Successful treatment of murine β-thalassemia intermedia by transfer of the human β-globin gene

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The β-thalassemias are caused by more than 200 mutations that reduce or abolish β-globin production. The severity of the resulting anemia can lead to lifelong transfusion dependency. A genetic treatment based on globin gene transfer would require that transgene expression be erythroid specific, elevated, and sustained over time. We report here that long-term synthesis of chimeric hemoglobin (mω2-huβ) could be achieved in mice with β-thalassemia intermedia following engraftment with bone marrow cells transduced with a lentiviral vector encoding the human β-globin gene. In the absence of any posttransduction selection, the treated chimeras exhibit durably increased hemoglobin levels without diminution over 40 weeks. Ineffective erythropoiesis and extramedullary hematopoiesis (EMH) regress, as reflected by normalization of spleen size, architecture, hematopoietic colony formation, and disappearance of liver EMH. These findings establish that a sustained increase of 3 to 4 g/dL hemoglobin is sufficient to correct ineffective erythropoiesis. Hepatic iron accumulation is markedly decreased in 1-year-old chimeras, indicating persistent protection from secondary organ damage. These results demonstrate for the first time that viral-mediated globin gene transfer in hematopoietic stem cells effectively treats a severe hemoglobin disorder. (Blood. 2002;99:1902-1908)
HS2, HS3, and HS4 regions of the human β-globin LCR.\textsuperscript{21,22} Following integration in mouse HSCs, human β-globin expression, normalized per vector copy, reached about 16% of endogenous hemizygous levels.\textsuperscript{19} To investigate hematologic correction and prevention of secondary organ damage, we turned to Hbb\textsuperscript{β-} mice,\textsuperscript{23} the most severe viable model of disease.\textsuperscript{24} In TNS9-treated mice, we show here long-term amelioration of anemia and normalization of hematopoiesis. In 1-year-old chimeras, EMH is averted as above, and cultured for 8 hours. Transduced bone marrow cells (5 × 10\textsuperscript{5}) were seeded in 10-cm diameter cell culture dishes 24 hours before transfection in Dulbecco modified Eagle medium (DMEM; Mediatech, Herndon, VA) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Rockville, MD), in a 5% CO\textsubscript{2} incubator, and colonies were counted at days 2 (CFU-Es) and 4 (BFU-Es). Granulocyte-macrophage colony-forming units (CFUs-GM) were analyzed with an Olympus BX60 immunofluorescence microscope and the images acquired with a Sony DCK-5000 digital camera. A minimum of 300 blood cells per slide was scored.

**Methods**

**Vector construction and production**

The lentiviral vectors TNS9 and pH\textsuperscript{R}eGFP have been previously described.\textsuperscript{19} Briefly, TNS9 was constructed by subcloning the human β-globin gene (from position 618 to +2484, thus encompassing the 3’ enhancer\textsuperscript{20}) into the pH\textsuperscript{R} vector.\textsuperscript{25} The β-globin gene is partially deleted within intron 2 as described for MβG.\textsuperscript{26} The 3.2-kb LCR assembled into TNS9 consists of a 840-base pair (bp) HS2 fragment (Sna\textsuperscript{II}-Bst\textsuperscript{II}), and a 1069-bp HS4 fragment (BamHI-BamHI). Viral stocks were generated by triple transfection of TNS9 or pH\textsuperscript{R}eGFP, pCMV\textsubscript{ΔR8.9,27} and pMD.G\textsuperscript{25} into 293T cells. The 293T cells (5 × 10\textsuperscript{5}) were seeded in 10-cm diameter cell culture dishes 24 hours before transfection in Dulbecco modified Eagle medium (DMEM; Mediatech, Herndon, VA) with 10% fetal bovine serum, 100 μg/mL streptomycin (Gibco BRL, Rockville, MD), in a 5% CO\textsubscript{2} incubator, and the culture medium was changed 2 hours prior to transfection. A total of 20 μg DNA was used for transfection of one dish: 3.5 μg of the envelope plasmid pMD.G, 6.5 μg of the packaging plasmid pCMV\textsubscript{ΔR8.9,9,10} and 10 μg of the vector plasmid. The precipitate was formed by adding the plasmids to a final volume of 437.5 μL 0.1 times TE (0.1 times TE is 10 mM Tris [pH 8.0] plus 1 mM EDTA) and 62.5 μL 2 M CaCl\textsubscript{2}, mixing well, then dropping 500 μL of 2 times HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM Na\textsubscript{2}HPO\textsubscript{4} [pH 7.12]) while vortexing and immediately adding the precipitate to the cultures. The medium (10 mL) was replaced after 14 to 16 hours; the viral supernatant was collected after another 24 hours, replaced, and again collected after 24 hours, cleared by low-speed centrifugation, and filtered through 0.45-μm pore size cellulose acetate filters. Viral concentration was performed by ultracentrifugation in a swing-bucket rotor at 25 000 rpm at 4°C for 90 minutes in 25 × 89-mm thick-walled polycarbonate tubes (Beckman Instruments, Palo Alto, CA). Viral pellets were resuspended overnight in X-VIVO-15 serum-free medium (Gibco BRL) at 4°C.

**Bone marrow chimeras**

Donor bone marrow was flushed from the femurs of 8- to 16-week-old male C57/BL6 or Hbb\textsuperscript{β-} mice\textsuperscript{23} obtained from Jackson Laboratories (Bar Harbor, ME), and had been injected intravenously (IV) 6 days earlier with 5-flurouracil (5-FU) 150 mg/kg body weight obtained from Pharmacia (Piscataway, NJ). Bone marrow cells were resuspended in X-VIVO-15 serum-free medium (Gibco BRL) at 4°C.

**Peripheral blood analyses**

Red blood cell lysates from freshly collected peripheral blood were analyzed by cellulose acetate electrophoresis obtained from Helena Laboratories ( Beaumont, TX). Hemoglobin bands were visualized by Ponceau S staining and quantitated by densitometry as previously described.\textsuperscript{19} To measure the fraction of peripheral blood cells expressing human β\textgreekalpha, smears of RBCs were fixed for 2 minutes in 3:1 acetic-aceton-methanol, then soaked for 2 minutes in wash buffer (isotonic phosphate–buffered saline [PBS]). The smear was covered with 10% goat serum in PBS 1×, and incubated for 15 minutes at room temperature in a moisture chamber. After staining, the slide was covered with a solution of 1 μg R-phycocerythrin (PE)–conjugated antimer TERY-119 antibody (Pharmingen, Franklin Lakes, NJ) and 1.5 μg fluorescein isothiocyanate (FITC)–conjugated monoclonal antibody to human hemoglobin A (EGG& W WALLAC, Turku, Finland) with 10% goat serum in PBS 1× for 30 minutes at room temperature in a moisture chamber. The slides were then washed with stirring for 10 minutes, drained, and mounted for examination. The slides were analyzed with an Olympus BX60 immunofluorescence microscope and the images acquired with a Sony DCK-5000 digital camera. A minimum of 300 blood cells per slide was scored.

**Tissue pathology**

Bone marrow chimeras were killed 40 weeks after BMT, at the age of 48 to 56 weeks. The tissues were fixed in 10% formalin, routinely processed, and embedded in paraffin. Tissue sections 4 μm thick were stained with hematoxylin and eosin and examined under light microscopy. Slides of control and treated mice were assessed in a blind manner. The sections of the spleen were evaluated for the amount of red and white pulp based on percentage of cross-sectional area of the tissue section. In the spleen the amount of EMH was visually estimated based on the percentage of nucleated erythroid precursor cells and mature erythroid cells seen. In the liver, the amount of EMH was evaluated semiquantitatively as marked, moderate, mild, or absent. In addition, 4-μm sections were stained for iron using Gomori iron stain (Poly Scientific, Bayshore, NY). The amount of iron deposition in the spleen, liver, and kidney tissues was characterized semiquantitatively on a scale of 0 (no iron present) to + (maximum amount of iron identified in a given organ).

**Statistical analysis**

We used the permutation rank sum statistic to determine whether hematologic parameters differed between treated and mock-treated groups. A low P value is evidence that the 2 proportions are different.

**Results**

**Persistent production of chimeric hemoglobin in thalassemic mice**

To investigate long-term expression of the transduced human β-globin gene and its therapeutic efficacy, we generated bone
marrow chimera analyzed 40 weeks after transplantation. Original magnification \times 40.

Figure 1. Sustained production of human \(\beta\)-globin protein (\(\beta^A\)) in the peripheral blood of TNS9-transduced bone marrow chimera. (A) The percent of hemoglobin tetramers that incorporate \(\beta^A\) (Hbb\(^{\beta^A}\), filled squares), and the percent of peripheral RBCs that stain positive for \(\beta^A\) chain (filled circles) remained stable up to 40 weeks after BMT. (B) Cellulose acetate gel electrophoresis shows Hbb\(^{\beta^A}\) levels in 3 TNS9-transduced bone marrow chimera (TNS9) 40 weeks after transplantation. Control lanes contain normal C57BL/6 (B6) and transgenic mouse line A85.68 (Tg\(^{28}\)) blood samples. The fraction of Hbb\(^{\beta^A}\) relative to total hemoglobin (Hbb\(^{\beta^A}/\text{Hbb}^{\mu}\) + Hbb\(^{\mu}\)) is indicated below each sample. (C) Peripheral blood erythrocytes from TNS9-transduced bone marrow chimera were stained for TER-119 (red) and Hbb\(^{\beta^A}\) (green, becoming yellow when superimposed on the red signal), then analyzed under an immunofluorescence microscope. Upper left panel shows normal C57BL/6 RBCs; upper right panel shows 50:50 mix of RBCs from normal C57BL/6 and A85.68 mice; lower panel is a representative blood sample from a TNS9-treated bone marrow chimera analyzed 40 weeks after transplantation. Original magnification \times 40.

Correction of EMH

To determine the impact of sustained human \(\beta\)-globin gene expression on hematopoiesis, we studied the degree of splenomegaly (enlargement of the spleen) and EMH in 1-year-old chimera and age-matched control mice. Spleen weights measured in TNS9-treated Hbb\(^{\beta^A}\)/\(\mu\)-chimeras were indistinguishable from recipients of eGFP-transduced normal bone marrow, as were the total number of cells per spleen (Table 1). In contrast, mice engrafted with eGFP-transduced Hbb\(^{\beta^A}\)/\(\mu\) bone marrow cells showed spleen weights and total cell numbers that were about 3-fold greater. The correction of spleen weight in TNS9 bone marrow chimera corresponded to a concomitant normalization in total hematopoietic progenitor cell content. Spleen CFU-Es, BFU-Es, and CFU\(_s\)-GM were reduced to levels measured in recipients of eGFP-transduced Hbb\(^{\beta^A}\)/\(\mu\) bone marrow (Figure 2B), whereas they remained elevated in control chimera engrafted with eGFP-transduced Hbb\(^{\beta^A}\)/\(\mu\) bone marrow cells and in age-matched Hbb\(^{\beta^A}\)/\(\mu\) mice, as previously observed in another murine model of \(\beta\)-thalassemia.\(^{29}\)

The regression of EMH was corroborated by morphologic examination of spleen and liver in long-term chimera and age-matched controls. The histopathology of spleens of mice that received transplants of eGFP-transduced Hbb\(^{\beta^A}\)/\(\mu\) marrow was virtually identical to that of spleens from control Hbb\(^{\beta^A}\)/\(\mu\) mice. Specifically, the red pulp was significantly expanded, accounting for 80% to 90% of the cross-sectional area, and densely occupied by nucleated erythroid precursors (Figure 3A and Table 1). The white pulp, based on cross-sectional area, was relatively decreased and the marginal zones were obscured by the large number of nucleated RBCs, reflecting major expansion of the red pulp and erythroid precursors. In TNS9-treated chimera, the amount of red
Hb b th3/

Table 1. Summary of morphologic findings

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Spleen red pulp</th>
<th>Spleen total cell no.*</th>
<th>Spleen weight (mg)</th>
<th>Liver EMH</th>
<th>Liver iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hbb+/+</td>
<td>Area 40%-50%; nRBCs ≥ 20%</td>
<td>190 ± 21</td>
<td>113 ± 21</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Hbbb-th3/</td>
<td>Area 70%-90%; nRBCs 80%-90%</td>
<td>509 ± 69</td>
<td>383 ± 33</td>
<td>Marked†</td>
<td>1-3+</td>
</tr>
<tr>
<td>Hbbb-th3/-GFP</td>
<td>Area 70%-90%; nRBCs 80%-90%</td>
<td>465 ± 99</td>
<td>343 ± 15</td>
<td>Moderate</td>
<td>1-4+</td>
</tr>
<tr>
<td>Hbbb-th3/-TNS9</td>
<td>Area 50%-60%; nRBCs 20%-70%</td>
<td>204 ± 37</td>
<td>120 ± 27</td>
<td>None</td>
<td>0-1+</td>
</tr>
</tbody>
</table>

nRBCs indicates nucleated red blood cells.

*Cell number × 10⁶.
†Two of 3 Hbbb-th3/- mice showed EMH.

The spleen was considerably decreased, accounting for only about 50% to 60% of the cross-sectional area (Figure 3A). In addition, the number of nucleated erythroid precursors in the red pulp was decreased (Figure 3B and Table 1). Other immature hematopoietic cells present in the red pulp, but much less frequently than in the spleens of control Hbbb-th3/- thalassemic mice (Figure 3B). The spleens from TNS9-treated chimeras were similar to those of the normal control mice in that no EMH was detected (Figure 4A, lower right panel). In contrast, livers from mice engrafted with eGFP-transduced Hbbb-th3/- bone marrow cells showed several small foci of intrasinusoidal EMH (Figure 4A, lower left panel).

Hepatic iron accumulation is markedly decreased

Toxic iron accumulation in the organs of thalassemic patients is a consequence of RBC destruction and increased gastrointestinal iron uptake. To determine whether sustained expression from the TNS9 vector reduced iron overload, we studied tissue sections of liver and heart, stained using Gomori iron stain. No iron deposition was seen in the livers of normal Hbb+/+ control mice, whereas Hbbb-th3/- mice showed variable amounts of iron, including some large aggregates (Figure 4B, upper left and right panels, respectively). TNS9-transduced treated chimeras demonstrated low to undetectable levels of iron in the livers (Figure 4B, lower right panel), whereas iron was readily detected in the livers of all mice that received transplants of eGFP-transduced Hbbb-th3/- bone marrow cells (Figure 4B, lower left panel, and Table 1). No iron accumulation was found in the heart of treated or control mice, as previously observed in another murine model of β-thalassemia,30 in contrast to what is found in the human disease.1,3

Discussion

Our findings indicate that stable engraftment with TNS9-transduced HSCs results in sustained amelioration of anemia, regression of splenomegaly and EMH, and a marked decrease in iron accumulation. Hepatic iron content, often measured to estimate total body iron,31 was low to undetectable by histochemical analysis. Further quantitative analyses of iron accumulation in chimeras treated at different ages will be needed to elucidate whether the remaining iron reflects either active iron accumulation or irreversible damage preceding transplantation. The most spectacular response achieved is the regression of splenomegaly and EMH. Spleen size, total cellularity, BFU-E, CFU-E, and CFU-GM content are all normalized. Foci of EMH in the liver, highly prevalent in age-matched control mice and mock-treated chimeras, are not found in chimeras expressing human β-globin. Altogether, these findings indicate that the chimeric Hbbh-th3/- hemoglobin is functional and that anemia is improved to a sufficient degree that EMH is abolished in the liver and greatly reduced in the spleen of TNS9-treated mice. This suggests that the human β-globin expression afforded by the TNS9 vector substantially improved erythroid maturation, suppressing ineffective erythropoiesis and restoring predominantly IMH.

There is currently no therapy in humans that leads to pathophysiologic correction of hemolysis, ineffective erythropoiesis, and secondary organ damage, short of allogeneic HSC replacement. Anemia is reduced by chronic transfusion, administered every 2 weeks in severely affected patients; RBC destruction is alleviated by splenectomy.2,3 Current inducers of γ-chain expression show activity in some patients, though their effects are often limited to increases of

Figure 2. Sustained amelioration of hematologic parameters in bone marrow chimeras reconstituted with TNS9-transduced Hbbb-th3/- bone marrow cells.

(A) Hemoglobin levels, hematocrit, RBC counts, and reticulocyte counts are shown at weeks 15, 30, and 40 after transplantation. All measured parameters show significant increases in recipients of TNS9-transduced Hbbb-th3/- versus eGFP-transduced Hbbb-th3/- bone marrow cells at week 40 after transplantation (P = .03 for each parameter). (B) Colony-forming assays were performed using spleen cells isolated from age-matched Hbb+/+ (black fill) and Hbbb-th3/- mice (horizontal lines), along with cells from chimeras engrafted with eGFP-transduced Hbb+/+ (white fill), Hbbb-th3/- (gray fill) bone marrow cells. CFU-E, BFU-E, and CFU-GM colonies were analyzed 40 weeks after transplantation (TNS9-transduced Hbbb-th3/- versus eGFP-transduced Hbbb-th3/- CFU-E, BFU-E, and CFU-GM, P = .03).
hemoglobin levels on the order of 1 to 2 g/dL. Iron accumulation is treated by iron-chelating agents such as deferoxamine. The treatment is, however, not devoid of complications, and requires strict compliance to a demanding regimen. Furthermore, iron chelation is not available to all patients, especially in developing countries. Newer drugs aiming to either improve iron chelation or augment production of fetal hemoglobin are sorely needed.

In this context, a genetic treatment based on globin gene transfer is highly desirable. In addition to circumventing limitations of allogeneic BMT, it offers the prospect of correcting both the anemia and secondary complications, as we show here in a murine model of β-thalassemia intermedia. These findings suggest that this approach may also be effective in sickle cell disease and in β-thalassemia major. Engraftment with TNS9-transduced bone marrow cells increased hemoglobin levels by 3 to 4 g/dL, a magnitude that would undoubtedly be of great benefit in human patients. This would require, however, that highly efficient gene transfer be achieved in human HSCs, which remains a challenge despite recent progress, and that the TNS9 vector function at least as well in human cells. In this regard, studies in nonhuman primates will be highly valuable. Furthermore, safety features of the gene delivery system will have to be further ascertained. Lentiviral vectors have not yet been approved for human use by the United States Food and Drug Administration. It is encouraging that no replication-competent retrovirus has been reported to date in patients treated with oncoretroviral vectors.

The genetic therapy of inherited disorders is still in early stages of research and it is too early to predict what place it will eventually occupy in the treatment of blood disorders. Remarkable results...
were recently obtained in children with severe combined immunodeficiency. In this instance, engraftment of autologous CD34+ cells transduced with a non–tissue-specific vector encoding the interleukin receptor common \( \gamma \) chain 36 allowed for the generation of T lymphocytes in unconditioned transplant recipients. Selective pressure very likely favored lymphocyte generation and lymphoid repopulation in these recipients. The possible occurrence of mechanisms facilitating selective erythroid reconstruction in \( \beta \)-thalassemic recipients remains to be further studied and eventually exploited. 37 Our data are consistent with moderate selection, insofar that chimeras harboring comparable vector copies showed 54% ± 12% \( \beta^+ \) cells in Hbb\(^{1/-}\) recipients 43 and 74% ± 7% in Hbb\(^{3/-}\) mice, as shown here. However, host conditioning is likely to remain a requirement for productive stem cell engraftment if the competitive advantage is confined to the erythroid compartment. Importantly, the toxicity of the conditioning regimen may be markedly reduced if the genetically modified cells are endowed with enhanced repopulating capacity. 10,12,38,39 Ultimately, the genetic treatment of \( \beta \)-thalassemias and other inherited blood disorders will have to combine phenotypic correction with safe transplantation conditions to be broadly applicable.

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References

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