means ± SDs are shown. *P < 0.05 for pairwise t test comparison of data from Hbb-b2Plt12 or Hbb-b2Plt12 mice with Hbb-b2+ controls or recipients of Hbb-b2Plt12 bone marrow with that from recipients of Hbb-b2+ marrow, after correction for multiple testing.

Discussion

Diverse mutations in hemoglobin-encoding genes have been described that cause a range of hematological disorders. We describe here Hbb-b2Plt12, a mouse germline mutation that converts GAT to GGT at codon 100 of the gene encoding Hb β minor that is predicted to cause a substitution of aspartic acid by glycine. This mutation results in production of a high O₂-affinity form of hemoglobin: Hbb-b2Plt12. These mice displayed abnormally low blood O₂ partial pressure and high oxygen saturation values. The Plt12 mouse is a model of the precise mutation referred to as hemoglobin Hotel Dieu, GAT to GGT at codon 99 in human, a rare mutation associated with erythrocytosis (8, 9).

Mutations at position 99 in the β chain are archetypal examples of variants that affect the intersubunit β1-α2 contacts that control the transition between the high- and low-affinity Hb forms. Located in the G-helix region of the β Hb chain, Asp99 forms a hydrogen bond with Tyr42 within the α chain C helix, forming a major contact in the α1-β2 interaction. All possible mutations at Hb β codon 99 have been described and any change in Asp99 results in a marked increase in O₂ affinity (4).

Heterozygosity for the Hbb-b2Plt12 allele resulted in a mild erythrocytosis that was significantly exacerbated in mice homozygous for the mutant allele. Semidominant erythrocytosis has also been observed in mice with an ENU-induced mutation causing substitution of tyrosine for cysteine at amino acid 145, a model of hemoglobin Rainier (6). Although this latter study did not characterize the erythrocytosis extensively, the spleen enlargement and expanded splenic erythropoiesis reported reconcile with our observations that Hbb-b2Plt12 mice had elevated circulating concentrations of EPO and a profound erythrocytosis with splenomegaly and excess CFU-E, consistent with tissue hypoxia causing overproduction of red blood cells driven by excess EPO-driven erythropoiesis. In humans heterozygous for mutations affecting the homologous residue in Hb β, EPO measurements have not always yielded consistent results, but high concentrations have been reported (16), suggesting a similar mechanism drives erythrocytosis in these patients. Genetic modifiers influenced the lethal phenotype in Hbb-b2Plt12 mice. On both genetic backgrounds, substantial preweaning lethality was observed, but whereas BALB/c weanlings soon succumbed to disease, C57BL/6 survivors remained healthy. Histopathological examination of these ostensibly healthy young adult mice revealed that cardiac hypertrophy accompanied the splenomegaly and active bone marrow hematopoiesis and lung pathology was also evident. A histopathological examination of moribund BALB/c Hbb-b2Plt12 mice revealed fatty degeneration of the liver in the majority of mice, in addition to the emphysema, cardiac, and hematopoietic abnormalities evident in healthy cohorts. Lethal cardiopulmonary abnormalities have previously been associated with erythrocytosis in transgenic mice overexpressing EPO (17), suggesting that the similar pathologies in Hbb-b2Plt12 mice might account for the postweaning lethality observed. Although similar lesions may underlie the preweaning lethality evident in both BALB/c and C57BL/6 Hbb-b2Plt12 mice, a phenotype also described in hemoglobin Rainier mouse model (6), we have not examined the pathophysiology of this early disease.

In Plt12 mutant mice, the erythrocytosis commonly associated with high O₂-affinity variants of hemoglobin was accompanied by thrombocytopenia: although mild in Plt12 heterozygotes, the number of platelets in Hbb-b2Plt12 mice was 20% of that observed in wild-type controls. Thrombocytopenia has not been reported in human subjects with erythrocytosis caused by high O₂-affinity mutations in Hb-encoding genes. Although this may result from differences in human and mouse pathophysiology,

![Fig. 4. Erythrocytosis in Hbb-b2Plt12 mice. (A) Similar kinetics of loss of labeled red blood cells from the circulation (Left) but increased emergence of unlabeled cells following pulse labeling (Right) in Hbb-b2Plt12 mice compared with wild type, indicative of normal cellular half life but increased rate of erythrocyte production. (B) Elevated serum erythropoietin (EPO) concentration in Hbb-b2Plt12 mice of 1.5–12 mo of age. Mean values are indicated with error bars denoting 1 SD. *P < 0.05 for comparison of data from Hbb-b2Plt12 mice with that of Hbb-b2+ controls.](image-url)
the overwhelming majority of patients are heterozygous for mutant alleles and, as observed in Plt12 mice, thrombocytopenia may only significantly manifest when mutations are homozygous. The thrombocytopenia in Hbb-b2Plt12/Plt12 mice was not attributable to deficient megakaryocyte production. There was no significant reduction in the frequency of megakaryocyte progenitor cells or morphologically recognizable megakaryocytes in the bone marrow and spleen; indeed, given that the spleens of Hbb-b2Plt12/Plt12 and wild-type mice. Comparable half-life of platelets in the circulation of Hbb-b2Plt12/Plt12 and wild-type mice. Numbers of circulating platelets before surgery and 28 and 85 d after splenectomy showing persistent thrombocytopenia in Hbb-b2Plt12/Plt12 mice. Mean values are indicated with error bars denoting 1 SD.

Fig. 5. Thrombocytopenia in Hbb-b2Plt12/Plt12 mice. (A) Histological sections of spleens showing von Willebrand factor-stained megakaryocytes at similar numbers but of smaller size and with scant cytoplasm in Hbb-b2Plt12/Plt12 mice compared with wild type. (B) Comparable half-life of platelets in the circulation of Hbb-b2Plt12/Plt12 and wild-type mice. (C) Numbers of circulating platelets before surgery and 28 and 85 d after splenectomy showing persistent thrombocytopenia in Hbb-b2Plt12/Plt12 mice. Mean values are indicated with error bars denoting 1 SD.

Materials and Methods

Isolation of the Plt12 Allele of Hbb-b2. Male BALB/c mice were injected intraperitoneally with a total dose of 255 mg/kg of ENU (Sigma-Aldrich) divided into three weekly injections, as described (27). Treated mice were allowed to recover fertility for 4 wk before mating with untreated BALB/c females to yield first-generation (G1) progeny. At 7 wk of age, blood from G1 mice was collected from the retroorbital plexus into Microtainer tubes containing EDTA (Becton Dickinson) and analyzed using an Advia 120 automated hematological analyzer (Siemens).

Hematology. For differential cell counts, blood was collected into tubes containing EDTA (Sarstedt) and analyzed with an Advia 120 analyzer (Bayer). Cytocentrifuge preparations were stained with May-Grumwald Giemsas before microscopic examination. Femurs were fixed in 10% buffered formalin, embedded in paraffin, and 1–3-μm sections were stained with hematoxylin/ eosin for megakaryocyte enumeration via light microscopy. Blood gas analysis was performed with a Radiometer ABL 715 blood gas analyzer at the Royal Melbourne Hospital, Australia. Chromatographic analysis of blood was performed with Paragon Hb and Paragon Acid Hb gels (Beckman Coulter). For splenectomy, 10-wk-old Hbb-b2Plt12/Plt12 and Hbb-b2Plt12/Plt12 mice were anesthetized and a small incision was made in the skin. The peritoneal membrane was opened to expose the spleen. The spleen was surgically removed intact, and wounds were sealed. The peritoneal membrane was sealed with sutures and skin with wound clips.
Measurement of Platelet and Red Blood Cell Half-Life. Blood cell half-life studies were performed on cohorts of mice at least 4 wk following transplantation with Hbb-b2Plt12/Plt12 or Hbb-b2+/+ bone marrow. Mice were injected i.v. with 1.5 mg NHS-biotin (Sigma) as described previously (28), and repeated tail-blood samples were collected. Biotinylated platelets and red blood cells were visualized by flow cytometry with fluorochrome-conjugated streptavidin in conjunction with platelet-specific anti-CD41 antibody or red blood cell-specific anti-Ter119 antibody (BD Biosciences). Counting beads (BD Biosciences) were added into each sample, and samples were analyzed with LSRII flow cytometer (Becton Dickinson).

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13. Samakoglu S, et al. (2001) beta2Minor-globin messenger RNA accumulation in reticulocytes governs improved erythropoiesis in beta thalassemic mice after erythropoi- 