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Excessive iron absorption is one of the main features of β-thalassemia and can lead to severe morbidity and mortality. Serial analyses of β-thalassemic mice indicate that while hemoglobin levels decrease over time, the concentration of iron in the liver, spleen, and kidneys markedly increases. Iron overload is associated with low levels of hepcidin, a peptide that regulates iron metabolism by triggering degradation of ferroportin, an iron-transport protein localized on absorptive enterocytes as well as hepatocytes and macrophages. Patients with β-thalassemia also have low hepcidin levels. These observations led us to hypothesize that more iron is absorbed in β-thalassemia than is required for erythropoiesis and that increasing the concentration of hepcidin in the body of such patients might be therapeutic, limiting iron overload. Here we demonstrate that a moderate increase in expression of hepcidin in β-thalassemic mice limits iron overload, decreases formation of insoluble membrane-bound globins and reactive oxygen species, and improves anemia. Mice with increased hepcidin expression also demonstrated an increase in the lifespan of their red cells, reversal of ineffective erythropoiesis and splenomegaly, and an increase in total hemoglobin levels. These data led us to suggest that therapeutics that could increase hepcidin levels or act as hepcidin agonists might help treat the abnormal iron absorption in individuals with β-thalassemia and related disorders.

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Hepcidin as a therapeutic tool to limit iron overload and improve anemia in β-thalassemic mice

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Excessive iron absorption is one of the main features of β-thalassemia and can lead to severe morbidity and mortality. Serial analyses of β-thalassemic mice indicate that while hemoglobin levels decrease over time, the concentration of iron in the liver, spleen, and kidneys markedly increases. Iron overload is associated with low levels of hepcidin, a peptide that regulates iron metabolism by triggering degradation of ferroportin, an iron-transport protein localized on absorptive enterocytes as well as hepatocytes and macrophages. Patients with β-thalassemia also have low hepcidin levels. These observations led us to hypothesize that more iron is absorbed in β-thalassemia than is required for erythropoiesis and that increasing the concentration of hepcidin in the body of such patients might be therapeutic, limiting iron overload. Here we demonstrate that a moderate increase in expression of hepcidin in β-thalassemic mice limits iron overload, decreases formation of insoluble membrane-bound globins and reactive oxygen species, and improves anemia. Mice with increased hepcidin expression also demonstrated an increase in the lifespan of their red cells, reversal of ineffective erythropoiesis and splenomegaly, and an increase in total hemoglobin levels. These data led us to suggest that therapeutics that could increase hepcidin levels or act as hepcidin agonists might help treat the abnormal iron absorption in individuals with β-thalassemia and related disorders.

Introduction

β-thalassemia is one of the most common congenital anemias arising from partial or complete lack of β-globin synthesis. β-thalassemia major, also known as Cooley anemia, is the most severe form of this disease and is characterized by ineffective erythropoiesis (IE) and extramedullary hematopoiesis (EMH) in the liver and spleen. Patients require regular blood transfusions to sustain life (1). In the milder form, termed β-thalassemia intermedia, blood transfusions are not always necessary, yet iron overload still occurs due to progressive iron absorption from the gastrointestinal tract (2). In β-thalassemia intermedia patients, studies show that the rate of iron absorption from the gastrointestinal tract is approximately 3 to 4 times greater than normal, varying between 2 and 5 g per year depending on the severity of erythropoiesis (1). Regular transfusions may double the rate of iron accumulation. In these patients, IE often worsens over time, exacerbating anemia, iron absorption, and splenomegaly (3). Increased gastrointestinal iron absorption may also play a role in transfused β-thalassemia major patients, increasing when hemoglobin (Hb) levels decrease (4). Progressive iron overload affects multiple organs and is the primary cause of death in patients with β-thalassemia syndromes (1).

The th3/+ mouse, a model of β-thalassemia, harbors a heterozygous deletion of βmajor and βminor genes (5, 6) and exhibits features comparable to those of patients affected by β-thalassemia intermedia, including Hb levels between 7 and 9 g/dl (5–7), IE, EMH, increased production of immature erythrocytes, aberrant erythrocyte morphology, and hepatosplenomegaly. Serum iron, transferrin (Tf) saturation, and nontransferrin-bound iron (NTBI) levels are elevated in th3/+ mice (8, 9). Iron accumulates primarily in the spleen and in the Kupffer cells of the liver. The Hb levels in th3/+ mice decrease with time, while the spleen size, the number of nucleated erythrocytoid cells, and the ratio of liver to spleen iron all increase (8). Thus, over time these mice exhibit some of the features associated with the most severe forms of the disease.

Hepcidin (HAMP) (9, 10), a peptide produced in the liver, regulates iron metabolism by triggering the degradation of ferroportin (FPN1) (11), an iron-export protein localized on absorptive enterocytes, hepatocytes, and macrophages. Altered expression of hepcidin is responsible for the modifications of iron metabolism that characterize several diseases, including β-thalassemia (8, 12). th3/+ mice have relatively low hepatic Hamp1 expression, suggesting that insufficient hepcidin may be responsible for the high iron levels in these mice (8, 13, 14). Similarly, low HAMP levels have been measured in the urine of patients with β-thalassemia (15), further supporting this hypothesis.

The iron absorbed by patients with β-thalassemia intermedia or th3/+ mice is excessive relative to the amount of iron needed to maintain a Hb of 9 g/dl (2, 3, 8). In this case, liver parenchymal cells store the surplus iron. Therefore, we postulate that (a) limiting the dietary
Iron intake of th3/+ mice would decrease organ iron with no effect on erythropoiesis, and that (b) similar results could be achieved by limiting dietary iron intake via increased Hamp1 expression. Since Fpn1 is also localized on macrophages, upregulation of Hamp1 in normal mice affects both dietary iron absorption and the recycling of iron, ultimately worsening their anemia. Since endogenous hepcidin in th3/+ mice is low relative to the amount of liver iron available for erythropoiesis, moderate hepcidin supplementation might limit iron absorption without interfering with the release of iron for erythropoiesis. Thus, our study reveals a potential role for Hamp1 or Hamp1 agonists in the development of new pharmacological approaches to treating the abnormal iron absorption in β-thalassemia and related disorders.

Results

Low dietary iron intake markedly affects erythropoiesis in normal mice. WT and th3/+ mice were generated by breeding and by engraftment of WT and th3/+ HSCs into normal mice after complete myeloablation (8). Using the engraftment of HSCs instead of breeding, we were able to obtain a larger number of mice for analysis in a shorter time. Here we present the results obtained using transplanted mice, there being no major differences observed between th3/+ mice on diets containing 35 and 2.5 ppm iron generated by the 2 methods (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI41717DS1).

A commercial rodent diet contains 200 ppm iron, more than necessary for normal physiological requirements. Therefore, we investigated defined diets containing either 35 ppm iron (iron-sufficient diet) or 2.5 ppm iron (iron-deficient diet).
The hematological values of WT mice fed the 35- and 2.5-ppm iron diets were similar (Figure 1, A and B) and essentially identical to those of mice analyzed on standard commercial diet (not shown). However, the amount of iron in the livers and spleens of WT mice fed the 2.5-ppm diet was reduced compared with mice fed the 35-ppm diet (Figure 1C). The serum iron levels were not significantly different from those of controls, although the corresponding Tf saturations were reduced (Figure 1D) with increased serum erythropoietin (Epo) levels (Supplemental Figure 2A).

After 5 months, WT mice fed the 35-ppm diet did not show any hematological changes (Figure 1, A and B) and exhibited a decrease in the total amount of iron in the spleen (Figure 1C) compared with mice analyzed at 1 month. The iron in the kidneys and heart was reduced (Supplemental Figure 3, A and B). Mice fed the 2.5-ppm diet for 5 months, however, exhibited a dramatic decrease in Hb, mean cell Hb (MCH), mean corpuscular volume (MCV), and mean corpuscular Hb concentration (MCHC) levels (Figure 1, A and B).

Serum iron levels and Tf saturation were reduced (Figure 1D). In addition, Hamp1 expression was extremely low (Figure 1E), while Epo levels were increased (Supplemental Figure 2A). FACS analysis showed that there were no statistically significant differences in the percentages or absolute numbers of immature (CD71+ Ter119+) and mature (CD71– Ter119+) erythroid progenitor cells in the spleen (Figure 2, A and C) or BM (not shown) of WT mice on either the 2.5- or 35-ppm diet for 1 and 5 months. In summary, this analysis indicated that WT mice on an iron-restricted diet exhibit reduced Hb and rbc synthesis.

Low dietary iron intake positively affects erythropoiesis while reducing tissue iron levels in th3/+ mice. As in the case of normal mice, the hematological values of th3/+ mice fed the 35- and 2.5-ppm iron diets for 1 month were similar (Figure 1, A and B) and identical to those of mice analyzed on a standard commercial diet (not shown). As in WT mice, a reduction was seen in the amount of iron in the liver and spleen (Figure 1C) and also in the kidneys and heart (Supplemental Figure 3, A and B) of the mice fed the 2.5-ppm diet. The serum iron levels and Tf saturation of th3/+ mice fed the 2.5-ppm iron diet were unchanged relative to controls (Figure 1D). Epo levels were increased (Supplemental Figure 2A) and Hamp1 expression was reduced in the th3/+ mice fed the 2.5-ppm diet (Figure 1E). This suggests that Hamp1 expression is more sensitive to the sup-
pressive effect of iron restriction in states of increased erythropoietic activity than in th3/± mice. The size of the spleen in th3/± mice fed the 2.5-ppm diet for 1 month was reduced (Figure 2D), while that of the liver was unchanged (Supplemental Figure 4A).

After 5 months on the 35-ppm diet, th3/± mice exhibited decreases in Hb levels compared with th3/± mice at 1 month (from 9.0 ± 0.7 g/dl to 6.9 ± 0.9 g/dl; Figure 1A). After the same length of time, th3/± mice fed the 2.5-ppm diet exhibited low Hamp1 expression (Figure 1E) together with low serum iron levels and Tf saturations (Figure 1D), while Epo levels were not statistically different (Supplemental Figure 2A). Organ iron content was considerably reduced (Figure 1C and Supplemental Figure 3, A and B). Both the spleen and liver weights were reduced (Figure 2D and Supplemental Figure 4A, respectively). However, th3/± mice on the 2.5-ppm iron diet did not experience a drop in their Hb concentrations (Figure 1A and B). FACS analysis and measurement of organ cell numbers revealed that th3/± mice fed the 2.5-ppm diet for 5 months exhibited a decrease in both the percentage and total number of immature erythroid progenitor cells (CD71+Ter119+) in their spleens together with an increase in the population of mature cells (CD71+Ter119+; Figure 2, A–C). In conclusion, these findings demonstrate that th3/± mice on a low iron diet, despite relative systemic iron deficiency, do not exhibit a worsening anemia although its secondary effects, such as hepatosplenomegaly, are mitigated.

Overexpression of transgenic Hamp1 leads to sequestration of iron in the spleen and erythropoietic effects in normal mice. We used mice that uniformly overexpress a transgenic form of Hamp1 in the liver (Tg-Hamp mice, C57BL/6 background) (16). We engrafted these mice with th3/± HSCs (C57BL/6 background) generating Tg-Hamp/th3 mice. HSCs from WT mice were transplanted into Tg-Hamp mice to generate positive controls. Mice that did not overexpress the transgenic Hamp1 were designated Tg−. Tg− mice were also transplanted with WT or th3/± HSCs to generate Tg−/th3-negative controls, respectively. All mice were maintained on the 35-ppm diet.

After 1 month on this diet, most Tg-Hamp mice showed reduced Hb, MCH, MCV, and MCHC levels and increased rbc and reticulocyte counts (Figure 3, A and B) as well as an increased number of

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**Figure 3**

Hematological and iron-related parameters in Tg-Hamp and Tg-Hamp/th3 mice fed a diet containing 35 ppm iron for 1 and 5 months. (A) Hb, rbc, and reticulocyte values. (B) MCH, MCV, and MCHC values. (C) Total iron content of liver and spleen as measured by atomic absorption. Groups of Tg-Hamp mice were compared with Tg− mice, and Tg-Hamp/th3 mice with Tg−/th3 controls. (D) Northern blot analysis of the endogenous (bottom band) and transgenic (upper band) Hamp1 mRNA transcripts. 18S, ribosomal subunit 18S loading control. *P < 0.05; **P < 0.01; ***P < 0.001. Data are presented as mean ± SD.
immature erythroid progenitor cells (CD71+Ter119+) in the spleen (Figure 4C). In addition, the serum iron levels and the Tf saturations were reduced, becoming nearly undetectable in Tg-Hamp mice compared with Tg− controls (not shown). Epo values did not change significantly (Supplemental Figure 2B).

We employed both Northern blot and real-time quantitative PCR (Q-PCR) assays to quantify the total Hamp1 transcribed (Figure 3D). Identification of the endogenous and transgenic mRNAs by Northern blot is possible because Tg-Hamp mRNA contains the β-globin poly(A) sequence instead of the endogenous Hamp1 3′ UTR and poly(A) elements, resulting in an increased length of the transcript (16). The Northern blot assay indicated that both the Tg-Hamp and Tg-Hamp/th3 mice expressed only the transgenic Hamp1 (Figure 3D).

In Tg-Hamp mice, Northern blot analysis indicated that the level of transgenic Hamp1 is increased approximately 2-fold compared with the mean expression of endogenous Hamp1 in Tg− mice. Consistently, the total amount of iron in the liver was reduced in Tg-Hamp mice compared with Tg− mice. However, the total amount of iron in the spleen of Tg-Hamp mice was increased after 1 month on the diet (Figure 3C). In 2 mice, designated high Hamp1 expressers (Figure 3D), we observed very low levels of Hb (6.8 and 5.2 g/dl, respectively; Figure 3A) compared with an average value of 11.2 g/dl in control mice. This correlates with higher levels of Hamp1 expression (approximately 4-fold when compared with the average expression in Tg− mice). In these mice, the iron content of the liver was also reduced, but that in the spleen was more than 6.2-fold higher than that in Tg− mice and 3.2-fold higher than in the other Tg-Hamp mice. These individual differences indicate that very high levels of hepcidin can markedly inhibit the release of iron from macrophages (Figure 3C and Supplemental Figure 5A), leading to a dramatic inhibition of rbc production. To confirm that the Hamp1 transgenic mRNAs did not carry any mutation acquired during transgenesis that could modify or alter the correlation between Hamp1 expression and the abnormal parameters observed in these mice, the mRNAs were isolated from the livers of the HHE-1 and HHE-2 mice, and the transgenic Hamp1 was sequenced. No mutations were identified in any of these transcripts.

After 5 months, although the transgenic Hamp1 remained elevated (not shown), no significant differences were observed in hematological parameters (Figure 3, A and B) or the iron content of the liver, spleen (Figure 3C), and heart (Supplemental Figure 3D), when comparing Tg-Hamp to Tg− mice. The only significant reduction of the iron content was observed in the kidneys (Supplemental Figure 3C). The fact that no major differences were present indicates that, after a few months, a yet-undescribed feedback
mechanism in Tg-Hamp mice compensated for the increased level of transgenic Hamp1 expression, leading to normalization of organ iron contents and erythropoiesis.

Limited overexpression of transgenic Hamp1 reduces tissue iron levels with a beneficial effect on erythropoiesis in th3/+ mice. In the majority of Tg/Hamp/th3 mice, moderate overexpression of Hamp1 (on average a 4-fold increase relative to endogenous Hamp1) led to a reduction of the organ iron contents to levels similar to those observed in normal mice on the 35-ppm iron diet. Moreover, the hematological parameters in the majority of Tg-Hamp/th3 mice improved compared with those of Tg/th3 mice after both 1 and 5 months. Specifically, Tg-Hamp/th3 mice exhibited higher Hb levels with lower MCH and MCV (Figure 3, A and B). Epo values did not change (Supplemental Figure 2B). Additionally, the reticulocyte counts (Figure 3A) and both the percentage and the absolute number of immature erythroid progenitor cells (CD71+Ter119+)
in the spleen decreased (Figure 4, A–C). In a subset of thalassemic mice, we analyzed erythroid cells from both spleen and BM by FACS analysis using a recently described technique that enables a more discrete resolution of the distinct stages in erythroid differentiation using the Ter119 and CD44 antibodies. This assay allows the separation of erythroid cells into distinct populations corresponding to proerythroblasts (fraction I), basophilic (II), polychromatic (III), orthochromatic cells, and reticulocytes (IV), and mature rbc (V) (Supplemental Figure 6, only spleen is shown; similar results were observed in the BM) (18). This analysis confirmed that both the percentage and the absolute number of immature erythroid progenitor cells in the spleen decreased as seen using the CD71 and Ter119 markers.

Low serum iron and Tf saturation levels were very low to undetectable at 1 month. Furthermore, iron content in the livers, spleens, and kidneys was reduced (Figure 3C and Supplemental Figure 3C). We also observed a reduction of splenomegaly (Figure 4D), a more normal architecture of the spleen (Supplemental Figure 7A), and a reduction of EMH in the liver (Supplemental Figure 7B). A single Tg-Hamp/th3 animal (Figure 3D) exhibited high hepcidin expression, approximately 9 times that of the average observed in the Tg/th3 mice. This HHE mouse also displayed a low level of Hb (4 g/dl; Figure 3A) and reduced iron in the liver but no change in its splenic iron content compared with control mice (Figure 3C). Iron present in the spleen was mostly sequestered in macrophages, indicating that high levels of hepcidin prevent normal iron recycling, resulting in a markedly negative effect on rbc production. The Hamp1 transgenic transcript of the HHE-3 mouse was sequenced in order to exclude the presence of mutations acquired during transgenesis (not shown).

We then quantified the mRNA levels in the Northern blots shown in Figure 3D and correlated the results with a series of parameters, such as Hb, liver and spleen iron, and splenomegaly (Figure 5). This analysis indicated that moderate overexpression of Hamp1 (up to 4-fold that of endogenous levels, excluding the HHE mouse) had a positive effect or no effect on Hb synthesis (Figure 5A) and correlated inversely with a decreased liver and spleen iron load and amelioration of splenomegaly (Figure 5, B–D). These observations, together with those of the blood profile (Figure 3) and the decrease in the number of immature erythroid cells (Figure 4C), indicate that reduction of erythroid activity ameliorates the morphology of the spleen (Figure 5H and Supplemental Figure 7A) in these mice and reverses splenomegaly (Figure 5B). In contrast, analysis of the HHE mouse indicated that excessive hepcidin production
in β-thalassemia is detrimental, as indicated by the low Hb level (Figure 5A), iron overload in the spleen (Figure 5, D and G), and splenomegaly (Figure 5B), emphasizing the fact that a therapeutic benefit would depend upon the level of Hamp1 achieved.

At 5 months, the Tg-Hamp/th3 mice still exhibited reduced organ iron contents (although the heart was not affected; Figure 3C and Supplemental Figure 3C) and improved erythropoiesis compared with Tg–/th3 mice (Figure 3A). The Tg-Hamp/th3 mice exhibited increased levels of serum iron and Tf saturation, as did the WT mice overexpressing transgenic Hamp1 (not shown).

Overexpression of transgenic Hamp1 also improves erythropoiesis in th3/+ mice. To identify the causes leading to amelioration of erythropoiesis in these mice, we analyzed additional th3/+ mice maintained on the 2.5-ppm iron diet and Tg-Hamp/th3 mice on the 35-ppm iron diet and compared them to Tg–/th3 control mice (respectively, n = 5, 3, and 4 mice per group). As observed previously, th3/+ mice on the 2.5-ppm iron diet and Tg-Hamp/th3 mice exhibited improved erythropoiesis compared with Tg–/th3 control mice (not shown). In particular, a reduced MCH was corroborated by direct quantification of the heme content using the same number of rbc from each mouse (Figure 6A). This led us to hypothesize that a reduced heme level would limit the formation of insoluble globins (α chain/heme aggregates) and thereby reduce their potential toxicity when they adhere to rbc membranes and produce ROS (19). Thus, a reduction in insoluble α-globins could improve the quality and survival of erythroid cells, leading to a larger number of rbc in the circulation and amelioration of the IE and anemia. To test this hypothesis, we prepared membrane fractions and determined the amount of bound globins by triton acetic acid urea (TAU) gel electrophoresis. th3/+ mice on the 2.5-ppm diet and Tg-Hamp/th3 mice showed a reduction of membrane-associated α chains compared with Tg–/th3 control mice (Figure 6B); this was confirmed by HPLC using the same membrane fractions (Supplemental Figure 8).

Splenomegaly exacerbates anemia both by sequestering rbc and augmenting their removal by erythrophagocytosis. Since we observed that the spleen was reduced in th3/+ mice on the 2.5-ppm iron diet and Tg-Hamp/th3 mice, we devised a new method to evaluate the lifespan of rbc to avoid the potentially confusing factor of splenomegaly. We transfused blood from WT, th3/+ on the 2.5-ppm iron diet, Tg–/th3, and Tg-Hamp/th3 mice into GFP-transgenic mice, which exhibit normal spleen sizes. rbc from the latter group of mice are fluorescent and easy to distinguish from donor rbc by FACS analysis. The rbc from th3/+ mice on the 2.5-ppm diet and Tg-Hamp/th3 mice also exhibited longer life spans, similar to WT rbc (Figure 6C). The rbc from Tg-Hamp/th3 mice exhibited a more normal morphology (Figure 6E). Additionally, a ROS indicator, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA), was added to peripheral blood, spleen, and BM cells. This compound permeates into the cells and is oxidized in the presence of ROS or free heme. Oxidation was detected by monitoring the increase in fluorescence by flow cytometry. This analysis indicated that rbc (Figure 6D) from th3/+ mice on the 2.5-ppm diet and Tg-Hamp/th3 mice have...
reduced fluorescence, and by implication, reduced ROS and/or free heme, when compared with cells from Tg–/th3 control mice. Furthermore, we analyzed distinct erythroid populations from both spleen and BM by FACS analysis using the Ter119 and CD44 antibodies (Figure 7, A–C, only spleen is shown) and investigated in these fractions the level of ROS (18). The analysis of the distinct erythroid populations in the spleen indicates that ROS were reduced in both Tg-Hamp/th3 mice (both fractions IV and V) and th3/+ mice fed the low-iron diet (fraction V) compared with Tg–/th3 controls (Figure 7, D–F).

Furthermore, WT mice and untreated Tg–/th3 mice have different patterns of erythroid differentiation and apoptosis in the BM and spleen. The relative percentage of erythroid cells increases, approximately 2-fold at each stage of differentiation (from fraction I to IV), likely reflecting 1-cell divisions, in WT mice (Figure 8A). In contrast, untreated Tg–/th3 mice increase 3 to 6 times (Figure 8B) between fractions I and II as well as between II and III. This observation corroborates previous studies that indicate that β-thalassemic erythroid cells exhibit increased cell proliferation and reduced cell differentiation compared with those from normal mice (20). However, comparing the FACS profiles of WT and Tg–/th3 mice (Figure 8, A and B, respectively) with those of Tg-Hamp/th3 mice (Figure 8C), we observed that the latter group of mice exhibited an improved profile both in the spleen and BM (not shown), as indicated by a reduction in the number of cells in fractions II to IV, a more balanced ratio of the number of cells between these fractions and a relative increase in terminally differentiated cells in fraction V.

Although we observed similar results in th3/+ mice on the 2.5-ppm iron diet (Figure 8D), further analysis using annexin V identified a large proportion of erythroid precursors undergoing apoptosis (orthochromatic cells and reticulocytes in fraction IV) or exhibiting an abnormal rbc membrane (mature rbc in fraction V, comparing Figure 8, E–G with H). This was not the case in Tg-Hamp/th3 mice.

In conclusion, our data suggest that modest overexpression of hepcidin limits the formation of toxic α chain/heme aggregates, reduces free heme and/or ROS formation, and improves both rbc lifespan and anemia. Moreover, it reduces IE by restoring to normal the relative proportion of cells at different stages of erythroid differentiation rather than by triggering cell death of erythroid precursors.

**Discussion**

Iron balance must be carefully regulated to provide iron as needed while avoiding the toxicity associated with its excess. Tissue iron overload is a primary focus of β-thalassemia management, and if not prevented or adequately treated, is fatal in both transfused and nontransfused patients. NTBI in the circulation damages the heart, endocrine organs, and liver (21). NTBI serves as a catalyst for the formation of ROS, which can cause myocyte damage, arrhythmias, and congestive heart failure, the main causes of death in patients with β-thalassemia (22). Therefore, development of new strategies to reduce excessive iron absorption and tissue iron overload is one of the primary goals of improved management for β-thalassemic patients.

*Figure 8*

Different stages of erythroid differentiation in the spleens of th3/+ mice fed the 2.5-ppm iron diet or overexpressing Hamp1. FACS analysis of representative mice (A) WT, (B) Tg–/th3, (C) Tg-Hamp/th3, and (D) th3/+ on the 2.5-ppm diet mice. Numbers indicate the percentage of cells measured in each distinct erythroid population. E, F, G, and H show representative analyses of membrane phosphatidylserine exposure for stages IV (left panel) and V (right panel) using annexin V. Data are presented as mean ± SD.
For this reason, we considered the possibility that iron overload could be avoided by limiting the amount of iron absorbed. Such an approach might serve as a substitute for or adjunct to iron chelation therapy in patients affected by β-thalassemia intermedia who exhibit increased iron absorption. In fact, it might be superior to iron chelation since it would be expected to prevent exposure to excessive and possibly toxic iron, obviating the need to eliminate an excess sequestered in vital organs. Although it is unclear how much iron is acquired through increased intestinal absorption in patients who require chronic blood transfusions, even minimal iron absorption would be potentially damaging. Therefore, in chronically transfused patients, we propose that limiting or blocking dietary iron absorption will increase the efficacy of their iron chelation therapy.

We hypothesize that the amount of iron absorbed from a standard iron diet is in excess of that required for erythropoiesis in thβ3/− mice. The unchanged Hb levels (~8 g/dl; Figure 1A) in both WT and thβ3/− mice fed the 2.5-ppm diet supports this notion. In other words, β-thalassemic mice require less iron to produce 8 g/dl of Hb than what is required by normal mice to produce 15 g/dl of Hb. Theoretically, then, iron intake in β-thalassemic patients who do not require chronic blood transfusions might be restricted so as to reduce organ iron levels without any detrimental effect on Hb levels.

Because the main cause of increased iron absorption in β-thalassemia intermedia is the low expression of Hamp1 (13, 14), we utilized transgenic mice overexpressing hepcidin to limit iron overload as a complementary approach to dietary iron restriction. Taken together, our data indicate that a moderate increase in the expression of Hamp1 in thβ3/− mice led to hepatic iron levels identical to those in normal mice while splenic iron levels were 4 times less than those in untreated β-thalassemic mice (Figure 3C). These reduced organ iron levels were associated with amelioration of anemia, splenomegaly, and EMH compared with untreated thβ3/− mice (Figure 3A, Figure 4D, and Supplemental Figure 7B). Taken together, these observations demonstrate that iron overload may play an important role in exacerbating IE, increasing splenomegaly, and decreasing Hb levels over time, perhaps by interfering with erythroid maturation or rbc formation.

After 5 months, both normal and thβ3/− mice that overexpressed Hamp1 exhibited increased serum iron levels compared with mice evaluated after 1 month. This occurred despite similar levels of transgenic Hamp1 expression in all mice indicating that other factors, in addition to the level of Hamp1 expression, control ongoing iron absorption, at least in mice. One possibility is that the level of Fpn1 increases over time, allowing more iron to enter through the duodenum. However, Fpn1 expression did not increase in mice overexpressing Hamp1 (Supplemental Figure 9). We cannot exclude the possibility that additional factors modify the translation, maturation, secretion, and ultimately, the serum levels of Hamp.

Both thβ3/− mice and Tg-Hamp/thβ3 mice increased their Hb levels, decreased reticulocyte counts, and reversed IE and splenomegaly. Additionally, reduced MCH and heme levels were observed in these mice (Figures 1–4 and Figure 6A). Thus, even though the total heme and Hb content in individual rbc decreased, anemia was reduced because of increased production of rbc. Moreover, while the number of rbc increased, the number of reticulocytes was reduced. This indicates that the IE in these mice was less severe than in untreated mice. We conclude that the toxicity of free heme and α chains is reduced, thereby making erythropoiesis more efficient.

Therefore, by limiting the availability of iron to erythroid precursors, hepcidin agonists might improve the efficiency of erythropoiesis and the survival of the resulting reticulocytes and erythrocytes, by decreasing the synthesis of heme and, perhaps, α-globin chains. Recently, thβ1/thβ1 mice, a model of β-thalassemia intermedia (23) similar to thβ3/−, treated with apo-Tf demonstrated a significant reduction of splenomegaly and IE, an increase in Hb and rbc concentrations, and higher hepcidin expression, suggesting that misdistribution of iron in β-thalassemia might also contribute to IE (24). In this study, MCH was also decreased. These complimentary observations suggest that decreasing iron availability for erythropoiesis may be beneficial in limiting abnormal rbc production. Decreased iron availability likely results in more effective erythropoiesis, as less iron is available during erythroid development to generate free heme or α-globin precipitates, factors associated with shortened rbc survival. Previously presented data demonstrate that the absence of heme-regulated inhibitor (HRI) kinase, which controls Hb synthesis (25), exacerbates the β-thalassemia phenotype (26), while lack of heme exporter feline leukemia virus subgroup C cellular receptor (FLVCR), which controls heme export (27), impairs rbc formation (28). These observations, along with our new data, suggest that an excess of iron and/or heme (in addition to α-globin) in erythroid cells might be deleterious to erythropoiesis. Moreover, modulation of erythroid iron intake or heme synthesis might also affect the stability of excess α-globin chains or selectively influence the synthesis of α versus β-globin chains. In the first scenario, in the absence of heme molecules, α-globin chains might be extreme-
ly unstable and rapidly eliminated, thereby obviating any toxicity. Alternatively, under conditions of reduced erythroid iron intake, α chains might be produced at a lower rate than β chains, with HRI potentially playing a role in this process. However, Q-PCR analysis of the α-globin mRNA transcript in control and experimental mice did not show any difference (not shown). Additional experiments are required to evaluate the stability and/or degradation of free α chains and their rate of synthesis under condition of low iron.

Furthermore, while the number of rbc were increased in Tg-Hamp/th3 mice, the number of reticulocytes, the proportion of immature erythroid precursors, and the total number of erythroid precursors in the spleen were reduced (Figures 3 and 4). Moreover, the Epo levels in these mice were unchanged from those in th3/+/- mice (Supplemental Figure 2B). Accordingly, we can also hypothesize that an excess of iron might alter the ratio between proliferation and differentiation of erythroid cells when the synthesis of globin chains is impaired. Additional studies will be required to determine whether reduced iron intake can variably modulate the synthesis of α- and β chains, the role of heme and ROS in erythroid differentiation/proliferation, and the mechanisms by which hepcidin agonists affect erythropoiesis.

In our study we also identified a small subset of mice, indicated as HHE (both normal and th3/+), that exhibited reduced Hb levels and elevated iron deposition in splenic macrophages. These findings were associated with elevated Hamp1 expression levels. Thus, although our data demonstrates as proof of concept that increased hepcidin levels can reduce tissue iron overload and improve erythropoiesis in β-thalassemia intermedia mice, this approach will require titration of HAMP or a HAMP agonist to avoid sequestration of iron in macrophages and thus exacerbation of anemia (Figure 9). Further studies are necessary to explore the potential use of hepcidin agonists/activators to prevent iron overload or reverse its toxic effects in β-thalassemia. Nevertheless, our data represent a proof of concept that increased hepcidin levels can reduce tissue iron overload and improve erythropoiesis in β-thalassemia intermedia and support our hypothesis that hepcidin therapy may be beneficial in this disorder.

**Methods**

**Generation of thalassemic mice and transgenic mice overexpressing Hamp1.** WT and th3/+/- embryos were genotyped at 13.5-15.5 days of gestation and the hematopoietic fetal liver cells (HFLCs) harvested as described (29). These HFLCs were then transplanted into WT syngeneic recipients (C57BL/6) (29). Transgenic mice overexpressing Hamp1 in their hepatocytes (Tg-Hamp mice, C57BL/6 background) have already been described (16). The studies described were carried out using defined diets containing 35 and 2.5 ppm of iron (Harlan-Teklad). Pathological analysis of tissues was as previously described (8). All studies were conducted under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Weill Cornell Medical College.

**Hematological studies.** Blood samples were obtained by retro-orbital puncture under anesthesia. Complete blood counts (CBC) were measured on an Advia 120 analyzer (H-System; Bayer Corp.).

**Measurement of tissue iron content, serum iron, heme, and Tf saturation.** The iron content of the liver and spleen as well as serum iron and Tf saturation levels were determined as described in our previous publications (8, 26). The amount of heme in the rbc was quantified using the Quantichrom Heme colorimetric assay (BioAssay Systems) according to the manufacturer’s instructions. Q-PCR. RNA samples extracted from the liver and duodenum of adult mice were retrotranscribed by using the SuperScript II First Strand Kit (Invitrogen). cDNAs were analyzed by Q-PCR with primers specific for Hamp1 (Fw: 5′-TGAGCAGCCACACTTATC-3′ and Rev: 5′-ACT-GGAATTGTTACAGCATTT-3′; GenBank sequence NM_032541), Hfe (Fw: 5′-GCAATCTGAGCATGATTTA-3′ and Rev: 5′-ATCTCAAC-CAAGAAGATGCC-3′; GenBank NM_010424), and fn1 (Fw: 5′-GCAAGAT-GAGCTCTGACCA-3′ and Rev: 5′-GCCAACAACAAACT CAGTCT-3′; GenBank NM_016917). Mouse Gapdh (Applied Biosystems) or mouse β-actin (Fw: 5′-GTTGGGCGGGCTCTAGGCACA-3′ and Rev: 5′-CGGT TGGCCCTAGGGTCCA-3′; GenBank NM_007393) were used as Q-PCR endogenous controls. Q-PCR reactions were performed by using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems), with TaqMan (TaqMan PCR 2X Master Mix; Applied Biosystems) and SYBR Green (iTaq SYBR Green Supermix; Bio-Rad Laboratories) chemistry.

**Sequencing.** In order to sequence the transgenic Hamp1 mRNA, we utilized 2 primers that resulted only in the retrotranscription of the transgenic Hamp1. One oligonucleotide (Fw: 5′-TCCCTTAGACTGACACAG- CAGAA-3′) recognizes a sequence at the beginning of Hamp1, in exon 1, before the ATG start codon. The second oligonucleotide (Rev: 5′-AAT-CAAAGGTCTCCCCAATCT-3′) hybridizes with a sequence in the β-globin poly(A) sequence, which is present only in the transgenic mRNA.

**Northern blot analysis.** RNA samples are analyzed as described by Roy et al. (16). Band intensity is quantified using the ImageQuant TL software (GE Healthcare/Amersham).

**CD71, Ter119, CD44, ROS, and annexin V analyses by flow cytometry.** BM and spleen cells were incubated with FITC-labeled anti-mouse CD71 and APC-conjugated anti-mouse Ter119 antibodies (BD Biosciences—Pharmingen). In a subset of mice, the cells were also stained with a PE-conjugated anti-mouse CD44 marker (BD Biosciences—Pharmingen) together with the aforementioned antibodies. ROS were detected in peripheral blood and in immature erythroid cells from the spleen and BM with the indicator CM-H2DCFDA (Invitrogen). The FITC Annexin V kit (BD Pharmingen) was utilized according to the instructions provided by the manufacturer. For all the analyses, cells were sorted using a FACSCalibur instrument (BD) and the results analyzed with FlowJo software (Tree Star, Inc.).

**Measurement of rbc lifespan.** To evaluate whether the lifespan of rbc from th3/+/- mice fed the 2.5-ppm iron diet, and Tg-Hamp/th3 mice fed the 35-ppm diet was extended compared with that of Tg/th3 mice, we injected 400 µl of blood from each th3/+/- mouse (n = 3 per group) into mice whose erythrocyes express GFP and that were previously phlebotomized, withdrawing the same volume of blood. The percentage of GFP-negative rbc from the donor mice was measured by FACS analysis 24 hours after injection (time 0) and again after 1 and 2 weeks. Blood from WT mice injected into GFP mice was used to measure the lifespan of normal erythrocytes. WT mice were also used to measure the rbc lifespan with a sulfo-NHS-biotin reagent (Pierce) that binds to the surface of erythrocytes in order to establish that both methods were comparable. A single dose of 1 mg of sulfo-NHS-biotin was injected into mice intravenously. After 24 hours, a drop of blood was collected from the tail and incubated with a fluorochrome-conjugated streptavidin, then analyzed by flow cytometry. FACS analyses were performed for 2 weeks after injection to measure the progressive clearance of the biotin-labeled erythrocytes from circulation. We did not observe any difference in the lifespan of WT rbc determined by the 2 methods (not shown).

**Analyses of α- and β-globin chains in plasma and on rbc membranes.** To visualize soluble as well as membrane-bound globins, we utilized TAU gel electrophoresis, which resolves α- and β-globin subunits under denaturing conditions (19, 30). The fraction of sample loaded on the TAU gel was adjusted relative to the number of rbc from CBC, so that the same number of erythrocytes (150 × 10⁶) is represented in each lane.

**Statistics.** Data are presented as mean ± SD. Unpaired 2-tailed Student’s t test was performed using Microsoft Excel, Mac 2008 software. P < 0.05 was considered statistically significant.
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