REGULAR ARTICLE

blood advances

Single-dose MGTA-145/plerixafor leads to efficient mobilization and in vivo transduction of HSCs with thalassemia correction in mice

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Key Points

- Mobilization of primitive HSCs with MGTA-145/plerixafor could allow for a portable and potentially safer in vivo HSC gene therapy.

We have developed an in vivo hemopoietic stem cell (HSC) gene therapy approach without the need for myelosuppressive conditioning and autologous HSC transplantation. It involves HSC mobilization and IV injection of a helper-dependent adenovirus HDAd5/35^{++} vector system. The current mobilization regimen consists of granulocyte colony-stimulating factor (G-CSF) injections over a 4-day period, followed by the administration of plerixafor/AMD3100. We tested a simpler, 2-hour, G-CSF–free mobilization regimen using truncated GRO-β (MGTA-145; a CXCR2 agonist) and plerixafor in the context of in vivo HSC transduction in mice. The MGTA-145+plerixafor combination resulted in robust mobilization of HSCs. Importantly, compared with G-CSF+plerixafor, MGTA-145+plerixafor led to significantly less leukocytosis and no elevation of serum interleukin-6 levels and was thus likely to be less toxic. With both mobilization regimens, after in vivo selection with O^6-benzylguanine (O^{6}BG)/BCNU, stable GFP marking was achieved in >90% of peripheral blood mononuclear cells. Genome-wide analysis showed random, multiclonal vector integration. In vivo HSC transduction after mobilization with MGTA-145+plerixafor in a mouse model for thalassemia resulted in >95% human γ-globin$^+$ erythrocytes at a level of 36% of mouse β-globin. Phenotypic analyses showed a complete correction of thalassemia. The γ-globin marking percentage and level were maintained in secondary recipients, further demonstrating that MGTA145+plerixafor mobilizes long-term repopulating HSCs. Our study indicates that brief exposure to MGTA-145+plerixafor may be advantageous as a mobilization regimen for in vivo HSC gene therapy applications across diseases, including thalassemia and sickle cell disease.

Introduction

Current hemopoietic stem cell (HSC) gene therapy protocols are based on the transplantation of ex vivo-transduced HSCs, which requires myeloablative conditioning to enable efficient engraftment of gene-modified cells, especially in patients with hemoglobinopathies. Ex vivo HSC manipulation is also associated with stem cell loss which can result in the loss of transduced cells over time in transplant recipients. Furthermore, the process of ex vivo HSC manipulation and transplantation is costly and must be performed in specialized, accredited centers, therefore significantly limiting access to patients with common genetic diseases. To simplify HSC gene therapy, we recently developed an approach for in vivo HSC transduction. Without HSC

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mobilization, direct transduction of bone marrow HSCs with IV-injected HDAd5/35^{++} vectors is very inefficient because of physical barriers formed by the bone marrow stroma.[1] Therefore, our approach involved subcutaneous (SC) injections of granulocyte colony-stimulating factor (G-CSF)/plerixafor; AMD3100) to mobilize HSCs from the bone marrow into the peripheral blood stream, followed by IV injection of an integrating helper-dependent adenovirus (HDAd5/35^{++}) vector system that targets CD46, a receptor expressed on primitive HSCs.[1] A large fraction of transduced HSCs returned to the bone marrow where they persist for the long term. Random integration is mediated by an activity-enhanced Sleeping Beauty transposase (SB100x).[2] Targeted integration can be achieved via homology-dependent DNA repair.[3] We have recently shown that in vivo HSC transduction in G-CSF/plerixafor-mobilized mice results in therapeutic efficacy in several disease models.[4-6] The initial data in nonhuman primates have shown that the in vivo HSC gene therapy approach is safe when combined with glucocorticoid, interleukin-6 (IL-6)– and IL1-β–receptor antagonist pretreatment to suppress innate immune responses after IV HDAd5/35^{++} injection.[7]

The mobilization regimen (4 days of G-CSF injection followed by an injection of plerixafor on day 5) robustly mobilizes HSCs from the bone marrow to the periphery and is therefore widely used for harvesting HSCs for transplantation.[8,9] G-CSF induces cell division in the bone marrow and leads to an expansion of the stem cell pool, as well as overall proliferation of hematopoietic cells. However, the use of G-CSF as a mobilizing agent has several disadvantages: (1) it requires multiple doses; (2) is known to alter the function of the HSC niche, as well as bone formation; (3) can cause bone pain and spleen enlargement and, on rare occasions, results in splenic rupture, myocardial infarction, or cerebral ischemia; (4) is contraindicated in some cases, such as sickle cell disease (SCD); (5) mobilizes a graft enriched in progenitor cells, rather than HSCs; and (6) has variable yields of HSCs after mobilization, requiring multiple apheresis sessions in some cases.[10,11]

Another class of mobilizing drugs is antagonists of C-X-C chemokine receptor type 4 (CXCR-4), most prominently the US Food and Drug Administration–approved drug plerixafor, a synthetic, small-molecule drug. Plerixafor leads to more rapid mobilization of HSCs than G-CSF[12,13] and is thought to cause mobilization solely through disruption of the stromal cell–derived factor 1 (SDF-1)-CXCR-4 axis, without inducing HSC proliferation. Plerixafor has been shown to synergize with G-CSF mobilization,[13] indicating that G-CSF can only partially interfere with the SDF-1-CXCR-4 axis. When combined with G-CSF, plerixafor increases the number of CD34^{+} cells in the periphery two- to three-fold, compared with G-CSF alone.[14,15] Plerixafor alone, however, mobilizes a variable number of HSCs. Therefore, a G-CSF-free regimen, safely enabling single-day mobilization, would be of high value.

Growth-related oncogene protein-β (GRO-β) and its novel N-terminal–truncated form, GRO-β(5-73) (GRO-βT), are agonists for CXCR-2, a receptor expressed on granulocytes and monocytes.[16,17] Granulocytes and monocytes are necessary for mobilization to occur and are the first cells to egress from the bone marrow into peripheral blood. Furthermore, studies by DiPersio et al indicate that the combination of GRO-β and plerixafor triggers cross talk between endothelial cells and neutrophils, leading to CXCR-2 activation that boosts VLA-4 antagonist-induced HSC egress.[18,19] Furthermore, GRO-β/GRO-βT and plerixafor can act through their respective CXCR-2 and -4 receptors on neutrophils, resulting in enhanced MMP-9 release and rapid mobilization.[20] Notably, GRO-βT is a potent mobilizer alone, as well as in combination with G-CSF.[16,21] In clinical studies, the combination of MGTA-145 (human GRO-βT) and plerixafor resulted in more efficient mobilization of primitive (CD34^{+}/CD90^{+} cells) from healthy donors.[22,23] CD34^{+} cells, mobilized by this drug combination and transplanted into humanized mice, engrafted more rapidly and at >10-fold higher levels than that achieved with G-CSF- or plerixafor-mobilized cells.[23] These studies also documented the safety of MGTA-145/plerixafor in healthy donors. Furthermore, it was previously published that CXCL2 antagonists did not mediate vaso-occlusion in a humanized mouse model of SCD.[24]

In this study, we tested MGTA-145/plerixafor mobilization in combination with in vivo HSC transduction in CD46-transgenic mice, as well as in a murine model of thalassemia intermedia (Hbb^{th3}/CD46$^{+/+}$).

Methods

Reagents

MGTA-145 (human GRO-βT) was provided by Magenta Therapeutics. G-CSF (filgrastim [Neupogen]; Amgen Thousand Oaks, CA), plerixafor (Sanofi), AMD3100 (Sigma-Aldrich, St Louis, MO), O^6-benzylguanine (O^{6}BG), and carmustine (BCNU; Sigma-Aldrich) were used for in vivo transduction and selection.

HDAd5/35^{++} vectors

HDAd-SB, HDAd-mgmt/GFP, and HDAd-γ-globin/mgmt have been described before.[25,26] Helper virus contamination levels were found to be <0.05%. Titers were 6×10^{12} to 12×10^{12} viral particles (vp)/mL. All HDAd vectors used in this study contain chimeric fibers composed of the Ad5 fiber tail, the Ad35 fiber shaft, and the affinity-enhanced Ad35^{++} fiber knob.[27]

Animal studies

Experiments involving animals were conducted in accordance with the institutional guidelines set forth by the University of Washington. The University of Washington is an Association for the Assessment and Accreditation of Laboratory Animal Care International–accredited research institution, and all live animal work conducted at this university is in accordance with the Office of Laboratory Animal Welfare Public Health Assurance policy, US Department of Agriculture Animal Welfare Act and Regulations, the Guide for the Care and Use of Laboratory Animals, and University of Washington's Institutional Animal Care and Use Committee protocol 3108-01. The mice were housed in specific-pathogen–free facilities.

hCD46-transgenic mice. C57Bl/6-based transgenic mice, which contain the human CD46 genomic locus and provide CD46 expression at a level and in a pattern similar to those in humans, have been described earlier.[28]

HDAd vectors. Stable transduction studies were performed with helper-dependent HDAd5/35^{++} vectors using a hyperactive Sleeping Beauty transposase (SB100x) system for integration of an

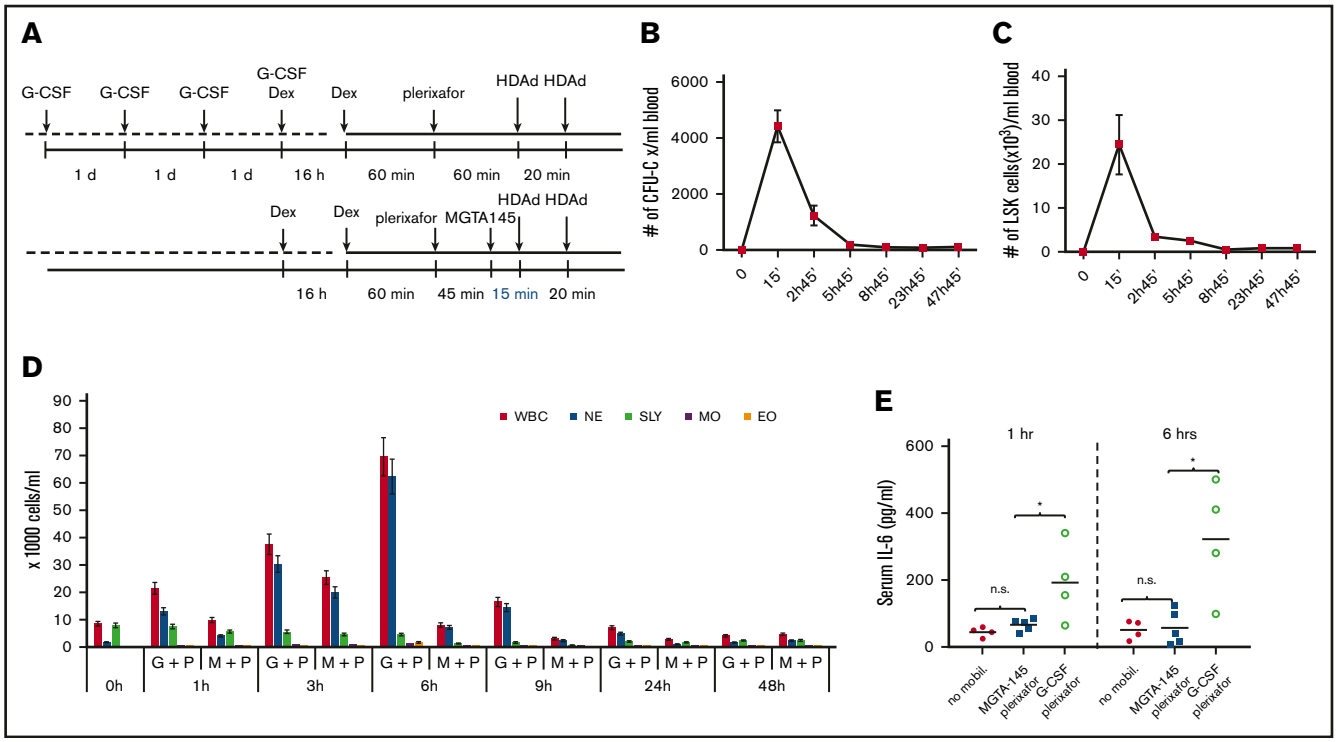

Figure 1. G-CSF/plerixafor vs MGTA-145/plerixafor mobilization and leukocytosis in CD46-transgenic mice. (A) Treatment regimen. CD46-transgenic mice were mobilized with G-CSF/plerixafor or with MGTA-145/plerixafor and then IV injected with HDAd-mgmt/GFP+HDAd-SB vectors at the indicated time points. (B-C) Kinetics of HSPC mobilization. Blood samples were collected at the indicated time points after MGTA-145 and either used for progenitor colony assays (B) or analyzed by flow cytometry for LSK cells (C). The number of LSK cells was calculated as leukocyte count in blood × LSK cell percentage by flow. (D) Blood cell counts. Peripheral blood samples were collected 1, 3, 6, 9, 24, and 48 hours after the plerixafor injection (n = 3). (E) Serum IL-6 levels at 1 and 6 hours after virus injection. *P <.05. EO, eosinophils; G+P, G-CSF+plerixafor; LY, lymphocytes; M+P, MGTA-145+plerixafor; MO, monocytes; NE, neutrophils; n.s., nonsignificant; WBC, white blood cells.

mgmt^{P140K}/GFP transgene cassette. This vector system consists of 2 vectors. The first, the transposon vector (HDAd-mgmt/GFP), carries the transgene cassette flanked by inverted transposon repeats (IRs) and *frt* sites. The second vector (HDAd-SB) provides both Flpe recombinase and SB100x transposase in *trans*.[1] The mgmt^{P140K} -based in vivo selection approach, requires only short-term exposure to O^6-BG/BCNU at doses that are neither immunosuppressive nor myeloablative.[29]

Mobilization and in vivo transduction.

G-CSF+PLERIXAFOR. Human CD46-transgenic mice were mobilized with 4 SC injections of G-CSF (250μg/kg, daily), followed by 1 injection of plerixafor (5 mg/kg) on day 5. One hour after plerixafor, mice were IV injected with HDAd5/35^{++} mgmt/GFP and HDAd5/35^{++}-SB100x (1:1; 2 injections 20 minutes apart, each 4 × 10^{10} vp). The animals received dexamethasone (10 mg/kg) intraperitoneally (IP) 16 and 2 hours before virus injection to control cytokine-related side effects.

MGTA-145+PLERIXAFOR. Mice received an SC injection of plerixafor (5 mg/kg) followed 45 minutes later by an SC injection of MGTA-145 (2.5 mg/kg). The first HDAd dose was injected 15 minutes after MGTA-145. The mice were pretreated with dexamethasone.

In vivo selection. Two or 4 weeks after the last HDAd injection, the mice were injected with O^6-BG (15 mg/kg, IP) 2 times, 30 minutes apart. One hour after the second injection of O^6-BG, the mice were injected with BCNU (5 mg/kg, IP). For the second, third, and fourth selection cycles with a 2-week interval, the O^6-BG dose remained the same, whereas the BCNU dose was increased to 7.5, 10, and 10 mg/kg, respectively. In agreement with our previous studies,[4,5,26,30] efficient marking in peripheral blood mononuclear cells (PBMCs)/red blood cells (RBCs) was achieved with a BCNU dose of 10 mg/kg. An intraindividual dose-escalation strategy was used to reach the effective BCNU dose, while allowing for the expansion of mgmt^{P140K}-expressing cells (and avoiding myelotoxicity) in animals with low HSC transduction rates or low mgmt^{P140K} expression levels.

Secondary bone marrow transplantation. Recipients were female C57BL/6 mice, 6 to 8 weeks old, from the Jackson Laboratory. On the day of transplantation, the recipient mice were irradiated with 1000 rad. Bone marrow cells from in vivo–transduced CD46tg or Hbb^{th3}/CD46$^{+/+}$ mice were isolated aseptically, and lineage-depleted cells were isolated by magnetic-activated cell sorting. Six hours after irradiation, the cells were injected IV at 1 × 10^6 cells per mouse. The secondary recipients were kept for 16 weeks until the time for euthanasia.

Tissue analysis

Spleen and liver tissue sections of 2.5-μm thickness were fixed in 4% formaldehyde for at least 24 hours, dehydrated, and embedded in paraffin. Staining with hematoxylin-eosin was used for histological evaluation of extramedullary hemopoiesis. Hemosiderin was detected in tissue sections by Perls' Prussian blue staining.

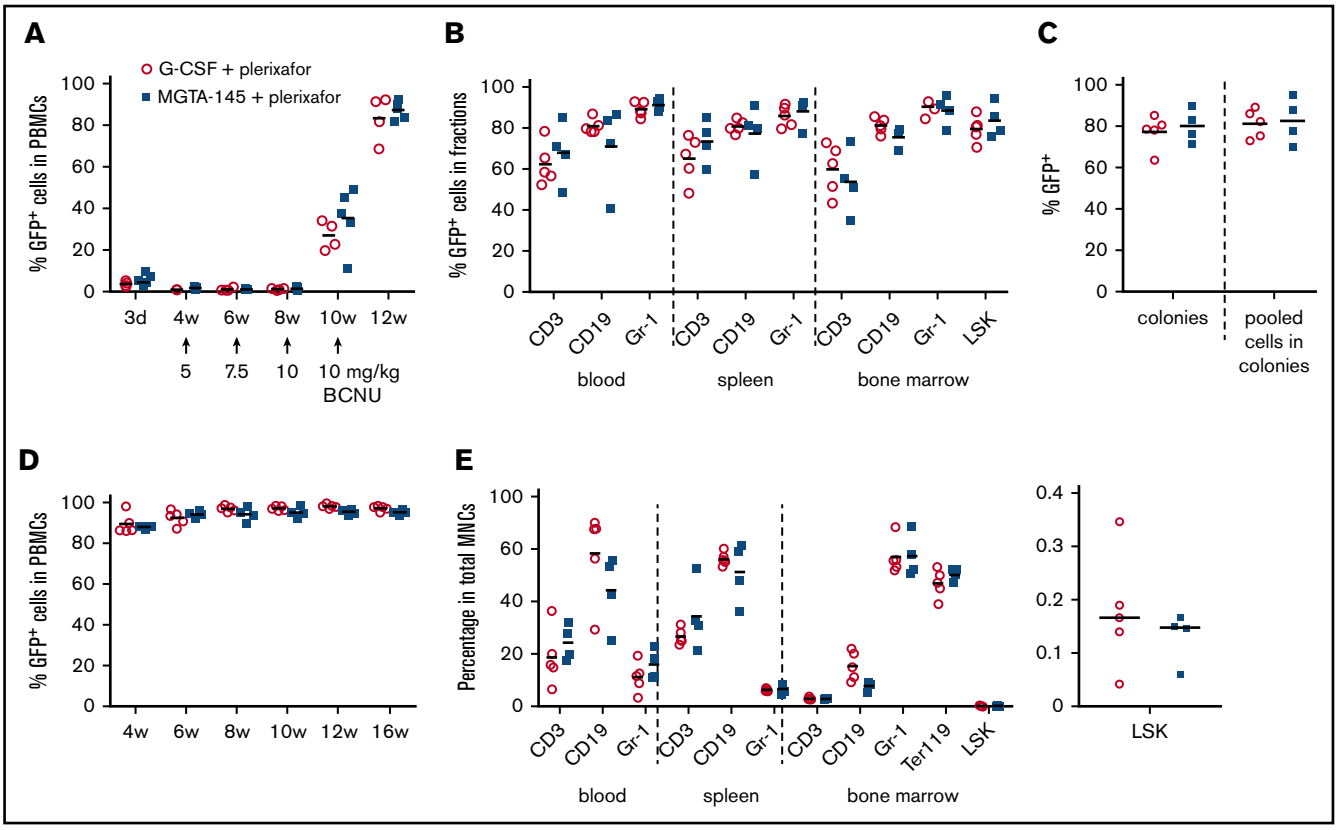

Figure 2. In vivo HSC transduction with a GFP-expressing HDAd5/35^{++} vector after G-CSF/plerixafor vs MGTA-145/plerixafor mobilization. (A) Analysis of in vivo transduction based on GFP marking in PBMCs. Arrows indicate in vivo selection with O^{6}BG/BCNU. With each cycle, the BCNU concentration was increased from 5 mg/kg, to 7.5 mg/kg, to 10 mg/kg. (B) Multilineage GFP marking in blood, spleen, and bone marrow cells harvested at week 12 after in vivo transduction. (C) GFP marking in progenitor colonies. Lin$^-$ cells from bone marrow harvested at week 12 were plated. (D-E) Secondary recipients. Mice were killed 12 weeks after in vivo transduction and bone marrow Lin$^-$ cells were harvested for transplantation into secondary recipients (lethally irradiated C57Bl/6 mice), and the animals were monitored for 16 weeks. (D) GFP marking in PBMCs. (E) Lineage composition in blood, spleen, and bone marrow at week 16 in secondary recipients. The percentage of LSK cells in bone marrow mononuclear cells is shown in a different scale (right). The difference between the 2 mobilization regimens was not significant.

In brief, the tissue sections were treated with a mixture of equal volumes (2%) of potassium ferrocyanide and hydrochloric acid in distilled water and then counterstained with neutral red. Spleens from untreated mice with similar body weight were used as controls for assessment of size.

Blood analysis

Blood samples were collected into EDTA-coated tubes, and analysis was performed on a HemaVet 950FS (Drew Scientific, Waterbury, CT). Peripheral blood smears were stained with Giemsa/May-Grünwald (Merck, Darmstadt, Germany) for 5 and 15 minutes, respectively. Reticulocytes were stained with Brilliant cresyl blue. The investigators who counted the reticulocytes on blood smears were blinded to the sample group allocation. Only the animal number appeared on the slides (5 slides per animal, 5 random 1-cm^2 sections).

Statistical analyses

For comparisons of multiple groups, 1- and 2-way analyses of variance with Bonferroni post-hoc test for multiple comparisons were used. Statistical analysis was performed with Prism, version 6.01 (GraphPad Software Inc, La Jolla, CA).

Results

G-CSF/plerixafor vs MGTA-145/plerixafor mobilization, leukocytosis, and IL-6 release in CD46-transgenic mice

CD46-transgenic mice were mobilized with G-CSF for 4 days, followed by 1 injection of plerixafor on day 5 (G-CSF/plerixafor group) or by a single SC administration of plerixafor, followed 45 minutes later by SC administration of MGTA-145 (Figure 1A; MGTA-145/plerixafor group). Mobilization was measured by formation of colonies on day 12 after plating of PBMCs from 5 μL of blood on ColonyGE 1202 (Figure 1B) or by flow cytometric quantification of lineage-negative (Lin$^-$), Sca1$^+$, cKit$^+$ (LSK) cells (Figure 1C). In agreement with previous studies,[16,20] in both assays, robust mobilization was observed 15 minutes after administration of MGTA-145 (60 minutes after plerixafor), leading to a peak of ~25 000 LSK cells per milliliter of blood. By comparison, a 5-day mobilization regimen with G-CSF+plerixafor led to ~20 000 to 40 000 LSK cells per milliliter of blood.[1,31] Based on the rapid stem/progenitor cell mobilization achieved with MGTA-145+plerixafor, we decided to inject the HDAd vector 60 minutes after plerixafor for both regimens (Figure 1A).

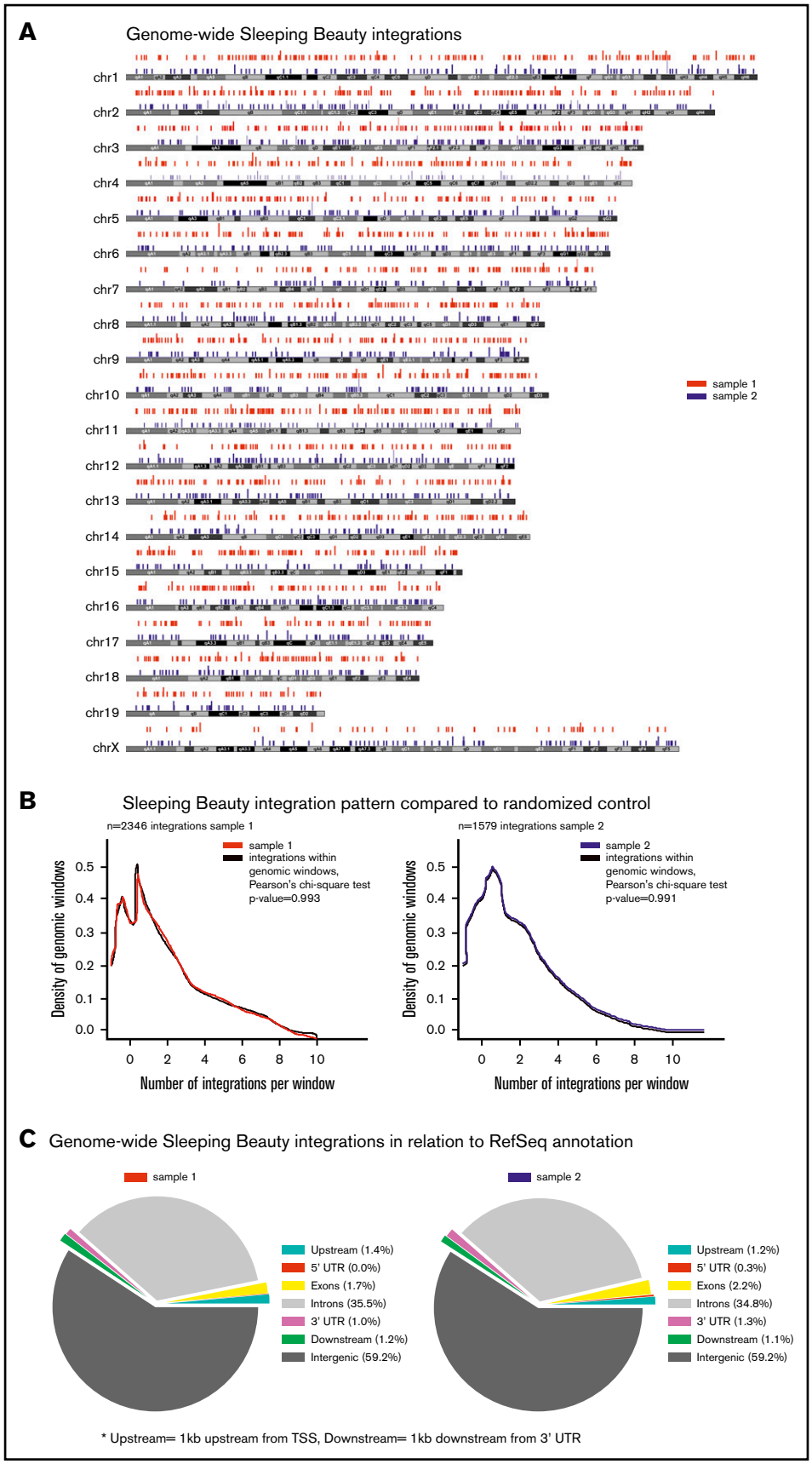

Figure 3. Integration site analysis. Genomic DNA from bone marrow mononuclear cells harvested at week 16 from secondary recipients (Figure 2D) was analyzed. One mouse for G-CSF/plerixafor and 1 mouse for MGTA-145/plerixafor were analyzed. (A) The integration distribution is represented on the chromosome level for both groups. Sample 1 was G-CSF/plerixafor with 2346 integration sites marked in red. Sample 2 is MGTA-145/plerixafor with 1579 integration sites marked in blue. (B) Integration patterns compared with randomized control. (C) Pie chart of SB100x-mediated integrations in relation to RefSeq genome annotations as a percentage of the total integrations. The integrations within 1 kb upstream and downstream from the transition start site (TSS); the 3′ and 5′ untranslated regions were also considered. More than 93% of the integrations in both libraries were intergenic or in introns.

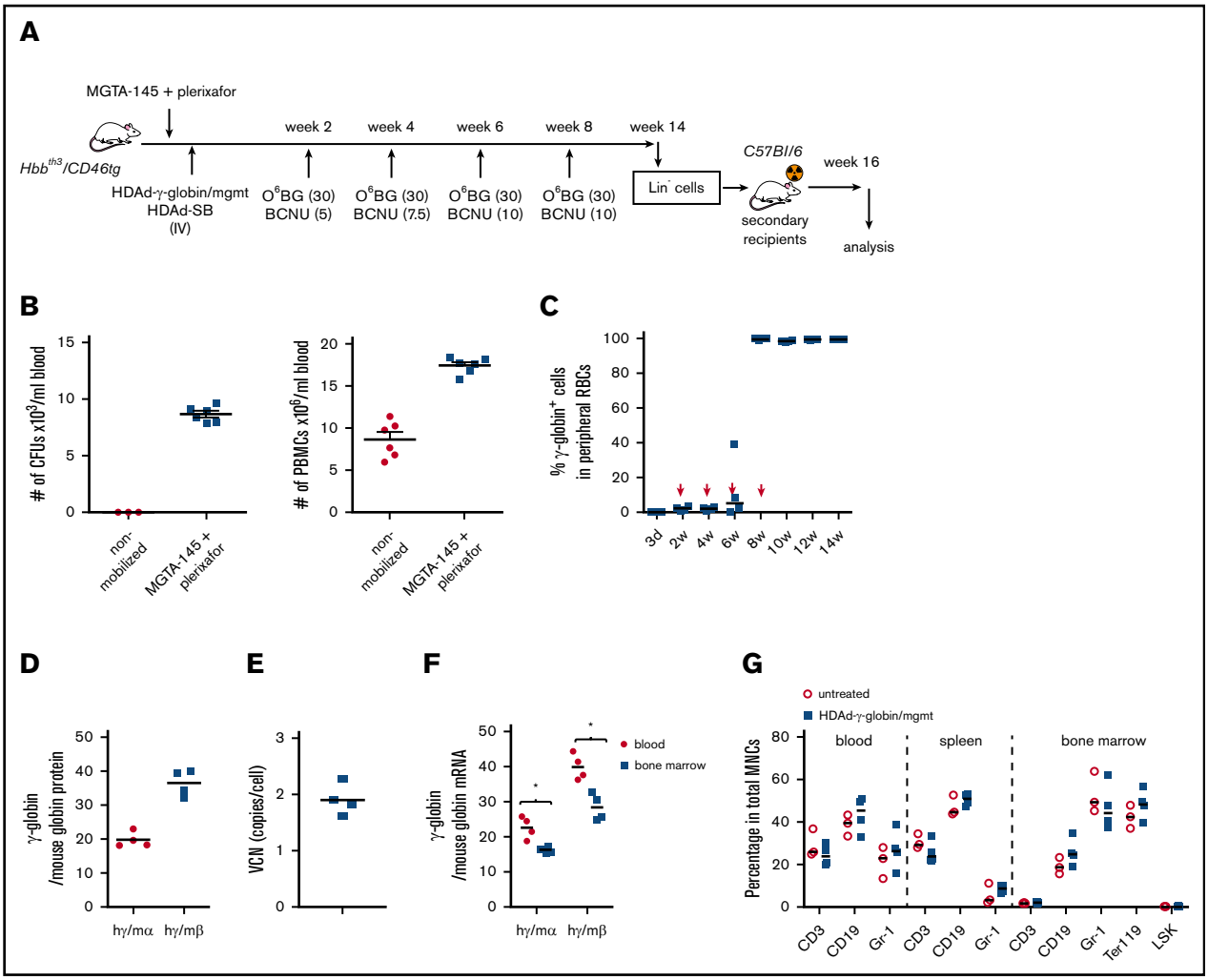

Figure 4. In vivo HSC gene therapy in MGTA-145/plerixafor-mobilized Hbb^{th3}/CD46$^{+/+}$ thalassemic mice. (A) HSCs were mobilized by 1 SC injection of plerixafor followed by MGTA-145 45 minutes later. Fifteen minutes after MGTA-145, animals were IV injected with a 1:1 mixture of HDAd-γ-globin/mgmt and HDAd-SB. At week 2, O^6-BG/BCNU treatment was started and repeated every 2 weeks (4 times). At week 14, animals were killed for tissue sample analysis and harvest of bone marrow Lin$^-$ cells for secondary transplantation into lethally irradiated C57BL/6 mice, which were then observed for another 16 weeks. (B) Efficacy of mobilization. The number of CFUs in peripheral blood at 15 minutes after MGTA-145 injection (left). The number of total mononuclear cells in peripheral blood at 15 minutes after MGTA-145 injection (right). (C) γ-globin expression in peripheral RBCs of in vivo–transduced Hbb^{th3}/CD46$^{+/+}$ mice measured, by flow cytometry. (D) γ-Globin protein levels measured in week 14 RBC lysates by HPLC. Shown is the percentage of γ-globin relative to mouse α_{major} and β_{major}. (E) The VCN measured at week 14 in PBMCs by qPCR. mgmt primers were used. (F) γ-Globin mRNA (relative to mouse α and β_{major} globin mRNA) measured in week 14 total blood cells and total bone marrow cells by qRT-PCR. (G) Cellular composition in blood, spleen, and bone marrow mononuclear cells at the time of euthanasia of primary mice. Untreated parental CD46tg mice were used as the control. The difference between the 2 groups was not significant.

Significant leukocytosis, caused by expansion of progenitor cells, is a major limitation for the use of G-CSF. Figures 1D and supplemental Figure 1 show blood cell counts for the 2 mobilization regimens. With G-CSF+plerixafor, the number of white blood cells, specifically neutrophils, increased 8- to 10-fold. After mobilization with MGTA-145/plerixafor, neutrophils increased ~3-fold and returned to normal levels by 6 hours, indicating that the elevated number of cells after administration of MGTA-145/plerixafor was transient relative to the 5-day mobilization regimen of G-CSF/plerixafor. When mobilization was combined with HDAd injection, the MGTA-145/plerixafor groups did not display elevated serum IL-6 levels, whereas mobilization with G-CSF/plerixafor led to significantly elevated IL-6 levels (Figure 1E). In summary, these data show that MGTA-145/plerixafor efficiently mobilized HSCs with an advantageous safety profile.

In vivo HSC transduction with HDAd-mgmt/GFP after mobilization with G-CSF/plerixafor or MGTA-145/plerixafor

HDAd5/35-mgmt/GFP contains a transposon consisting of the EF1α promoter driving the expression of a human mgmt^{P140K} gene linked to a GFP gene via a self-cleaving picornavirus 2A peptide (supplemental Figure 2). The transgene cassette can be integrated by a hyperactive Sleeping Beauty transposase expressed from a second vector (HDAd-SB). The mgmt^{P140K} gene confers resistance

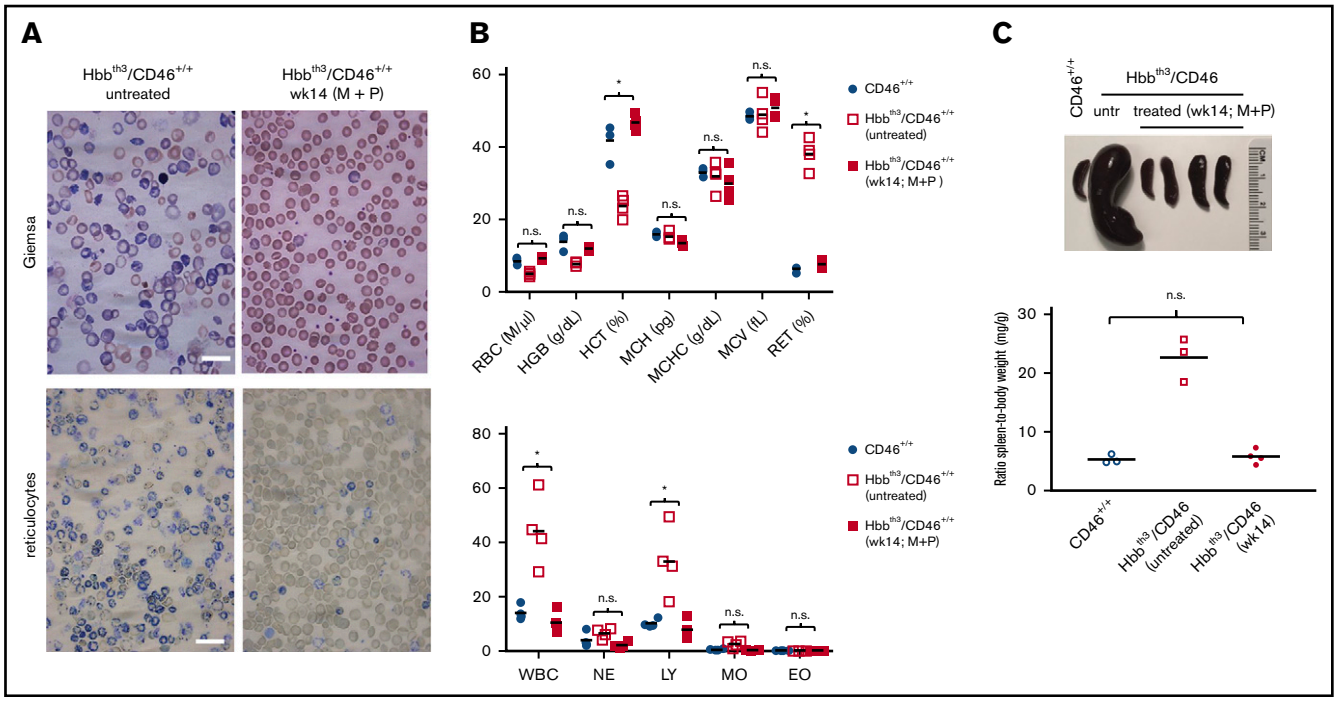

Figure 5. Phenotypic correction in blood after in vivo transduction of MGTA-145/plerixafor mobilized HSCs in Hbbth3/CD46$^{+/+}$ mice. (A) Representative blood smears stained with Giemsa/Grünwald showing the normocytic morphology of erythrocytes at week 14 after treatment (top). Blood smears stained for reticulocytes by Brilliant cresyl blue (bottom), a dye that stains remnants from nuclei and cytoplasmic organelles. (A quantification is shown in Figure. 4B, right column, right panel). Bars represent 20 μm. M+P: MGTA-145/plerixafor. All images were taken on a Leica microscope, type 020-519.0.10, model LB30J (Wetzlar GmbH). Leica Application Suite, version 4.1, was used for image acquisition. Perls and hematoxylin and eosin (H&E) stain; original magnification ×40; 2.24 ms exposure, 4.1× gain, 1.50 saturation, γ 0.60. Giemsa stain, original magnification ×100; 12.3-ms exposure, 4.5× gain, 1.35 saturation, and γ 1.63. Reticulocyte images were taken at original magnification ×100; 4.87 ms exposure, 4.5× gain, 1.35 saturation, and γ 0.81. (B) Hematological analysis of peripheral blood erythroid parameters (top) and blood cell counts (bottom). *P <.01 for the Hbb^{th3}/CD46$^{+/+}$ group, compared with the other 2 groups. The differences between the healthy control (CD46^{++}) and the treated Hbb^{th3}/CD46$^{+/+}$ (week 14) groups were not significant. HCT, hematocrit; LY, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RET, percentage of reticulocytes. (C) Spleen sizes. Photographs of spleens from healthy CD46tg and untreated Hbb^{th3}/CD46$^{+/+}$ mice were used as controls (top). Spleen weights relative to body weight (bottom).

to O^{6}BG/BCNU and allows for expansion of stably transduced progenitors. With both mobilization regimens, after in vivo selection with O^{6}BG/BCNU, >90% of PBMCs expressed GFP, and marking rates were stable in the long term (Figure 2A). Mice were euthanized 12 weeks after in vivo transduction. GFP marking was detected at comparable levels in all blood, spleen, and bone marrow lineages analyzed (CD3, CD19, and Gr-1; Figure 2B). Furthermore, ~80% GFP marking was measured in bone marrow LSK cells (Figure 2B, right) and in pooled cells from colony-forming units (CFU) (Figure 2C) for both groups. Bone marrow Lin$^-$ cells were harvested for transplantation into secondary recipients. GFP marking in secondary recipients (>90% at week 16 after transplantation) was also not significantly different between the 2 mobilization regimens (Figure 2D). Analysis of lineage composition in blood, spleen, and bone marrow, as well as the CFU capacity of Lin$^-$ cells at week 12 in primary mice and week 16 in secondary recipients, showed no significant differences between the 2 settings (supplemental Figure 3; Figure 2E).

The vector copy number (VCN) measured at week 16 in bone marrow mononuclear cells of secondary recipients was comparable (2-2.5 copies per cell; supplemental Figure 4). To exclude potential genetic transformation and consequent clonal expansion associated with SB100x-mediated integration and in vivo selection, we performed genome-wide analysis of integration sites using linear-amplification–mediated-polymerase chain reaction (PCR) and next-generation sequencing. Different integration sites (2346 and 1579 per mouse) were detected in animals in the G-CSF/plerixafor and MGTA-145/plerixafor groups, respectively (Figure 3A). This finding reflects the number of primitive HSCs that were initially stably transduced. The integration pattern in both mice was random (Figure 3B). More than 93% of integrations in both groups were intergenic or in introns. These data are in agreement with our previous studies in secondary mice at week 16 after transplantation, where we found ~2 integrated vector copies per cell with >1000 different integration sites per animal without signs of clonal expansion.[4,5,25,26] To reduce the risk of genotoxicity in future studies, we are planning to use HDAd5/35^{++} vectors containing long regulatory regions with insulator activity (eg, a 27-kb version of the human β-globin locus control region[5]) or HDAd5/35^{++} vectors mediating targeted integration into a safe genomic harbor.[3]

Overall, the results in this mouse model suggest that mobilization by MGTA-145/plerixafor mediated equally robust gene marking (as observed in the G-CSF/plerixafor mobilization setting) with a good safety profile.

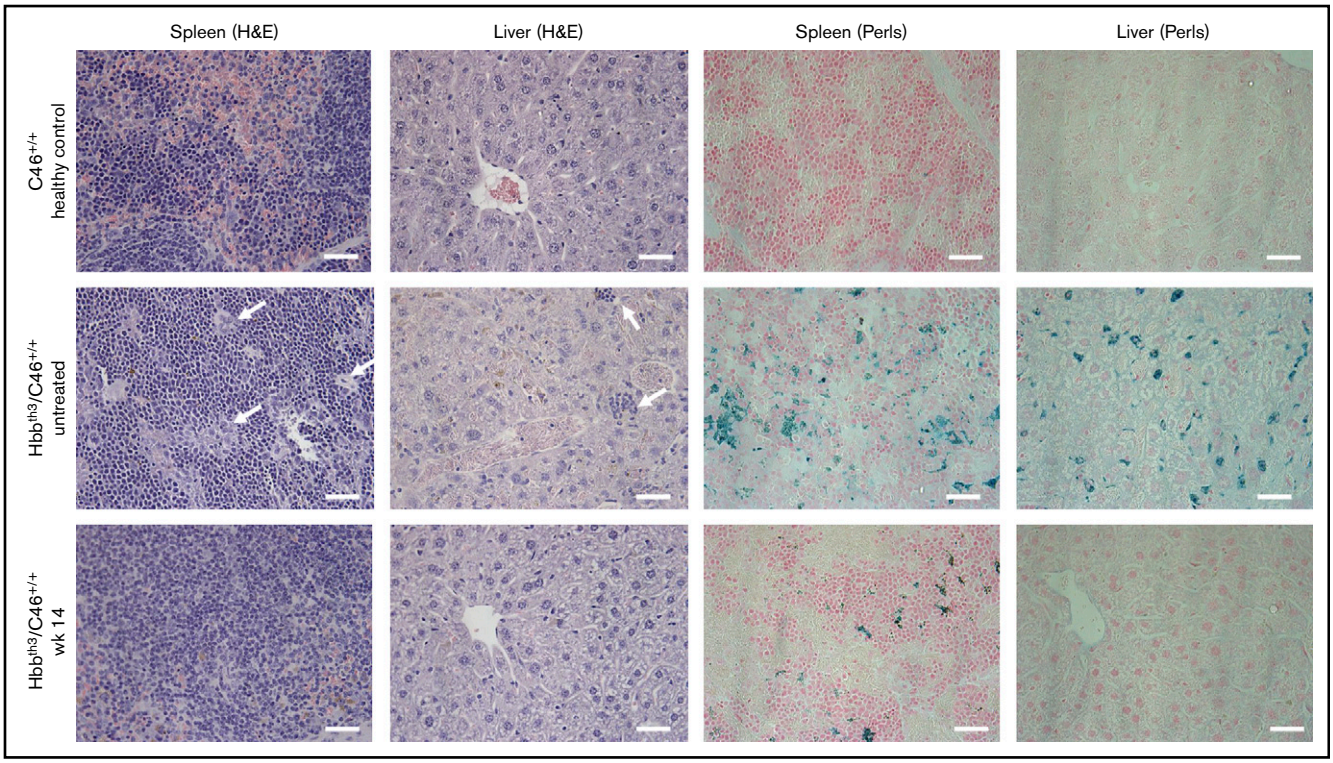

Figure 6. Phenotypic correction in spleen and liver. Extramedullary hemopoiesis identified by hematoxylin/eosin staining in spleen and liver sections (left 2 columns). Arrows indicate clusters of erythroblasts in the liver and megakaryocytes in the spleen of untreated $Hbb^{th3}/CD46^{+/+}$ mice. Also, the presence of deformed erythrocytes in liver blood vessels of untreated $Hbb^{th3}/CD46^{+/+}$ mice is noted. Representative images are shown. Iron deposition in spleen and liver. Hemosiderin deposition was detected in sections by Perls Prussian blue staining (right 2 columns). Bars represent 20 μm.

In vivo HSC gene therapy in MGTA-145/ plerixafor-mobilized thalassemic mice

We then tested the MGTA-145/plerixafor mobilization regimen for potential in vivo HSC gene therapy for thalassemia. We recently developed a thalassemic mouse model by backcrossing CD46tg ($CD46^{+/+}$) mice with Hbb^{th3} mice heterozygous for the mouse Hbb-β1 and -β2 gene deletion.[4,32] (CD46 is required for HDAd5/35^{++} transduction.[1]) $Hbb^{th3}/CD46^{+/+}$ mice represented a phenotype resembling human thalassemia intermedia. $Hbb^{th3}/CD46^{+/+}$ were mobilized with MGTA-145/plerixafor and IV injected with HDAd-γ-globin/mgmt and HDAd-SB (supplemental Figure 2) 15 minutes after MGTA-145 (60 minutes after plerixafor). HDAd-γ-globin/mgmt contains a mini β-globin locus control region controlling a human γ-globin gene and the $mgmt^{P140K}$ cassette (supplemental Figure 2). In vivo transduced mice were subjected to O^6BG/BCNU in vivo selection and observed for 14 weeks, after which Lin^- cells were transplanted into secondary recipients (Figure 4A). Administration of MGTA-145/plerixafor in $Hbb^{th3}/CD46^{+/+}$ mice led to a robust increase in the number of circulating CFUs at 15 minutes after MGTA-145 injection (Figure 4B; left) with a minimal (2-fold) increase in leukocyte counts (Figure 4B; right). After O^6BG/BCNU in vivo selection, at week 8 after transduction, 100% of RBCs were positive for γ-globin, as measured by flow cytometry (Figure 4C). This marking level remained stable until the end of the study (week 14). High-performance liquid chromatography (HPLC) analysis of RBC lysates (week14) showed human γ-globin levels of ~22% (human-γ/mouse-α) and 38% (human-γ/mouse-β_{major}; Figure 4D; supplemental Figure 5). The VCN in bone marrow mononuclear cells was ~2 copies per cell at week 14 (Figure 4E). We also performed quantitative reverse transcription-PCR (qRT-PCR) to measure the corresponding globin messenger RNA (mRNA) concentrations in total bone marrow and peripheral blood cells (Figure 4F). The level of human γ-globin expression was significantly higher in mature RBCs and was in the same range as the protein levels measured by HPLC. Overall, in vivo HSC transduction after MGTA-145/plerixafor mobilization had no effect on lineage composition in blood, spleen, and bone marrow (Figure 4G).

Whereas in untreated mice, the red cell morphology in blood smears was characterized by hypochromia, widely varying sizes and shapes (anisopoikilocytosis), and cell fragmentation, blood smears from mice mobilized with MGTA-145/plerixafor and injected with the HDAd-γ-globin/mgmt+HDAd-SB vectors displayed a normocytic red cell appearance at week 14 after treatment (Figure 5A; top). Disturbed erythropoiesis in $Hbb^{th3}/CD46^{+/+}$ mice was also reflected by the presence of ~38% reticulocytes in peripheral blood (Figure 5B). In sharp contrast, the percentage of reticulocytes in treated $Hbb^{th3}/CD46^{+/+}$ mice was comparable to that of parental healthy CD46tg mice (Figure 5A, bottom; Figure 5B, last data set). Furthermore, the number of RBCs, hemoglobin, hematocrit, mean corpuscular hemoglobin, and mean corpuscular volume were normal in treated $Hbb^{th3}/CD46^{+/+}$ mice (Figure 5B). Leukocytosis, another sign of abnormal hemopoiesis in $Hbb^{th3}/CD46^{+/+}$ mice was absent in treated animals (Figure 5B; bottom).

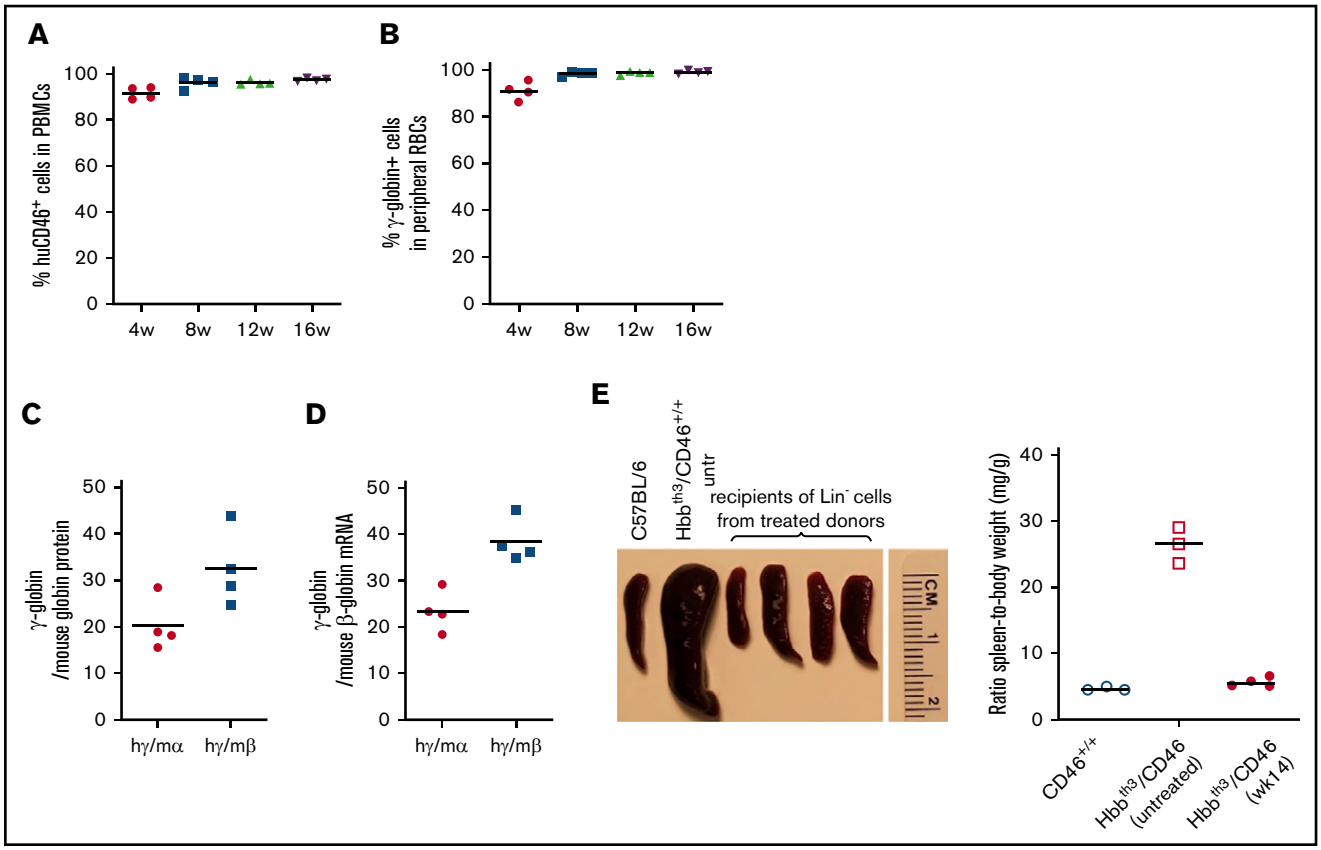

Figure 7. Secondary recipients of Lin$^-$cells from treated Hbb^{th3} /CD46$^{+/+}$ mice. (A) Engraftment of donor Lin$^-$ cells from Hbb^{th3}/CD46$^{+/+}$ mice based on the percentage of human CD46$^+$ PBMCs. (Recipient C57Bl/6 mice do not express human CD46). (B) γ-Globin marking in peripheral RBCs. (C) γ-Globin protein levels measured in week 16 RBC lysates by HPLC. Shown is the percentage of γ-globin relative to mouse α and β_{major} globins. (D) γ-Globin mRNA (relative to mouse α and β_{major} globin mRNA). (E) Spleen sizes. Photographs of untreated C57BL/6 and Hbb^{th3}/CD46$^{+/+}$ mice were used as controls (left). Spleen weights relative to body weight (right).

Extramedullary hemopoiesis resulted in splenomegaly in untreated Hbb^{th3}/CD46 mice (Figure 5C). In contrast, the spleen size in treated animals was normal.

Histological analysis of liver and spleen from Hbb^{th3}/CD46$^{+/+}$ mice revealed foci of extramedullary hemopoiesis containing clusters of erythroid precursors or megakaryocytes (Figure 6; middle row, leftmost 2 panels), whereas Perls' stain demonstrated marked parenchymal iron deposition (Figure 6; middle row, rightmost 2 panels). These pathological features, which recapitulate the human disease, were absent in treated Hbb^{th3}/CD46$^{+/+}$ mice (Figure 6, bottom row). Taken together, these data demonstrate that same-day mobilization by MGTA-145/plerixafor followed by in vivo HSC transduction/selection completely cured the thalassemia phenotype of Hbb^{th3}/CD46$^{+/+}$ mice.

Transduction of long-term repopulating HSCs after MGTA-145/plerixafor and IV HDAd-γ-globin/mgmt and HDAd-SB injection

Although the efficacy data are important in the context of simplifying our in vivo HSC approach; successful gene therapy outcomes are dependent on engraftment of long-term HSCs mobilized by MGTA-145/plerixafor. Studies in secondary recipients were performed as described for the GFP/mgmt vector (Figure 2D-E). Bone marrow Lin$^-$ cells harvested from treated Hbb^{th3}/CD46$^{+/+}$ mice at week 14 were injected into lethally irradiated C57Bl/6 mice and transplant-recipient animals were observed for 16 weeks. Engraftment (measured based on CD46$^+$ PBMCs) was >95% through 16 weeks after transplant (Figure 7A). Nearly 100% of erythrocytes were positive for γ-globin (Figure 7B). γ-Globin levels were 32% of mouse β_{major}-globin according to HPLC (Figure 7C), with consistent γ-globin mRNA measured by qRT-PCR (Figure 7D). No abnormalities were found in blood smears and spleen size (supplemental Figure 6; Figure 7E). These data demonstrate the in vivo transduction of primitive cells with durable, long-term repopulating capability after mobilization with MGTA-145/plerixafor.

Discussion

We have previously reported that G-CSF/plerixafor mobilization and in vivo HSC transduction/selection resulted in an amelioration of the thalassemic phenotype in the Hbb^{th3}/CD46 mouse model.[4] In the present study, we demonstrated that MGTA-145/plerixafor is at least as efficient as G-CSF/plerixafor in triggering the mobilization of long-term, repopulating, primitive HSCs, resulting in stable expression of transgenes. In a mouse thalassemia model, in vivo HSC transduction after MGTA-145/plerixafor mobilization resulted in >95% γ-globin$^+$ RBCs with levels of >30% γ-globin compared with adult globin after 4 cycles of O^{6}BG/BCNU selection. The

observation that γ-globin mRNA levels were higher in mature RBCs than in bone marrow erythroid progenitors could imply that a full conversion of the HSC population (ie, 100% γ-globin^{+} RBCs) is probably not necessary. Particularly in patients with severe hemoglobinopathies, it may therefore be possible to reduce the number of O^6BG/BCNU treatments.

The novel mobilization approach has several advantages: (1) MGTA-145/plerixafor is a G-CSF-free method to mobilize a large number of HSCs in a single day without leukocytosis, which is a major barrier for use of G-CSF (with or without plerixafor); (2) second, MGTA-145/plerixafor reliably mobilizes a large number of HSCs with durable long-term engraftment, as demonstrated by stable multilineage engraftment in primary and secondary recipients in CD46-transgenic and thalassemia mouse models; (3) mobilization and IV HDAd injection can be performed on the same day, within hours, and thus could allow for an outpatient in vivo HSC gene therapy approach that could greatly increase the accessibility and affordability of the technology; (4) use of MGTA-145/plerixafor increases the safety of IV HDAd5/35^{++} injection by limiting the number of cytokine-containing neutrophils that can come in contact with viral particles, which are present in a high number after mobilization with G-CSF alone or in combination with plerixafor; (5) the combination of MGTA-145/plerixafor and IV HDAd injection consistently resulted in high transgene-marking rates in all 11 animals treated, whereas a previous study indicated great variability when plerixafor was used alone,[26] an observation that was also made in clinical studies.[18]

GRO-βT has been shown to mobilize HSCs when administered in combination with other agents, such as VLA4 antagonists or the small synthetic molecule firategrast,[19] BIO5192,[33] or CWHM-823, for example.[34] Karpova et al demonstrated that BIO5192 in combination with GRO-βT targets primitive, serially repopulating HSCs with high efficiency.[19] Future studies may assess the use of the vasodilator sildenafil[35] and the Src family kinase inhibitor PP2.[20] Both molecules, when given in combination with plerixafor, increase the exit of HSCs from bone marrow.

Though not an objective of the current study, our in vivo/gene therapy marking approach may enable tracking of gene-marked HSCs in the blood, bone marrow, and spleen to help better understand basic biology and functional differences of HSCs that have been mobilized with different agents and combinations.

In summary, HSC mobilization with MGTA-145/plerixafor can facilitate the clinical translation of a portable, cost-efficient, durable, and G-CSF-free in vivo HSC gene therapy for hemoglobinopathies and other genetic diseases that are currently treated with ex vivo gene therapy or genome-editing approaches.

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Authorship

Contribution: A.L. provided the conceptual framework for the study; C.L. designed the experiments; C.L., S.G., Z.L., T.R., and A.P. performed the experiments; K.A.G., Z.I., T.P., J.C.D., and H.-P.K. provided critical input; and A.L. wrote the manuscript.

Conflict-of-interest disclosure: A.L. and H-P.K. are scientific cofounders of Ensoma, Inc. Z.I. is a cofounder of MDCell, a Helmholtz Innovation Laboratory. K.A.G. and J.C.D. are employees and shareholders of Magenta Therapeutics. The remaining authors declare no competing financial interests.

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