# Brief Report

## GENE THERAPY

# Plerixafor+G-CSF–mobilized CD34+ cells represent an optimal graft source for thalassemia gene therapy

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### Key Points

- Effective gene correction and long-term engraftment of human thalassemic CD34+ cells mobilized with different strategies.
- Plerixafor+G-CSF–mobilized CD34+ cells produce higher β-globin/VCN and superior early engraftment over single agent-mobilized cells.

Globin gene therapy requires abundant numbers of highly engraftable, autologous hematopoietic stem cells expressing curative levels of β-globin on differentiation. In this study, CD34+ cells from 31 thalassemic patients mobilized with hydroxyurea+granulocyte colony-stimulating factor (G-CSF), G-CSF, Plerixafor, or Plerixafor+G-CSF were transduced with the TNS9.3.55 β-globin lentivector and compared for transducibility and globin expression in vitro, as well as engraftment potential in a xenogeneic model after partial myeloablation. Transduction efficiency and vector copy number (VCN) averaged $48.4 \pm 2.8\%$ and $1.91 \pm 0.04$, respectively, whereas expression approximated the one-copy normal β-globin output. Plerixafor+G-CSF cells produced the highest β-globin expression/VCN. Long-term multilineage engraftment and persistent VCN and vector expression was encountered in all xenografted groups, with Plerixafor+G-CSF–mobilized cells achieving superior short-term engraftment rates, with similar numbers of CD34+ cells transplanted. Overall, Plerixafor+G-CSF not only allows high CD34+ cell yields but also provides increased β-globin expression/VCN and enhanced early human chimerism under nonmyeloablative conditions, thus representing an optimal graft for thalassemia gene therapy. (*Blood*. 2015;126(5):616-619)

## Introduction

β-Thalassemias are monogenic disorders causing partial or complete elimination of β-globin expression.[1] Allogeneic hematopoietic stem cell transplantation (allo-HSCT), although curative, is limited by the lack of matched donor availability.[2,3] Thus, most patients undergo palliative, lifelong transfusions and chelation[1] that compromise quality and duration of life.[4] Gene therapy (GT), by introducing the normal globin gene into patients' own CD34+ cells, is devoid of the complications usually accompanying allo-HSCT, and it is anticipated to permanently correct erythropoiesis, rendering patients transfusion free.[5,6] Mobilized blood represents the preferable source for HSCT and stem cell GT due to the high numbers of CD34+ cells provided through a minimally invasive procedure.[7,8] We previously defined Plerixafor+granulocyte colony-stimulating factor (G-CSF) as the optimal mobilization approach for thalassemia GT over other strategies (G-CSF, hydroxyurea [HU]+G-CSF, Plerixafor) in 2 clinical trials.[9,10] Because differently mobilized cells may possess distinct intrinsic characteristics, we compare here their potency regarding gene transfer and vector-encoded β-globin expression in vitro and in xenografts to define the optimal graft for thalassemia GT.

## Study design

The 2 mobilization trials in adults with thalassemia were previously described.[9,10] The bulk of the purified CD34+ cells was cryopreserved for future clinical-grade gene transfer, whereas small aliquots were frozen for the preclinical studies, described herein.

CD34+ cell aliquots with >85% purity, from 31 differently mobilized thalassemic patients (HU+G-CSF: n = 7, G-CSF: n = 7, Plerixafor: n = 13 and Plerixafor+G-CSF: n = 4; supplemental Table 1 available on the *Blood* Web site) were thawed, prestimulated overnight, and subjected to two 24-hour transductions (multiplicity of infection 25 × 2) with the research-grade TNS9.3.55 β-globin lenti-vector,[11] currently being clinically tested (#NCT01639690).[12] Transduced cells were seeded in clonogenic assays and erythroid differentiation culture[12] and, if in sufficient numbers, xeno-transplanted (supplemental Figure 1) to comparatively assess gene transfer rates, β-globin expression, and engraftment. Integration site (IS) analysis was performed on genomic DNA from erythroid differentiation liquid cultures derived from transduced CD34+ cells.
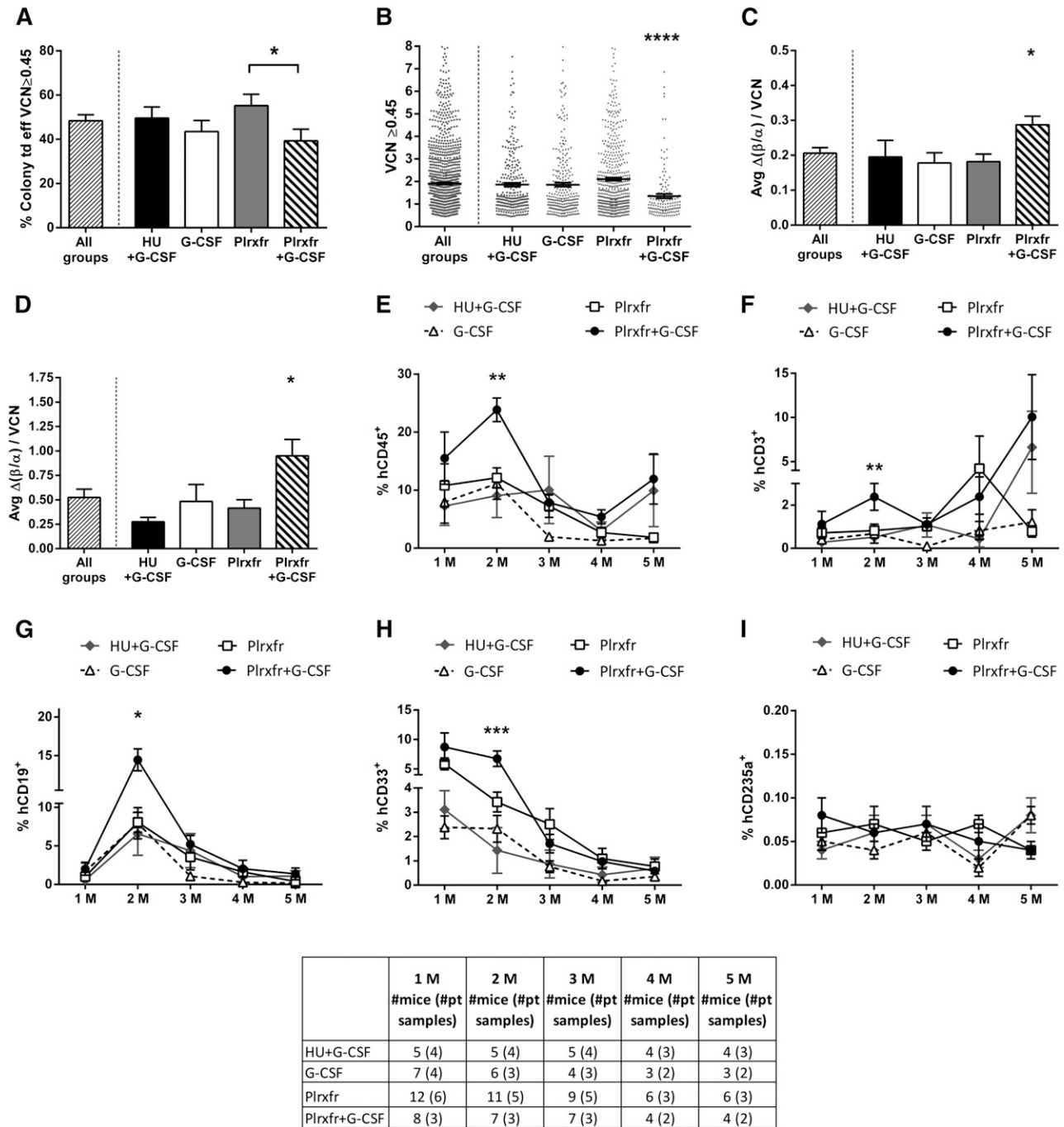
IL2Rγ[null] mice were conditioned with 100 mg/kg Busulfan, corresponding to the human equivalent, nonmyeloablative dose of 8 mg/kg (www.accessdata.

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|  | 1 M<br>#mice (#pt<br>samples) | 2 M<br>#mice (#pt<br>samples) | 3 M<br>#mice (#pt<br>samples) | 4 M<br>#mice (#pt<br>samples) | 5 M<br>#mice (#pt<br>samples) |
|---|---|---|---|---|---|
| HU+G-CSF | 5 (4) | 5 (4) | 5 (4) | 4 (3) | 4 (3) |
| G-CSF | 7 (4) | 6 (3) | 4 (3) | 3 (2) | 3 (2) |
| Plrxfr | 12 (6) | 11 (5) | 9 (5) | 6 (3) | 6 (3) |
| Plrxfr+G-CSF | 8 (3) | 7 (3) | 7 (3) | 4 (2) | 4 (2) |

**Figure 1. Comparison of differently mobilized CD34$^+$ cells in terms of susceptibility to transduction, globin expression, and engraftment rates.** (A) The mean frequency of vector positive colonies with vector copy number $\geq$0.45. The column on the left represents the average gene transfer efficiency achieved in all differently mobilized cells (n = 31). *$P$ = .04 (unpaired Student $t$ test). (B) Comparative vector copy number analysis among transduced CD34$^+$ cells obtained by different mobilization approaches, depicted as single colony distribution. The mean vector copy achieved in all groups of mobilization is also shown on the left. ****$P$ < .0001 (Kruskal-Wallis test). (C) The normalized vector-encoded β-globin expression per vector copy, overall (left bar) and per mobilization mode (HU+G-CSF: n = 6; G-CSF n = 5; Plerixafor: n = 12; Plerixafor+G-CSF: n = 4) in bulk erythroid cultures. *$P$ = .05 (Kruskal-Wallis test). (D) The normalized vector-encoded β-globin expression per vector copy, overall (left bar) and per mobilization mode (HU+G-CSF: n = 2; G-CSF n = 3; Plerixafor: n = 6; Plerixafor+G-CSF: n = 3) in pools of BFU-E colonies. *$P$ = .03 (1-way analysis of variance [ANOVA]). (E-H) Engraftment rates measured monthly by flow cytometry and expressed as a percentage of (E) human CD45$^+$ cells, **$P$ = .001 (1-way ANOVA); (F) human CD3$^+$ cells, **$P$ = .007 (1-way ANOVA); (G) human CD19$^+$ cells, *$P$ = .02 (1-way ANOVA), (H) human CD33$^+$ cells, ***$P$ = .0008 (1-way ANOVA), and (I) human CD235a$^+$ cells in the peripheral blood of xenografted mice (HU+G-CSF: gray line; G-CSF: dashed line; Plerixafor: black line with clear squares; Plerixafor+G-CSF: black line with black circles). No differences, among the differently mobilized cells, were encountered in the erythroid lineage where the engraftment, as expected for a xenograft model, was overall poor.[16] The mice surviving at each time point after transplantation with the TNS9.3.55-transduced mobilized CD34$^+$ cells from individual patients (displayed in parentheses) are shown in the table provided at the bottom of the figure. Only cells from patient samples that provided sufficient numbers for transplantation were injected. Mortality allowed a small number of animals to be evaluated long-term (supplemental Results). Plrxfr: plerixafor; Pt, patient.

**Table 1. High-performance liquid chromatography analysis before and after transduction in bulk erythroid culture and in pools of erythroid colonies**

| Genotype | n | β/α ratio untransduced | β/α ratio transduced | Δ(β/α) ratio | Fold increase | VCN | Δ(β/α)/VCN |
|---|---|---|---|---|---|---|---|
| **Bulk** | | | | | | | |
| $\beta^0/\beta^0$ | 11 | 0.02 ± 0.01 | 0.44 ± 0.03 | 0.41 ± 0.03 | 22 | 1.88 ± 0.16 | 0.24 ± 0.02 |
| $\beta^0/\beta^+$ | 6 | 0.12 ± 0.02 | 0.47 ± 0.03 | 0.35 ± 0.03 | 3.9 | 1.91 ± 0.31 | 0.22 ± 0.04 |
| $\beta^+/\beta^+$ | 10 | 0.32 ± 0.02 | 0.56 ± 0.03 | 0.23 ± 0.03 | 1.75 | 1.55 ± 0.16 | 0.17 ± 0.03 |
| Average | | 0.16 ± 0.03* | 0.49 ± 0.02* | 0.33 ± 0.02 | 9.2 ± 6.4 | 1.74 ± 0.11 | 0.21 ± 0.02 |
| | | | Output per copy | | | | |
| Normal | 3 | | 0.45 ± 0.02 | | | | |
| **BFU-E pools** | | | | | | | |
| $\beta^0/\beta^0$ | 2 | 0.01 ± 0.01 | 0.33 ± 0.2 | 0.32 ± 0.19 | 33 | 0.76 ± 0.35 | 0.39 ± 0.07 |
| $\beta^0/\beta^+$ | 5 | 0.14 ± 0.02 | 0.38 ± 0.03 | 0.23 ± 0.04 | 2.7 | 0.67 ± 0.3 | 0.58 ± 0.16 |
| $\beta^+/\beta^+$ | 7 | 0.27 ± 0.02 | 0.54 ± 0.05 | 0.28 ± 0.04 | 2 | 0.64 ± 0.13 | 0.53 ± 0.13 |
| Average | | 0.19 ± 0.03** | 0.45 ± 0.04** | 0.27 ± 0.03 | 12.5 ± 10.2 | 0.67 ± 0.12 | 0.52 ± 0.09 |
| | | | Output per copy | | | | |
| Normal | 2 | | 0.44 ± 0.08 | | | | |

The Δ(β/α) ratio is calculated by subtracting the residual expression measured in untransduced controls from their corresponding gene-corrected samples. The Δ(β/α)/VCN is calculated by subtracting the residual expression measured in untransduced controls from their corresponding gene-corrected samples and normalizing to vector copy number. Output per copy: the β/α ratio per allele of a normal genotype. Data represent individually analyzed samples and are expressed as means ± standard error of the mean. *,**$P < .0001$ (unpaired Student $t$ test).

UT, untransduced; Td, transduced.

fda.gov/scripts/cder/onctools/animalquery.cfm), and infused with $1 \times 10^6$ transduced $CD34^+$ cells, under a protocol approved by the institutional animal care and use committee. For more information, see supplemental Methods.

## Results and discussion

TNS9.3.55-transduced and untransduced $CD34^+$ cells provided similar clonogenic potential and expanded and differentiated successfully in erythroid liquid culture (supplemental Figure 2A-C), irrespective of the mobilization approach. The overall mean gene transfer in clonogenic assays was 48.4 ± 2.8% and the mean vector copy number (VCN) reached 1.91 ± 0.04 (Figure 1A-B, left). Plerixafor-mobilized cells exhibited higher colony transduction rates (Figure 1A, right) compared with Plerixafor+G-CSF ($P = .04$). This might be causally linked to the cycling status of Plerixafor-mobilized cells, as they comprised a greater fraction of cells in the G1 phase ($P = .01$; supplemental Figure 2D).[13,14] Interestingly, and apparently not linked to quiescent cell cycle kinetics (supplemental Figure 2D), Plerixafor+G-CSF–mobilized $CD34^+$ cells displayed significantly lower colony VCN compared with all other mobilized cells ($P < .0001$; Figure 1B, right). We showed that murine thalassemic Plerixafor+G-CSF–mobilized cells are immunophenotypically ($CD150^+/CD48^-/lin^-/sca$-$1^+/c$-$kit^+$) and functionally more primitive than differently mobilized cells,[13] and Genovese et al[15] demonstrated that the most primitive, and with the highest long-term repopulation potential, $CD34^+$ cell subset ($CD34^+/CD133^+/CD90^+$) displayed the lowest transduction efficiency; these findings may tie in with the comparably lower transducibility of Plerixafor+G-CSF cells in the current study, although the readout from our assays derives from progenitor cells, as it is usually the case with globin vectors.

The mean β-globin expression by high-performance liquid chromatography, either in terminally differentiated bulk erythroid suspension cultures or in pools of erythroid bursts (BFU-E), in individually analyzed samples from all the differently mobilized $CD34^+$ cells, approximated the normal hemizygous expression with an average of 1.74 and 0.67 VCN/cell, respectively (Table 1). Interestingly, although globin expression after transduction did not differ among the 3 different genetic backgrounds ($\beta^0/\beta^0$, $\beta^0/\beta^+$, and $\beta^+/\beta^+$; supplemental Figure 3A-B), the net increment of β/α ratio provided by the vector

was highly different among genotypes; the $\beta^0/\beta^0$ background induced the highest gain in expression ($P < .0001$), whereas the $\beta^+$ allele seemed to partially compromise the transgene-derived β-chain synthesis (Table 1). The mean overall net increase of β/α ratio after transduction was 0.33, and the normalized per VCN β-globin gain in expression was 0.21 in bulk and 0.52 in BFU-E (Table 1). In fact, the vector-encoded β-globin expression/VCN is underestimated in bulk culture, because the TNS9.3.55 lots were derived from research-grade preparations and the likely transient presence of plasmid may lead to an overestimation of VCN. These expression levels, albeit measured in vitro, allow us to anticipate that less than 2 VCN/cell will probably suffice to provide significant clinical benefit to both $\beta^0$- and $\beta^+$-thalassemia. Interestingly, and despite their lower transduction efficiency, Plerixafor+G-CSF–mobilized cells demonstrated higher β-globin expression per VCN compared with the other mobilization groups (bulk: $P = .05$; BFU-E: $P = .03$; Figure 1C-D). This difference is neither due to a confounding effect of the genotype, as it was not associated with a higher frequency of $\beta^0/\beta^0$ genotypes in the Plerixafor+G-CSF group (25% vs 67%, 60%, and 25% of the HU+G-CSF, G-CSF, and Plerixafor groups, respectively; supplemental Table 1) or a higher erythroid lineage transduction in Plerixafor+G-CSF–mobilized $CD34^+$ cells over the differently mobilized cells (supplemental Table 2). Differential gene expression and chromatin remodeling events may, for different mobilization treatments, bring about accessibility to alternative genomic regions for vector integration, and in the case of Plerixafor+G-CSF mobilization, select IS that favor high levels of expression. IS analysis demonstrated a highly polyclonal global integration pattern with similar distribution across all mobilization groups (supplemental Table 3). Plerixafor+G-CSF cell IS were slightly more enriched within genes and near gene promoters associated with globin expression (supplemental Table 4), but not significantly overrepresented. Gene ontology analysis negatively correlated Plerixafor+G-CSF cell IS with gene silencing and positively correlated with positive regulation of the cell cycle and telomere maintenance and organization (supplemental Table 5; supplemental Results).

$IL2R\gamma^{null}$ mice conditioned with a nonmyeloablative dose of Busulfan and infused with equal numbers of the differently mobilized $CD34^+$ cells demonstrated long-term hematopoietic repopulation (Figure 1E-I; supplemental Figure 4A). Gene marking in terms of VCN in blood, percent vector-positive colonies, and colony VCN per

cell, as well as β-globin expression derived from ex vivo expanded and terminally differentiated bone marrow cells, was persistent long-term (supplemental Figure 4B-C) and similar to that measured in the preinfusion product (supplemental Table 6). Plerixafor+G-CSF cells displayed enhanced short-term engraftment over all other differently mobilized grafts (Figure 1E-H), a feature that may be clinically translatable into faster hematologic reconstitution and abbreviated hospitalizations and therefore significantly reducing early transplant-related morbidity/mortality. Admittedly, our long-term engraftment data are limited by the small number of surviving mice; thus, we cannot draw conclusions in the xenograft model. Nevertheless, we previously demonstrated, in the less vulnerable to mortality Hbb[th-3] mouse model, a clear superiority in the long-term engraftment capacity of Plerixafor+ G-CSF cells over differently mobilized cells.[13] Genovese et al[15] showed that by modifying the culture process, the transduction rates of the CD34^{+} cell subset with the highest long-term repopulation potential but the lowest transduction efficiency were significantly increased, yet with preservation of the primitive cell nature. Similar manipulation for Plerixafor+G-CSF–mobilized cells might thus boost VCN, maintaining their high β-globin expression/VCN and their long-term repopulating potential.

Overall, the high CD34^{+} cell numbers obtained by Plerixafor+G-CSF mobilization through single collections, even in hard-to-mobilize thalassemic patients,[10] coupled with the superior short-term engraftment and increased β-globin production/VCN of Plerixafor+G-CSF cells, render them an optimal graft for thalassemia GT. Importantly, a given required expression level might be achieved by Plerixafor+G-CSF–mobilized cells at a lower VCN and, subsequently, higher biosafety for clinical applications. In summary, we provide data on the quality and preclinical efficacy of mobilized transduced HSCs from patients with thalassemia that may prove critical for the optimization of clinical GT protocols. Additional information can be found in supplemental Results and Discussion.

## Authorship

Contribution: G.K. designed and conducted experiments, collected and assembled data, performed data analysis and interpretation, and wrote the manuscript; N.P., J.E.A., F.Z., and N.Z. conducted experiments or/and data collection; C.W.L. conducted experiments and data collection and edited the manuscript; M.K., C.T. and A.A. provided administrative or/and financial support; M.S. and I.R. supplied study materials and provided administrative support; G.S. provided financial and administrative support and gave final approval of manuscript; and E.Y. conceived and designed the study, interpreted data, provided financial and administrative support, wrote the manuscript, and provided final approval of manuscript.

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## References

1. Weatherall DJ, Clegg JB. The thalassemia syndrome. Oxford, UK: Blackwell Scientific Publishers; 1981.

2. Lucarelli G, Galimberti M, Polchi P, et al. Marrow transplantation in patients with thalassemia responsive to iron chelation therapy. N Engl J Med. 1993;329(12):840-844.

3. Lucarelli G, Isgrò A, Sodani P, Gaziev J. Hematopoietic stem cell transplantation in thalassemia and sickle cell anemia. Cold Spring Harb Perspect Med. 2012;2(5):a011825.

4. Borgna-Pignatti C, Rugolotto S, De Stefano P, et al. Survival and complications in patients with thalassemia major treated with transfusion and deferoxamine. Haematologica. 2004;89(10):1187-1193.

5. Sadelain M, Boulad F, Galanello R, et al. Therapeutic options for patients with severe beta-thalassemia: the need for globin gene therapy. Hum Gene Ther. 2007;18(1):1-9.

6. Yannaki E, Emery DW, Stamatoyannopoulos G. Gene therapy for β-thalassaemia: the continuing challenge. Expert Rev Mol Med. 2010;12:e31.

7. Champlin RE, Schmitz N, Horowitz MM, et al; IBMTR Histocompatibility and Stem Cell Sources Working Committee and the European Group for Blood and Marrow Transplantation (EBMT). Blood stem cells compared with bone marrow as a source of hematopoietic cells for allogeneic transplantation. Blood. 2000;95(12):3702-3709.

8. Bensinger WI, Martin PJ, Storer B, et al. Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers. N Engl J Med. 2001;344(3):175-181.

9. Yannaki E, Papayannopoulou T, Jonlin E, et al. Hematopoietic stem cell mobilization for gene therapy of adult patients with severe β-thalassemia: results of clinical trials using G-CSF or plerixafor in splenectomized and nonsplenectomized subjects. Mol Ther. 2012;20(1):230-238.

10. Yannaki E, Karponi G, Zervou F, et al. Hematopoietic stem cell mobilization for gene therapy: superior mobilization by the combination of granulocyte-colony stimulating factor plus plerixafor in patients with β-thalassemia major. Hum Gene Ther. 2013;24(10):852-860.

11. May C, Rivella S, Callegari J, et al. Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. Nature. 2000;406(6791):82-86.

12. Boulad F, Wang X, Qu J, et al. Safe mobilization of CD34+ cells in adults with β-thalassemia and validation of effective globin gene transfer for clinical investigation. Blood. 2014;123(10):1483-1486.

13. Psatha N, Sgouramali E, Gkountis A, et al. Superior long-term repopulating capacity of G-CSF+plerixafor-mobilized blood: implications for stem cell gene therapy by studies in the Hbb(th-3) mouse model. Hum Gene Ther Methods. 2014;25(6):317-327.

14. Larochelle A, Krouse A, Metzger M, et al. AMD3100 mobilizes hematopoietic stem cells with long-term repopulating capacity in nonhuman primates. Blood. 2006;107(9):3772-3778.

15. Genovese P, Schiroli G, Escobar G, et al. Targeted genome editing in human repopulating haematopoietic stem cells. Nature. 2014;510(7504):235-240.

16. Hu Z, Van Rooijen N, Yang Y-G. Macrophages prevent human red blood cell reconstitution in immunodeficient mice. Blood. 2011;118(22):5938-5946.