LETTERS
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# Intrabone hematopoietic stem cell gene therapy for adult and pediatric patients affected by transfusion-dependent ß-thalassemia

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**ß-thalassemia is caused by ß-globin gene mutations resulting in reduced ($\beta^+$) or absent ($\beta^0$) hemoglobin production. Patient life expectancy has recently increased, but the need for chronic transfusions in transfusion-dependent thalassemia (TDT) and iron chelation impairs quality of life[1]. Allogeneic hematopoietic stem cell (HSC) transplantation represents the curative treatment, with thalassemia-free survival exceeding 80%. However, it is available to a minority of patients and is associated with morbidity, rejection and graft-versus-host disease[2]. Gene therapy with autologous HSCs modified to express ß-globin represents a potential therapeutic option. We treated three adults and six children with $ß^0$ or severe $ß^+$ mutations in a phase 1/2 trial (NCT02453477) with an intrabone administration of HSCs transduced with the lentiviral vector GLOBE. Rapid hematopoietic recovery with polyclonal multilineage engraftment of vector-marked cells was achieved, with a median of 37.5% (range 12.6–76.4%) in hematopoietic progenitors and a vector copy number per cell (VCN) of 0.58 (range 0.10–1.97) in erythroid precursors at 1 year, in absence of clonal dominance. Transfusion requirement was reduced in the adults. Three out of four evaluable pediatric participants discontinued transfusions after gene therapy and were transfusion independent at the last follow-up. Younger age and persistence of higher VCN in the repopulating hematopoietic cells are associated with better outcome.**

HSC gene therapy with lentiviral vectors has provided evidence of safety and long-term efficacy in different genetic diseases[3]. ß-thalassemia syndromes are among the most prevalent monogenic diseases worldwide, with an estimated 60,000 infants born yearly with severe TDT[4]. A pioneering gene therapy study and an ongoing clinical trial reported safety and clinical benefits, in which transfusion independence was reached by patients with less severe ß-thalassemia mutations (non-$ß^0/ß^0$) and three out of nine patients with $ß^0/ß^0$ or severe $ß^+$ mutations[5,6]. We previously showed that the GLOBE lentiviral vector ameliorates erythropoiesis in patients' $CD34^+$ cells[7] and corrects thalassemia in a murine model[8,9], with absence of vector-induced toxicity and tumorigenicity[10]. On the basis of these results, we designed a phase 1/2 gene therapy clinical trial for patients with TDT using reduced-toxicity conditioning with treosulfan–thiotepa. A staggered design based on safety evidence allowed inclusion of adolescents and children. To avoid trapping intravenously infused HSCs into filter organs, we delivered the cells intrabone, hypothesizing that there would be an early hematopoietic recovery with a favorable long-term outcome, as has been shown for cord-blood transplantation[11–15]. We developed a murine model to test this hypothesis. Lineage-negative cells expressing luciferase[16] were intrabone- or intravenously administered in recipients. We showed via bioluminescent imaging that intrabone injection favors direct homing to the bone marrow spaces, limiting unspecific loss in non-target organs (Extended Data Figs. 1 and 2a,b). In intrabone-injected mice, cells that expanded in the injected femur persisted for 7 days and were detectable in other hematopoietic districts at day 10. Less cell trapping in lung and liver–spleen and more efficient platelet rescue with a significantly higher engraftment at 2 and 4 months were evident in intrabone-injected mice relative to intravenously injected

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**Table 1 | Characteristics of participants, drug product and treatment**

| Participant ID number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Age at gene therapy (years) | 31 | 35 | 34 | 13 | 13 | 13 | 6 | 5 | 4 |
| Gender | M | F | M | M | M | M | M | F | F |
| Genotype | cod39/IVS I-110 ß$^0$/ß$^+$ | cod39/IVS I-110 ß$^0$/ß$^+$ | cod39/IVS I-110 ß$^0$/ß$^+$ | IVS I-110 homo ß$^+$/ß$^+$ | cod39 homo ß$^0$/ß$^0$ | IVS I-6/IVS I-110 ß$^+$/ß$^+$ | cod39 homo ß$^0$/ß$^0$ | IVS I-110 homo ß$^+$/ß$^+$ | IVS II-1 IVS I-110 ß$^0$/ß$^+$ |
| Transfusion requirement (ml kg$^{-1}$ per year) | 284 | 266 | 197 | 216 | 255 | 226 | 324 | 206 | 298 |
| Chelation | DFO | DFX | DFX | DFX | DFO | DFX | DFP | DFX | DFX |
| Serum ferritin (ng ml$^{-1}$) | 791 | 171 | 178 | 1,018 | 1,776 | 2,585 | 2,397 | 2,918 | 1,560 |
| Cardiac iron T2* (ms) | 32.10 | 45.11 | 46.60 | 44.00 | 38.00 | 26.30 | 62.84 | 32.00 | Not done§ |
| Cardiac iron overload | Not significant | Not significant | Not significant | Not significant | Not significant | Not significant | Not significant | Not significant | Not applicable |
| LIC (mg Fe per g dw) | 2.06 | 2.34 | 2.07 | 3.12 | 2.41 | 3.88 | 5.23 | 8.67 | Not done§ |
| Liver iron overload | Not significant | Not significant | Not significant | Not significant | Not significant | Mild | Mild | Moderate | Not applicable |
| Comorbidities | Gilbert syndr. | Multiple^ | Splenomegaly Gilbert syndr. | None | Growth hormone def. | None | None | None | None |
| Number of CD34$^+$cells collected (×10$^6$ kg$^{-1}$) | 30 | 23 | 21 | 53 | 47 | 45 | 50 | 31 | 30 |
| No. apheresis procedures | 2 | 2 | 3 | 1 | 2 | 2 | 1 | 1 | 2 |
| Treosulfan exposure (AUC mg h l$^{-1}$) | 1,380 | 2,038 | 1,502 | 1,684 | 1,573 | 1,311 | 1,231 | 1,875 | 1,782 |
| CD34$^+$cell dose (×10$^6$ kg$^{-1}$) | 19.4 | 18.7 | 18.0 | 19.5 | 16.3 | 19.5 | 19.7 | 20.0 | 19.8 |
| Drug product (ml) | 58.8 | 58.5 | 58.6 | 48.9 | 29.3 | 58.7 | 24.3 | 20.3 | 22.4 |
| Drug product VCN | 0.8 | 0.7 | 0.7 | 1.2 | 1.5 | 0.7 | 1.0 | 0.9 | 0.9 |
| Drug product transduction efficiency (%) | 60 | 63 | 68 | 53 | 77 | 62 | 59 | 55 | 38 |
| Neutrophil engraftment (d) | 21 | 16 | 25 | 18 | 19 | 17 | 22 | 15 | 34* |
| Platelet engraftment (d) | 16 | 11 | 21 | 13 | 15 | 10 | 15 | 16 | 24 |
| Follow-up (months) | 28.0 | 21.8 | 23.7 | 18.8 | 17.9 | 15.3 | 13.7 | 1.7 | 0.9 |

M, male; F, female; DFO, deferoxamine; DFX, deferasirox; DFP, deferiprone; homo, homozygous; ms, milliseconds; LIC (liver iron concentration) expressed as mg of iron per g dw (dry weight), calculated from liver T2* (ref. [33]); syndr., syndrome; def, deficiency; AUC, area under the curve; VCN, vector copy number/cell. °IVS I-110 is a ß$^+$ mutation usually resulting in very modest endogenous hemoglobin A production and therefore is addressed as severe. §Magnetic resonance imaging not done as iron overload very improbable owing to young age and regular chelation history. ^Participant 2 comorbidities: hypogonadism, hypothyroidism, osteoporosis, HCV antibody positivity with negative RNA, pernicious anemia. *Neutrophil engraftment on 8 January 2018, outside data cut off (31 December 2017).

mice (Extended Data Fig. 2). Comparative studies in mice and non-human primates have shown that intrabone injection resulted in a higher repopulating cell frequency than intravenous injection[17] and significantly reduced trapping[18]. Thus, we moved to clinical application using the intrabone administration route.

Between September 2015 and December 2017, 10 patients were enrolled and 9 were treated (1 patient did not meet inclusion criteria). Efficacy outcome is reported for seven patients with >12 months of follow-up as of December 2017. All patients had a high pre-gene-therapy transfusion requirement (median 255 ml kg$^{-1}$ per year, range 197–324 ml kg$^{-1}$), severe mutations (β$^0$ or severe ß$^+$ IVS I-110) and a median follow-up of 18 months (range 1–28 months) (Table 1). Three participants had mild or moderate liver iron overload. A median of 31 × 10$^6$

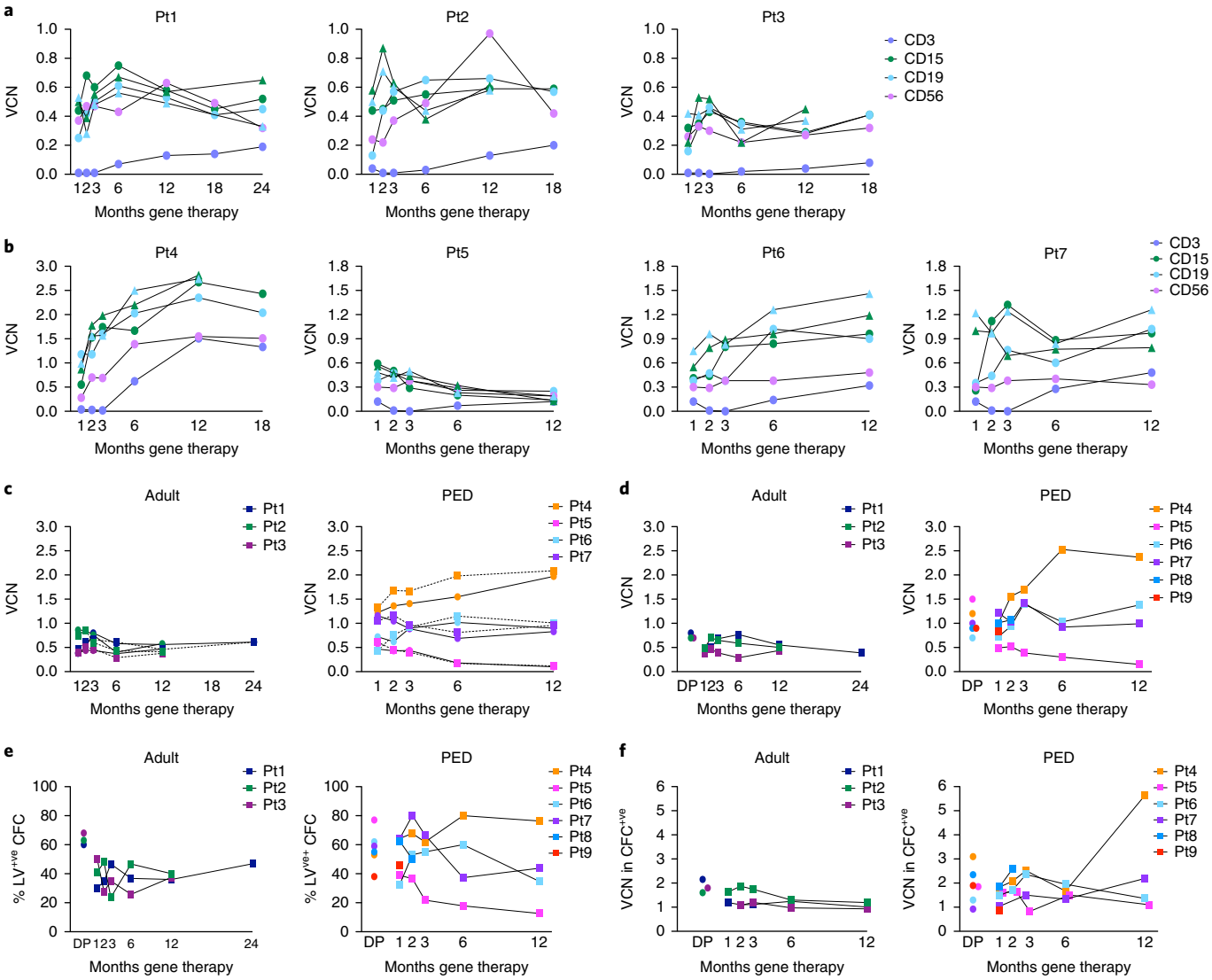



**Fig. 1 | Engraftment of transduced cells in peripheral blood and bone marrow of adult and pediatric participants. a**,**b**, VCN per genome in different purified lymphoid (CD3$^+$, CD19$^+$, CD56$^+$) and myeloid (CD15$^+$) lineages from peripheral blood (circle) and bone marrow (triangle) of adults (**a**) and pediatric (**b**) participants. **c**, VCN in erythroid glycophorin A$^+$ cells (circle and solid line) and CD36$^+$ cells (square and dashed line) purified from bone marrow of adult and pediatric (PED) participants. **d**, VCN in CD34$^+$ cells purified from bone marrow of adult and pediatric participants. **e**, Transduction efficiency was assessed as the percentage of lentiviral vector–positive CFCs . **f**, Median VCN values in lentiviral vector–positive CFCs are reported for adult and pediatric participants. Drug product (DP) indicates values evaluated on infused cells.

CD34$^+$ cells per kg were harvested following mobilization with lenograstim and plerixafor (Table 1). Participants were in the expected treosulfan exposure range[19], with a mean day 1 AUC of 1,597 mg h l$^{-1}$ (range 1,231–2,038 mg h l$^{-1}$). GLOBE lentiviral vector (Extended Data Fig. 3)–transduced CD34$^+$ cells (drug product) were administered intrabone at a median cell dose of $19.5\times10^6$ per kg (range 16.3–20.0 per kg) with a median VCN of 0.9 (0.7–1.5) and transduction efficiency of 60% (38–77%) (Table 1). All participants had grade 4 neutropaenia lasting a median of 16 d (range 12–31 d) and platelet transfusion–supported grade 4 thrombocytopenia. Neutrophil engraftment occurred on day 19 (median day 19, range day 15–34) and platelet engraftment on day 15 (median day 15, range day 10–24) (Table 1). Participants were discharged from the hospital after a median of 31 d (range 27–47 d) and were clinically well at the last visit, with a median follow-up of 18 months (range 0.9–28 months). Potential procedural risks of intrabone injection such as local bone damage, infection or hemorrhage, systemic embolization, respiratory depression or hypotension, as well as adverse reactions attributable to drug product, were absent. Chemotherapy-related toxicity was mild, consisting of grade 1–2 mucositis in five participants, grade 1–2 skin rash in four participants and grade 1 hyperbilirubinemia in participant 9. Five serious adverse events of infectious nature were reported (Supplementary Table 1) and resolved. On the basis of early studies in which non-myeloablative conditioning resulted in insufficient engraftment of gene-marked cells and minimal clinical benefit[20], we opted for myeloablative conditioning. The choice of treosulfan–thiotepa over busulfan was made given its reduced extramedullary toxicity[21,22]. In line with the experience in allogeneic stem cell transplantation[23], we observed neither veno-occlusive disease nor hepatic toxicity. The gene-therapy procedure was uneventful, and the time of neutrophil and especially of platelet engraftment was comparable with that of autologous stem cell transplant and shorter than that observed in other HSC gene-therapy studies[6,24–26]. Although this trial was not

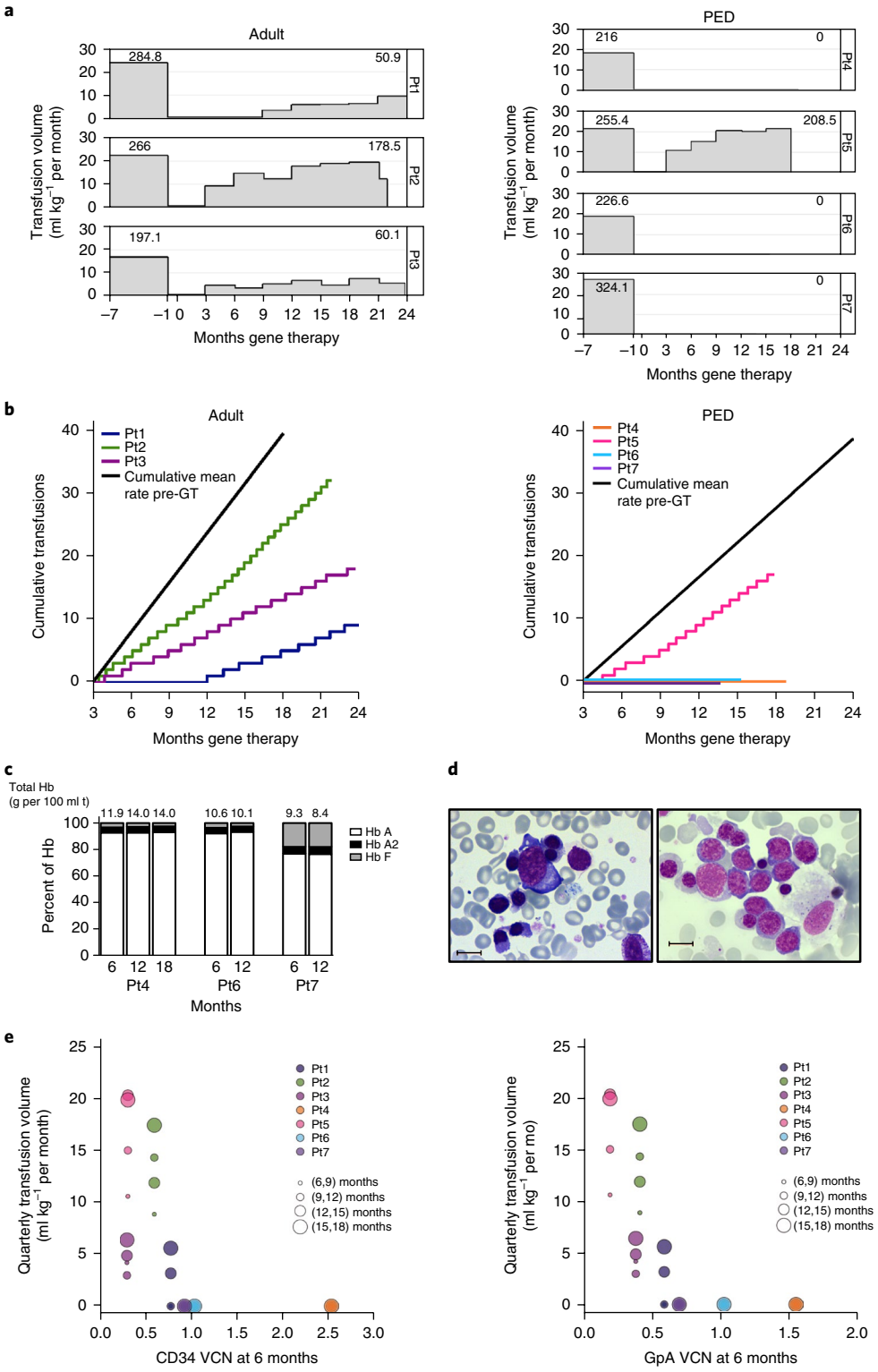
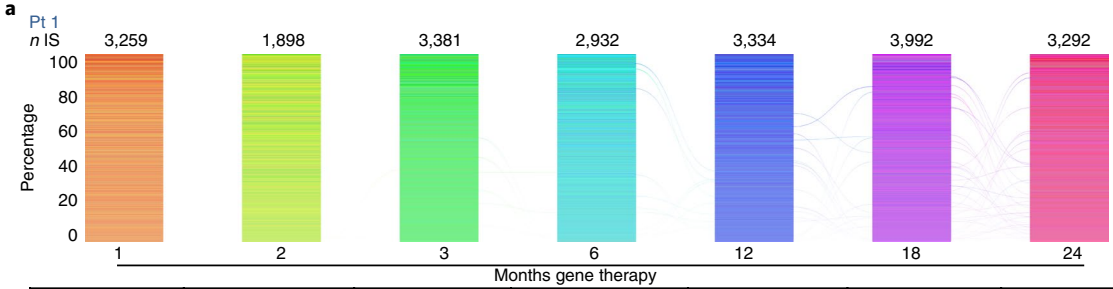
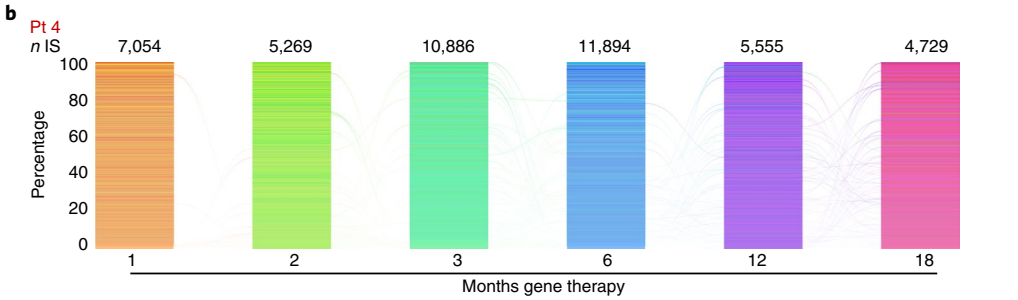



**Fig. 2 | Clinical outcome. a**, Graphical representation of quarterly transfusion volume pre- and post-gene-therapy in individual adults (left panel) and pediatric participants (PED, right panel). Pre- and post-gene-therapy yearly volumes (ml $kg^{-1}$ per yr) are reported. **b**, Transfusion rate pre-gene-therapy (mean reference: 2.2 transfusions/month, thick black line) and post-genetherapy in adults (left panel). Transfusion rate pre-gene-therapy (mean reference: 1.6 transfusions per month, thick black line) and post-gene-therapy in pediatric participants (right panel). **c**, Hemoglobin (Hb) fractions and total hemoglobin values (expressed as g per 100 ml above the corresponding bars) in transfusion-independent participants (Pt4, Pt6 and Pt7) at different follow-up times. **d**, Bone marrow morphology in participant 4 at baseline (left panel) and 12 months post-infusion (right panel). In the right panel, a reduced poikilocytosis of red blood cells, more homogeneous hemoglobinization of the cytoplasm of erythroblasts, reduction of binuclear erythroblasts and internuclear bridges can be seen. May–Grunwald–Giemsa staining, magnification ×1,000. Scale bar, 10 µm. **e**, Association between VCN at 6 months in bone marrow $CD34^+$ cells (left panel) and glycoforin-$A^+$ cells (right panel) and transfusion volume in the time intervals of (6,9), (9,12), (12,15) and (15,18) months. The size of the symbols increases with increasing time.




| 1 | | 2 | | 3 | | 6 | | 12 | | 18 | | 24 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene symbol | Rel. % | Gene symbol | Rel. % | Gene symbol | Rel. % | Gene symbol | Rel. % | Gene symbol | Rel. % | Gene symbol | Rel. % | Gene symbol | Rel. % |
| *ATXN2L* | 0.32 | *TMC3-AS1* | 0.38 | *NF1* | 0.37 | *LINC01122* | 0.38 | *PARN* | 0.29 | *KANSL1* | 0.19 | *AHNAK* | 0.32 |
| *EZH2* | 0.29 | *KDM4C* | 0.30 | *TRAIP* | 0.36 | *CENPC* | 0.27 | *SKAP2* | 0.25 | *MIR2054* | 0.19 | *MOB3A* | 0.26 |
| *HNRNPM* | 0.28 | *CENPL* | 0.27 | *VPRBP* | 0.35 | *ZNF627* | 0.26 | *ESF1* | 0.24 | *RABL6* | 0.18 | *XKR3* | 0.24 |
| *NF1* | 0.26 | *ZNF267* | 0.23 | *KCTD13* | 0.34 | *KL* | 0.26 | *GTF2I* | 0.24 | *WIZ* | 0.17 | *CCDC88A* | 0.23 |
| *PPP1R12C* | 0.25 | *ATP11C* | 0.23 | *USP14* | 0.32 | *YTHDF2* | 0.25 | *SPOP* | 0.23 | *ZMYM4* | 0.17 | *GPATCH8* | 0.22 |
| *GPN1* | 0.25 | *ZSWIM5* | 0.23 | *DHDDS* | 0.32 | *FAM169A* | 0.25 | *CPNE8* | 0.23 | *MALAT1* | 0.17 | *MUM1* | 0.21 |
| *TANC2* | 0.23 | *PTBP2* | 0.23 | *ADAM28* | 0.31 | *LOC103312105* | 0.25 | *SORL1* | 0.23 | *DYM* | 0.17 | *PDE4DIP* | 0.21 |
| *GPC6* | 0.22 | *AOAH* | 0.22 | *VGLL4* | 0.30 | *PCDH9* | 0.24 | *SKAP2* | 0.21 | *KDM6A* | 0.17 | *ISY1* | 0.20 |
| *PPP6R3* | 0.22 | *STAT5B* | 0.21 | *REPS2* | 0.30 | *FCHSD2* | 0.23 | *PABPC1* | 0.21 | *PPP2CB* | 0.17 | *BBX* | 0.20 |
| EFCAB13 | 0.22 | ZCCHC2 | 0.21 | TRAPPC6B | 0.30 | RAB28 | 0.23 | SNTG1 | 0.21 | PTK2 | 0.17 | CAPN1 | 0.20 |




| 1 | | 2 | | 3 | | 6 | | 12 | | 18 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene symbol | Rel. % | Gene symbol | Rel. % | Gene symbol | Rel. % | Gene symbol | Rel. % | Gene symbol | Rel. % | Gene symbol | Rel. % |
| *TNKS* | 0.21 | *SEC24A* | 0.38 | *KIF1B* | 0.14 | *S100A6* | 0.19 | *SPATA16* | 0.22 | *RNF216* | 0.21 |
| *INTS6* | 0.18 | *KIF11* | 0.25 | *VRK1* | 0.13 | *SSBP2* | 0.19 | *FKBP5* | 0.20 | *ARFGEF1* | 0.21 |
| *NF1* | 0.16 | *FAM216A* | 0.24 | *KAT8* | 0.12 | *RPTOR* | 0.18 | *CCNE1* | 0.19 | *FAM208B* | 0.17 |
| *ACAP2* | 0.16 | *PTPDC1* | 0.23 | *ACOX1* | 0.12 | *RERE* | 0.17 | *FXYD5* | 0.19 | *PTPRA* | 0.16 |
| *SRP68* | 0.16 | *SPECC1* | 0.23 | *STK19* | 0.11 | *MIR620* | 0.16 | *ERO1L* | 0.18 | *DNAJC8* | 0.16 |
| *RANBP10* | 0.16 | *GBF1* | 0.23 | *UBAP2L* | 0.11 | *METTL16* | 0.16 | *ZC3H4* | 0.16 | *EIF4G3* | 0.16 |
| *ARHGAP35* | 0.16 | *HNRNPR* | 0.22 | *TAF3* | 0.10 | *CEP83* | 0.16 | *PACS2* | 0.16 | *MROH1* | 0.15 |
| *ARID5B* | 0.15 | *LRPPRC* | 0.22 | *ATF7* | 0.10 | *ATP6V1H* | 0.16 | *WDR59* | 0.16 | *STXBP3* | 0.14 |
| *DIP2B* | 0.15 | *AKR1D1* | 0.22 | *FAM117B* | 0.10 | *HDAC5* | 0.16 | *ZBED5* | 0.15 | *CEL* | 0.14 |
| KAT6B | 0.14 | ZMYM3 | 0.22 | IFT140 | 0.10 | CDK15 | 0.16 | BLM | 0.15 | LOC101927817 | 0.14 |

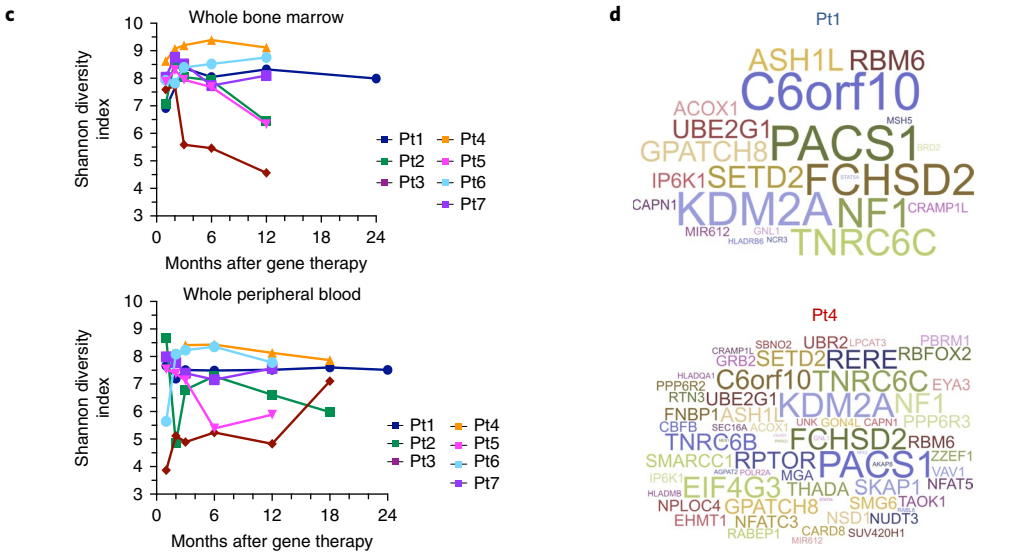



**Fig. 3 | Integration site analysis. a**,**b**, Clonal abundance from whole peripheral blood samples over time in an adult participant (Pt1) (**a**) and a pediatric participant (Pt4) (**b**). For each participant, the clonal abundance is represented over time (*x* axis, months after gene therapy) with a stacked bar plot in which each clone (each represented by a different color) and the height shows the percentage of genomes with a specific integration site over the total genomes; ribbons connect tracked clones between two consecutive time points. Below each plot, the ten most abundant clones annotated with the closest gene are reported. *n* IS, number of integration sites. **c**, Diversity index (Shannon entropy) computed for each time point. For participants 8 and 9, data are not available yet. **d**, Common integration sites (CIS) analysis. Word clouds representing the top targeted genes (selecting the common insertion site by $P < 0.05$ using the kernel-based statistical approach as previously described in de Ridder et al.[32] and the outliers of the distribution > 2σ) for participants 1 (*n* integration sites = 48,745) and 4 (*n* integration sites = 21,520) emerging as common insertion site statistics. The size of each gene symbol is proportional to the number of integration sites targeting that gene.

conceived to compare the intrabone and intravenous administration routes, the intrabone administration route could have contributed to a shorter time to platelet engraftment (median 15 d, range 10–24 d; compared with 39.5 d, range 19.0–191.0 d in HGB-204; 23 d, range 20–26 d in HGB-205 (ref. [6])). This is particularly relevant for patients with thalassemia who can be refractory to platelet transfusions owing to life-long exposure to transfusions and who therefore are at risk of life-threatening bleeding during aplasia[27].

Quantitative PCR analysis of VCN in bone marrow and peripheral blood lineages showed that vector-marked cells generally increased over the first 6 months and then stabilized in most participants (Fig. 1a–c). A lower and slowly increasing level of marked CD3$^+$ cells was detected, in line with previous HSC gene-therapy trials[28]. In the three adults, VCN in CD34$^+$ cells and proportion of lentiviral vector–positive colony-forming cells (CFCs) peaked in the second to third month and then decreased and stabilized from month 12 onward (VCN: median 0.50, range 0.44–0.55; percentage of lentiviral vector–positive CFCs: median 37.5%, range 36.0–40.0%). In children, the trend was more heterogeneous (VCN: median 1.19, range 0.15–2.37; percentage of lentiviral vector-positive CFCs: median 39.3%, range 12.6–76.4%) (Fig. 1d,e). Median VCN values in CD34$^+$ clonogenic progenitors were higher in pediatric compared with adult participants (Fig. 1f). VCNs in erythroid CD36$^+$ or GpA$^+$ cells were similar to those in CD34$^+$ cells, suggesting that there was lineage maintenance over time from the pool of transduced HSCs (Fig. 1c,d).

At the last follow-up, the three adults had a reduced transfusion requirement compared with baseline (Fig. 2a, left panel and Supplementary Table 2). Participant 1 had a 9-month transfusion-free interval followed by reintroduction of transfusion support because hemoglobin was <8 g per 100 ml, but frequency and blood volume requirement were reduced. Transfusion continued for participant 3 at a reduced frequency and volume to maintain a hemoglobin target of >8 g per 100 ml, while participant 2 continued transufsion with a mild reduction compared with that before gene therapy (Fig. 2a,b, left panels). In line with this clinical outcome, analysis of this patient's baseline residual ß-globin expression revealed the lowest value (Extended Data Fig. 4).

Among the four pediatric participants (<14 years of age) evaluable for efficacy, three out of four received the last transfusion shortly after gene therapy and remained transfusion free to the last follow-up, with different hemoglobin levels depending on genotype severity and VCN value (Fig. 2a,b, right panels and Supplementary Table 2). Total hemoglobin levels stabilized over time, although at different values (Fig. 2c), with a positive correlation with the VCN in CD34$^+$ progenitors. The highest hemoglobin level, which reached up to 14 g per 100 ml, was seen in participant 4 who had severe ß$^+$ mutations (homozygous IVS I-110) and also the highest frequency and VCN of gene-modified cells. In this participant, endogenous hemoglobin A production at age 7 months, before the first transfusion, was 0.28 g per 100 ml. Participant 6, who had β$^+$ mutations (IVS I-110/IVS I-6), reached a hemoglobin level of about 10 g per 100 ml. Participant 7, who had a β$^0$β$^0$ genotype, achieved transfusion independence and a hemoglobin level of 8.4 g per 100 ml at the last follow-up, with absence of erythroblasts in peripheral blood smears, suggesting no significant ineffective erythropoiesis. Despite having the highest VCN measured in the drug product and an adequate treosulfan exposure, participant 5, who had a β$^0$β$^0$ genotype, had an unexpected VCN drop in all lineages and a consequent lack of clinical benefit, requiring restoration of transfusion support. A clonogenic assay on the day of gene therapy revealed a higher number of colonies in this participant compared with the others (Supplementary Table 3), suggesting a possible inefficient bone marrow niche depletion. A low level of marked cells in patients after gene therapy has been reported by others[6], suggesting the contribution of additional factors other than VCN in a drug product to the gene therapy outcome in these patients. Among participants who achieved transfusion independence, there was a reduction in ineffective erythropoiesis and erythroid dysplastic changes in bone marrow morphology (Fig. 2d). In line with this result, immune phenotype analysis showed restored progression to terminal erythroid differentiation, and blood smear revealed amelioration of red blood cell morphology. This was particularly evident in participant 4, who produced the highest hemoglobin level (Extended Data Fig. 5). In most patients, an association was observed between VCN in bone marrow CD34$^+$ and glycophorin A$^+$ cells and clinical benefit in terms of a reduced transfusion requirement (Fig. 2e).

In patients who had been transplanted with allogenic donors and had stable mixed chimerism, a selective advantage of normal erythroid cells has been reported[29]. However, in our study the levels of marked erythroid and myeloid cells were similar. Our preliminary data suggest that a VCN > 0.8 in peripheral blood and bone marrow cells and above 40% engraftment of gene-corrected CD34$^+$ progenitors may be the threshold for achieving freedom from transfusions in patients affected by severe genotypes. Although we could not demonstrate an association between VCN in the drug product and clinical benefit, at least with the number of participants and the VCN range of this trial, a high VCN in vivo seems to be one of the contributors to clinical benefit. To reduce variability in the outcome of gene therapy, further optimization of HSC gene transfer is desirable so that levels observed in other studies can be achieved[24,28,30].

Recent results from 22 patients with TDT treated in two combined phase 1/2 multicenter studies with intravenous HSC gene therapy following busulfan myeloablation showed that most of the non-ß$^0$/ß$^0$ patients achieved transfusion independence, while ß$^0$/ß$^0$ patients mainly achieved a reduction of transfusion requirement[6]. Nine out of 15 of the mainly adult patients that became transfusion independent had the mild ßE mutation, which was not present in our trial. The transfusion requirement before gene therapy was much higher in our trial compared with that in reported studies[6] (median 255 ml kg$^{-1}$ per year versus 164 and 182 ml kg$^{-1}$ per year). Overall, comparison of different clinical trials is crucial for protocol improvements geared toward patient safety and benefit but is also hampered by study-specific confounding variables.

The analysis of vector integration sites allows monitoring of the extent of polyclonal hematopoietic reconstitution and the potential occurrence of clonal expansion. Recovery of a high number of unique integration sites from all participants, ranging from 21,520 to 174,845 (Supplementary Table 4), was indicative of polyclonal reconstitution without evidence of clonal expansion or dominance (Fig. 3a,b and Extended Data Fig. 6). The clonal complexity in the progenitors and hematopoietic lineages, calculated with the Shannon diversity index, was maintained over time, with no evidence of oligoclonality or exhaustion (Fig. 3c) and with positive correlation with the level of gene marking in bone marrow (Extended Data Fig. 7a). Clonality was significantly higher in bone marrow than in peripheral blood in all subjects, with a higher polyclonal profile in pediatric subjects than in adults (Extended Data Fig. 7b). This finding reassures that there is an adequate pool of gene-marked cells in bone marrow. Clonality profiles in bone marrow erythroid CD36$^+$ and glycophorin A$^+$ cells (Extended Data Fig. 8) confirmed this finding in other cell subpopulations. The lentiviral vector genomic distribution showed a preference for integration in gene-dense regions and within transcription units, comparable with the pattern observed in other clinical trials (Extended Data Fig. 9a,b). The most-targeted genes at common insertion sites for the lentiviral vector and the overrepresented gene classes were not enriched for oncogenes or cancer-related genes and were similar to those of previous gene-therapy trials with longer follow-up[24,28,30–32] (Fig. 3d and Extended Data Fig. 9c).

In conclusion, our findings support the safety and efficacy of intrabone HSC gene therapy following a reduced-toxicity myeloablative

conditioning, as well as in pediatric patients affected by severe mutations. Reassuring safety results support involving very young patients on the basis of more efficient hematopoietic reconstitution with gene-corrected cells and higher clinical benefits. We hypothesize that the superior clinical outcome in children could be related to biological differences in HSC repopulating capacity and bone marrow niche function that could be potentially impaired during aging. A longer history of ineffective erythropoiesis, blood transfusions, iron overload and comorbidities in adults may contribute to an impaired HSC long-term engraftment, resulting in a less favorable outcome. Longer follow-up and results from a larger cohort of patients will provide further evidence on the long-term efficacy and safety of this treatment, confirming whether gene therapy can cure patients affected by TDT.

## Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41591-018-0301-6.

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## Author contributions

S.M., M.P.C., F.G. and V.C. contributed to the study design, patient follow-up, data collection, interpretation and manuscript writing. S.S. contributed to laboratory experiments, molecular analysis, data collection, interpretation and manuscript writing. A. Assanelli, M.E.B., M.C., G.C., N. Masera, E.A., N. Mirra, R.O., I.T., S.P., G.G. contributed to patient follow-up, data collection and interpretation. A.B. and M.G. contributed to data collection and regulatory applications. M.R.L. designed and performed the studies of the experimental model of intrabone HSC injection and contributed to laboratory experiments and data interpretation. C.R. and A. Aprile contributed to laboratory experiments and data interpretation. A.C., F.B., G.S. and E.M. contributed to integration sites sequencing, mapping and analysis. R.M., S. Gattillo, M.C., G.V. and L.S. were responsible for stem cell collection, characterization and data interpretation. R.W. was responsible for treosulfan pharmacokinetics data. S. Galimberti and M.G.V. contributed to study design, data interpretation and were responsible of

statistical analysis. L.N., M.D.C., F.C. and A. Aiuti contributed to study design, data interpretation and manuscript writing. G.F. contributed to study design, supervision of molecular studies and laboratory experiments, data interpretation and manuscript writing.

## Competing interests

The San Raffaele Telethon Institute for Gene Therapy (SR-TIGET) is a joint venture between the Telethon Foundation and Ospedale San Raffaele. The Telethon Foundation and Ospedale San Raffaele are entitled to receive milestone payments and royalties on commercialization of this therapy. A. Aiuti is the principal investigator of the SR-TIGET clinical trial of gene therapy for ß-thalassemia and S.M., M.P.C., F.G., V.C., M.D.C. and F.C. are co-investigators or sub-investigators. G.F. is the scientific director of the study. L.N. is an inventor on patents on lentiviral vector technology filed by the Salk Institute, Cell Genesys, Telethon Foundation and/or Ospedale San Raffaele, and may be entitled to receive some financial benefits from the licensing of such patents. All authors declare no other competing interests.

## Additional information

**Extended data** is available for this paper at https://doi.org/10.1038/s41591-018-0301-6.

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41591-018-0301-6.

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**Correspondence and requests for materials** should be addressed to G.F.

## Methods

**Study design and oversight.** This is an ongoing, single-center, non-randomized, open-label, phase 1/2 clinical study (NCT02453477). Eligible participants had TDT and were aged ≥3 years and were of any genotype. Pediatric patients (≤18 years) were excluded from participation if they had a suitable HLA 10/10 matched related or unrelated donor. Complete inclusion criteria are shown in the Supplementary Methods. A staggered strategy for enrollment was adopted on the basis of age, evaluated by an independent monitoring board (Supplementary Methods).

We have complied with all the ethical regulations for retrieving biological materials from patients. The study was approved by the Institutional Review Board of San Raffaele Hospital and conducted in compliance with the Declaration of Helsinki. Written informed consent was obtained from patients and/or parents. The medicinal product received Orphan Drug Designation by the European Medicines Agency (EMEA/OD/053/05). The primary aim of the study is to assess safety and efficacy of intrabone administration of GLOBE lentiviral vector–modified HSCs in patients with TDT after myeloablative conditioning with treosulfan–thiotepa. Primary safety endpoints are overall survival, achievement of hematological engraftment before day +60, safety of GLOBE lentiviral vector–transduced HSCs, safety and tolerability of conditioning regimen, overall safety and tolerability and polyclonal engraftment of transduced cells by lentiviral vector integration analysis. Primary efficacy endpoint is reduction in transfusion requirement up to transfusion independence. Secondary efficacy endpoints are detailed in Supplementary Methods.

**Participants.** Out of a planned ten treated patients, ten patients were enrolled and nine patients treated (six males, three females) between September 2015 and December 2017. One patient did not meet the inclusion criteria. Nine treated patients are included in this interim report and efficacy outcome is reported for seven patients with more than 1 year of follow-up at 31 December 2017 (Supplementary Methods).

**Trial procedures.** Peripheral blood stem cells were collected by leukapheresis following mobilization with lenograstim (Myelostim, Italfarmaco S.p.A.) 5 µg per kg twice daily and plerixafor (Sanofi-Mozobil, Genzyme) 0.24 mg kg$^{-1}$ daily after 3 days of lenograstim administration. Manufacturing of clinical-grade GLOBE lentiviral vector–transduced CD34$^+$ cells was performed at Molmed SpA (Supplementary Methods). The drug product was cryopreserved prior to treatment. An unmanipulated back up of ≥$2\times10^6$ CD34$^+$ cells per kg was also cryopreserved. Patients received a myeloablative conditioning regimen consisting of thiotepa (Tepadina, Adienne) 8 mg kg$^{-1}$ (reduced to 6 mg kg$^{-1}$ in adults) on day −5 and treosulfan (Treosulfan, Medac) 14 g m$^{-2}$ per day on days −4, −3, and −2.

On day 0, transduced CD34$^+$ cells were thawed, washed and infused intrabone bilaterally after local anesthesia was applied in the posterior superior iliac crests via one injection site for each side. The maximum accepted drug product dose was $20\times10^6$ CD34$^+$ cells per kg. The procedure was performed under bedside moderate sedation using a standard fenestrated bone-marrow harvest needle. Before injection, a small volume of bone marrow was aspirated to confirm that the needle was accurately inserted in the marrow cavity. To maximize marrow retention fenestrated needles, we avoided using more than one ipsilateral intrabone access site to avoid leakage from the non-injected site and used small volumes and a slow infusion rate[34]. The entire procedure took 20–30 minutes. To minimize the potential procedural risk, the intrabone injection was performed by a transplant physician skilled in intrabone infusions of cord blood, and the sedation was performed by an anesthetist.

On day +3, autologous peripheral blood lymphocytes with a target CD3$^+$ cells of $5\times10^7$ per kg (accepted range $1\times10^7$–$10\times10^7$ per kg) were intravenously infused to favor immune reconstitution. From day 3 onwards, participants will be followed-up for 2 years in the frame of the current trial (Supplementary Table 5) and for an additional 6 years in a long-term follow-up study (GSK 207757, NCT03275051). Fertility preservation measures were offered to all female participants and post-puberal males, according to current practice[35].

**Laboratory studies.** Peripheral blood and bone marrow samples were obtained at different time points from patients before and after gene therapy. Measurements of lentiviral vector VCN and integration sites analysis in different subpopulations, frequency of transduced cells and transgene expression are described in the Supplementary Methods.

**Statistical analysis.** Means, medians and ranges are reported for descriptive purposes, as appropriate. The individual quarterly rate of transfusion (number of transfusions per person-month of observation) and transfusion requirement (ml of blood per kg body weight and person-month of observation) were estimated before gene therapy (from month −7 to −1) and after (from 3 months after gene therapy onward to 24 months). The pre-gene-therapy mean rates of transfusion in adults and children were separately estimated as a reference. Follow-up was updated as of 31 December 2017[36].

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Code availability

Software scripts developed for the integration site analysis will be made available upon request from the corresponding author.

## Data availability

All requests for raw and analyzed data and materials are promptly reviewed by the corresponding author to verify if the request is subject to any intellectual property or confidentiality obligations. Patient-related data not included in the paper were generated as part of clinical trials and may be subject to patient confidentiality. Any data and materials that can be shared will be released via a Material Transfer Agreement.

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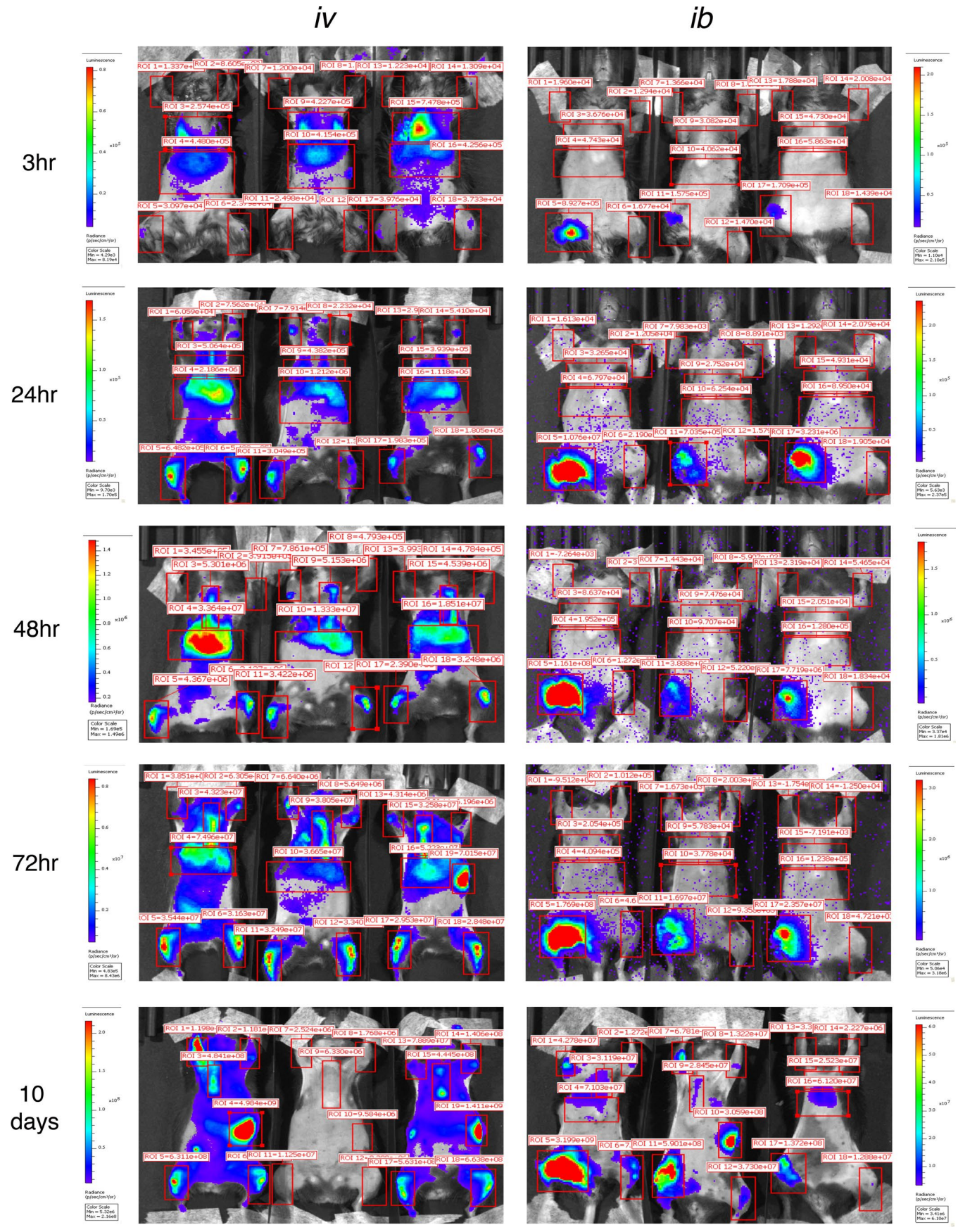
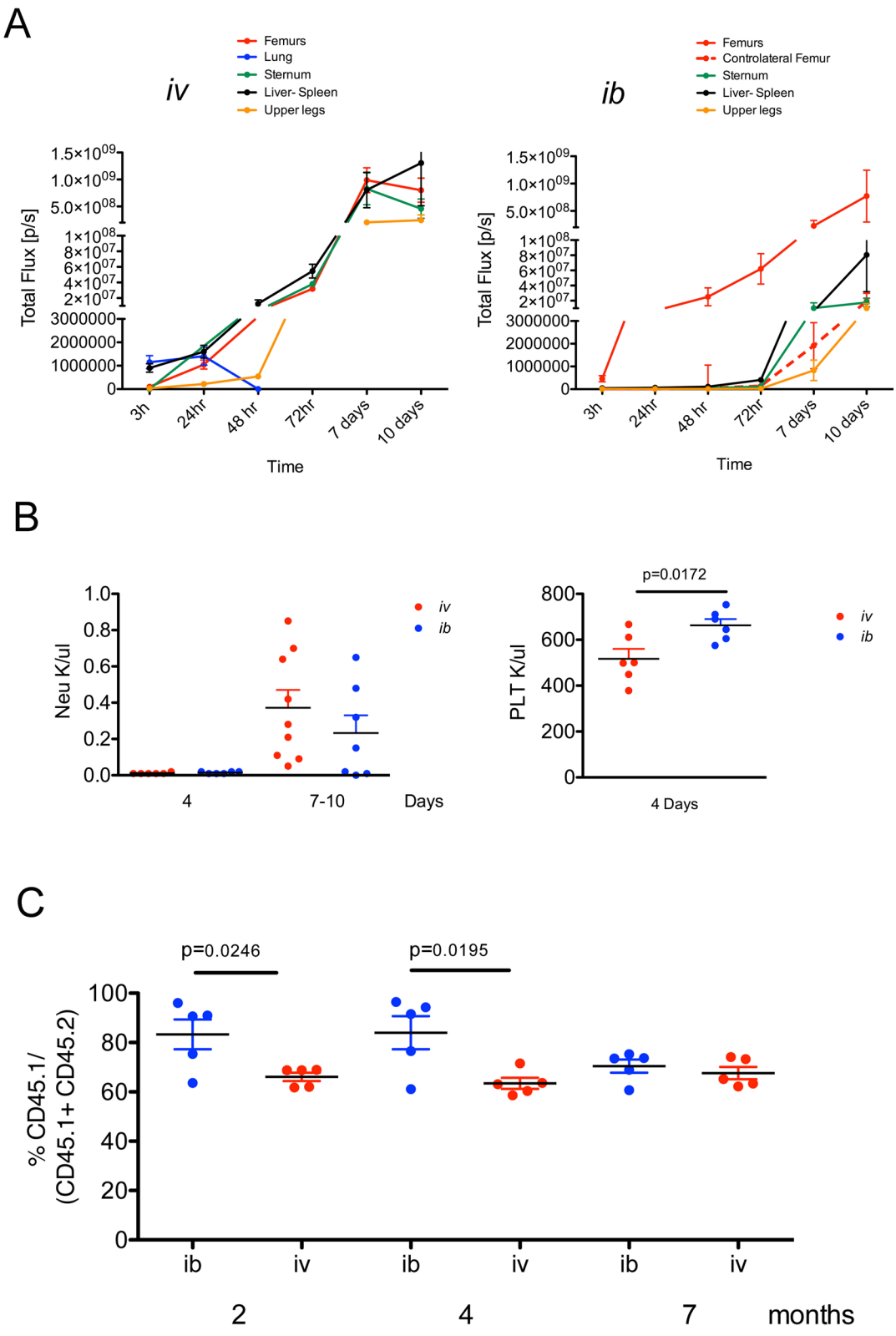
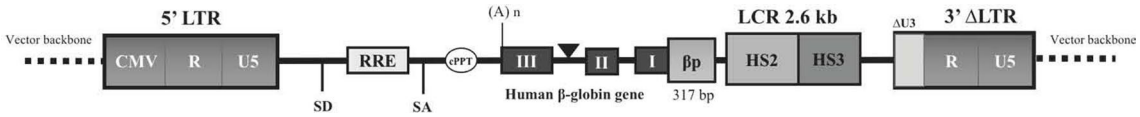


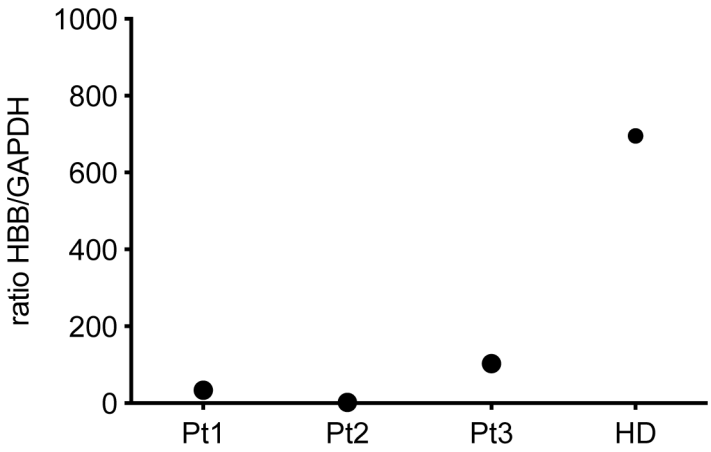

**Extended Data Fig. 1 | In vivo distribution of marked Lin- cells in different districts of the mouse body (intravenous versus intrabone).** HSPCs were injected in mice by intravenous or intrabone administration. Bioluminescence imaging analysis was used to evaluate the distribution of HSPCs expressing luciferase in the different body districts at the indicated time points. Representative images of three animals for each group.



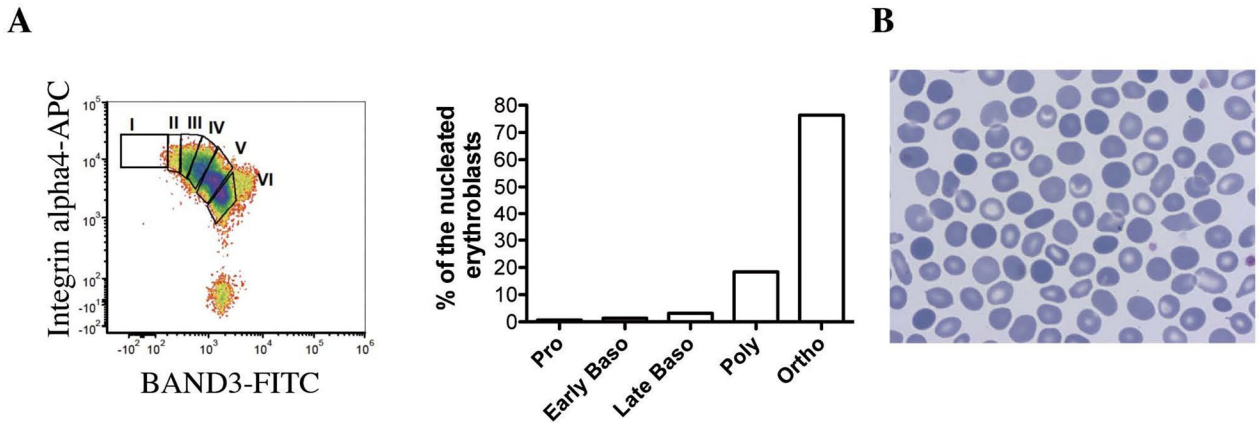

**Extended Data Fig. 2 | Analysis of biodistribution of transduced HSCs (intravenous versus intrabone). a**, Mice were analyzed using bioluminescence imaging to evaluate the dynamics of transduced of hematopoietic stem and progenitor cells (HSPCs) in the different districts of the animals at the indicated time points. Total flux was measured in the different districts of the animal body. At earlier time points, the levels of intravenous transplanted cells in the lung (3 h $P = 0.0120$, 24 h $P = 0.0216$) and in liver-spleen (3 h $P = 0.0042$, 24 h $P = 0.0014$ and 48 h $P = 0.0440$) were significantly higher than those of intrabone injected cells. (see Extended Data Fig. 2) **b**, Evaluation of hematological parameters (neutrophil, left panel and platelet count, right panel) in mice transplanted with HSPCs by intrabone ($N = 6$) or intravenous ($N = 6$) ($P = 0.0172$), a two-tailed *t*-test was applied. (see Extended Data Suppl. Fig. 2). **c**, Evaluation of hematopoietic reconstitution of HSPCs transplanted by intrabone or intravenous. Engraftment was analyzed by flow cytometry at the different time points in the peripheral blood (2–4–7 months) (see Extended Data Fig. 2). Engraftment was calculated as %CD45.1$^{pos}$ cells/ (CD45.1$^{pos}$ + CD45.2$^{pos}$) cells: 2 months ($P = 0.0246$) and 4 months ($P = 0.0195$). A two-tailed *t*-test was applied.



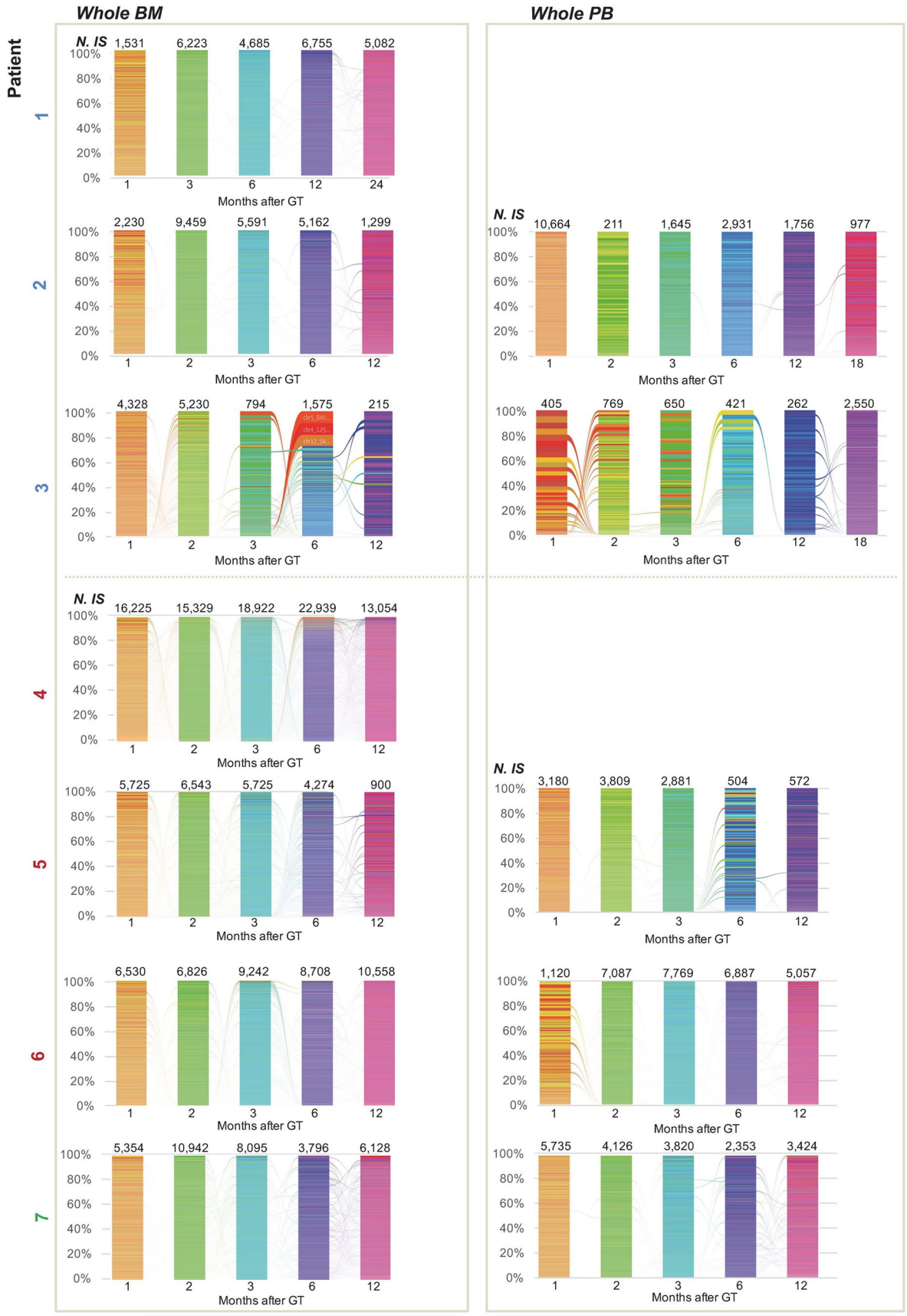

**Extended Data Fig. 3 | Structure of the GLOBE lentiviral vector.** Drug product was manufactured using clinical-grade GLOBE lentiviral vector lots. LTR, Long Terminal Repeat; CMV, enhancer/promoter region of cytomegalovirus; RRE, rev-responsive element; SD, splicing donor site; SA, splicing acceptor site; cPPT, central polypurine tract; βp, globin promoter; HS, DNase I-hypersensitive sites; LCR, Locus Control Region.



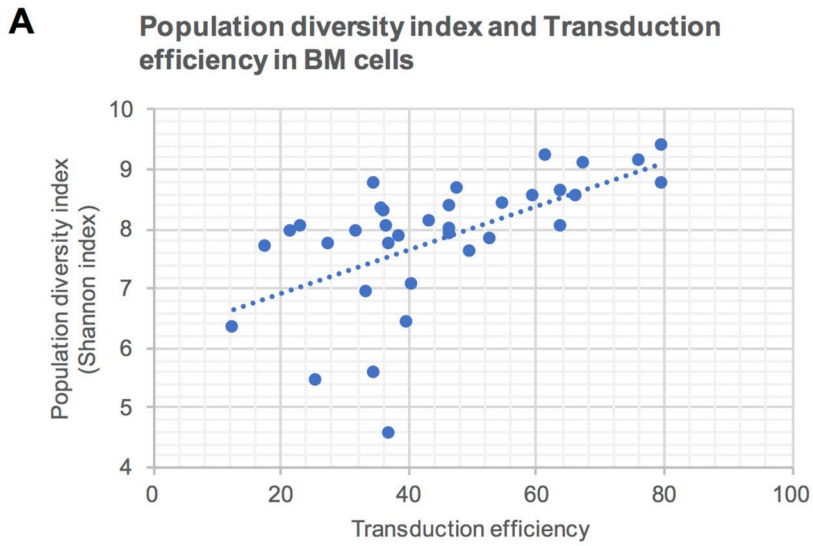

**Extended Data Fig. 4 | Basal level of expression in adult participants.** Levels of residual hemoglobin B expression measured by qPCR at screening in erythroid cultured cells from adult participants, expressed as a ratio on the housekeeping gene GAPDH. Results of patients are expressed as a mean of two replicates.

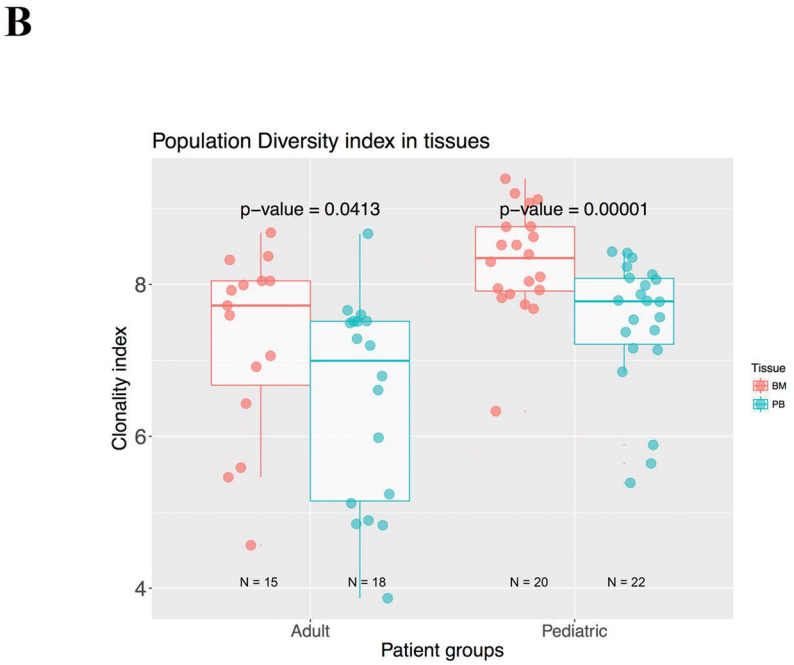



**Extended Data Fig. 5 | Terminal erythropoiesis in transfusion independent participant 4. a**, Gating strategy (left panel) and erythroid maturation (right panel) measured by flow-cytometry analysis on bone marrow isolated from participant 4 at 1 yr post-gene-therapy. Erythroid cells at different maturation stages are reported as percentage of total gated cells. Pro: proerythroblast; baso: basophilic erythroblast; poly: polychromatophilic erythroblast; ortho: orthochromatic erythroblast. In healthy donor, the physiologic progression of normal human terminal erythroid differentiation follows a ratio of Pro/Early Baso to Late Baso/Poly/Ortho, of 1/2/4/8/16 (as from Hu et al.[36]) **b**, Peripheral blood smear from Pt4 at 1 year follow-up. May–Grunwald–Giemsa staining, magnification ×1,000.

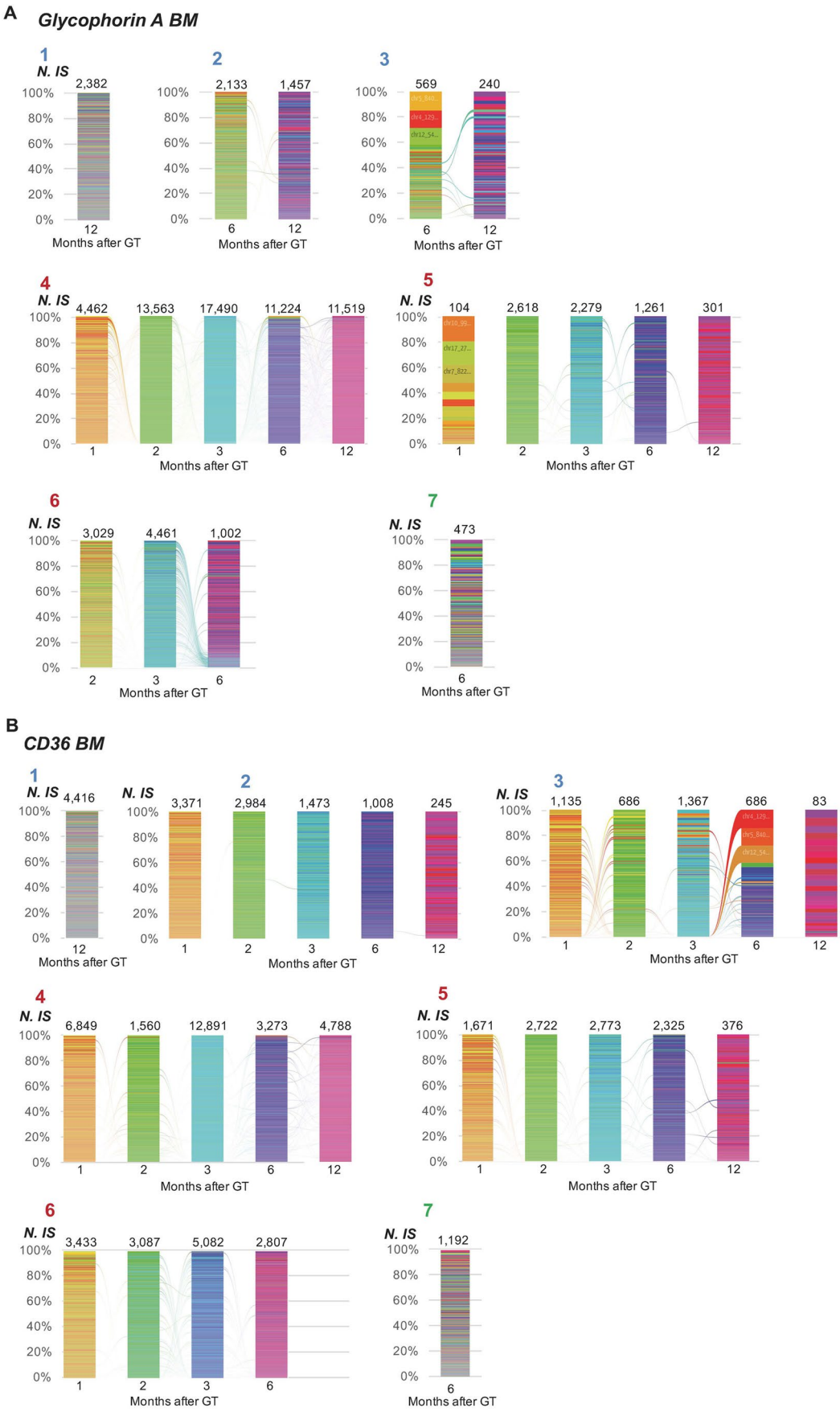



**Extended Data Fig. 6 | Clonal abundance over time.** For each participant, tissue and population analyzed (whole peripheral blood and whole bone marrow), the clonal abundance over time is represented (*x* axis, days after gene therapy) with a stacked bar plot in which each clone is a different color with the height in relative proportion with the number of retrieved cells (percentage); ribbons connect tracked clones between two consecutive time points. Clonal abundance of peripheral blood for participants 1 and 4 are shown in Fig. 3 of the main text. For participants 8 and 9 data are not available yet.

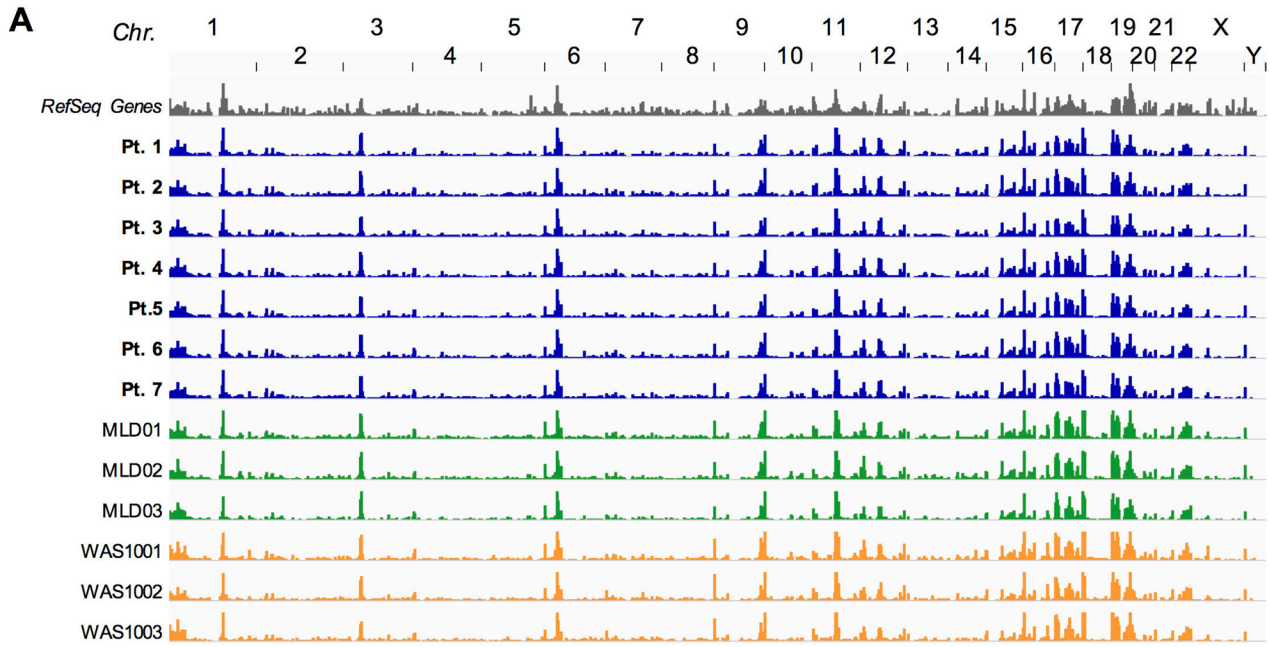


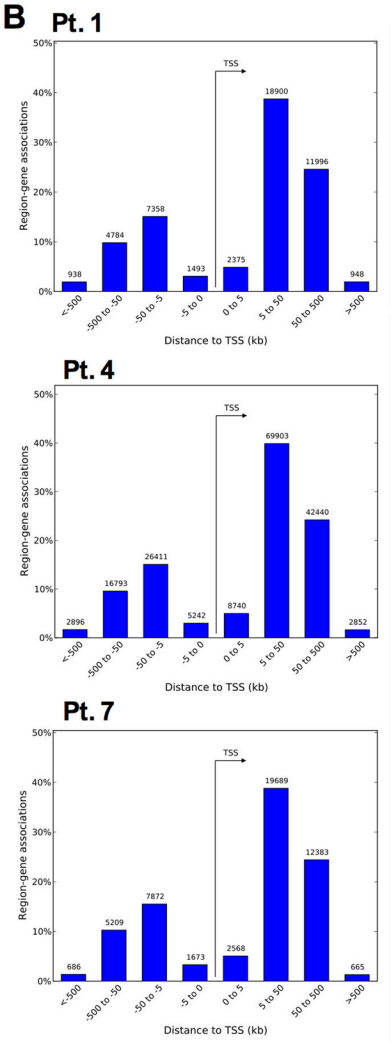



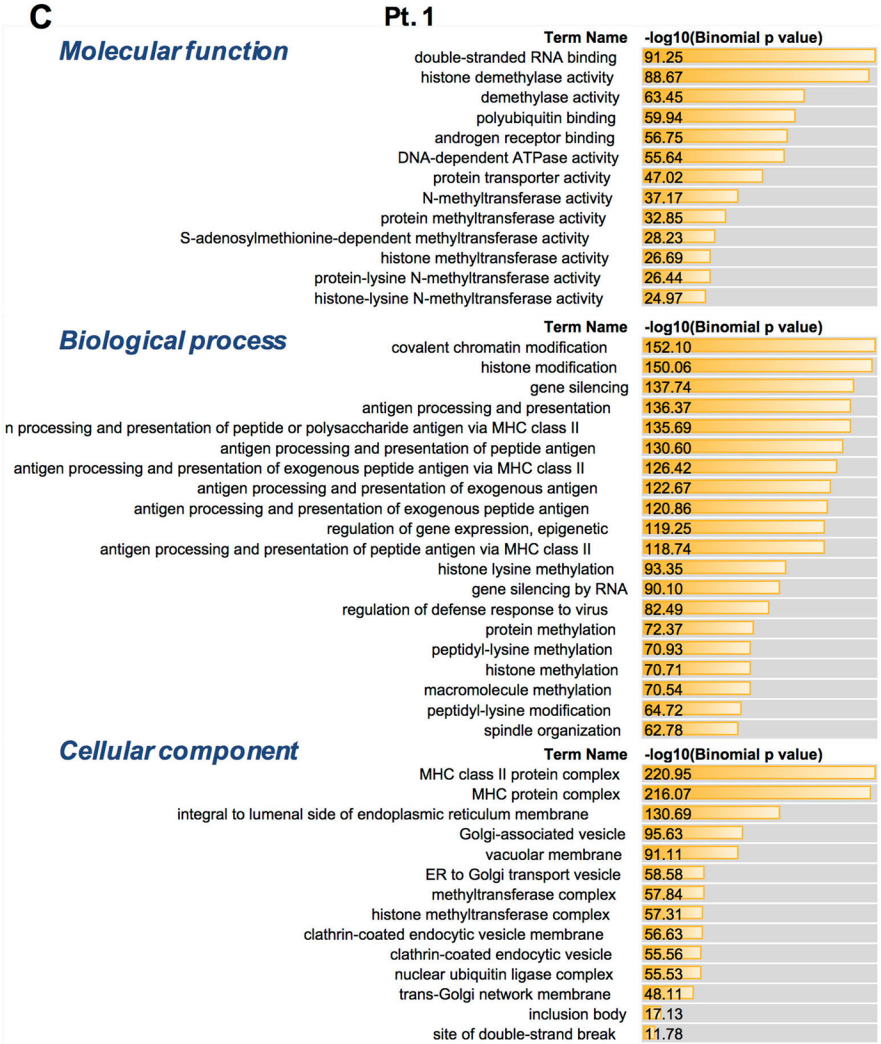

**Extended Data Fig. 7 | Correlation between transduction efficiency and the clonal population diversity index through integration site analysis.** **a**, Correlation between transduction efficiency in cells from bone marrow samples for all patients and all time points and the clonal population diversity index through integration site analysis. **b**, Clonality indexes obtained from whole bone marrow and peripheral blood cells of adults and pediatric subjects. Boxplots shows distributions of data (dots) in quartiles, where in each boxplot the upper/lower whiskers refer to 75th/25th percentile, the box refers to inter-quantile range and the median is shown with the solid line. A non-parametric exact Wilcoxon test was used to compare intregration site diversity between groups of patients (adults versus pediatrics) and a paired version was adopted to compare integration site diversity between tissues (bone marrow versus peripheral blood) (two-sided tests with alpha = 0.05).




**Extended Data Fig. 8 | Clonal abundance over time in erythroid bone marrow compartment. a**,**b**, Glycophorin A$^+$ (a) and CD36$^+$ (**b**) samples. Plots exploit a stacked bar plot representation (*x* axis, months post-gene-therapy; *y* axis, relative proportion of clonal abundance, number of cells per clone) in which each color is associated to a distinct clone, tracked with a ribbon between two consecutive time points. For participants 8 and 9 data are not available yet.




**Extended Data Fig. 9 | Genomic profile of vector integration sites.** Genomic profile of vector integration sites. **a**, Vector distribution profile in all chromosomes in comparison with that of other lentiviral vector trials participants. For participants 8 and 9 data are not available yet. **b**, Distribution of IS surrounding genes, centered on TSS for participants 1, 4 and 7. **c**, Gene ontology for participant 1 for the classes biological processes, cellular components, and molecular functions.

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# Reporting Summary

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## Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed | |
|---|---|---|
| ☐ | ☒ | The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☐ | ☒ | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☐ | ☒ | The statistical test(s) used AND whether they are one- or two-sided<br>*Only common tests should be described solely by name; describe more complex techniques in the Methods section.* |
| ☐ | ☒ | A description of all covariates tested |
| ☒ | ☐ | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☐ | ☒ | A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☐ | ☒ | For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted<br>*Give P values as exact values whenever suitable.* |
| ☒ | ☐ | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☒ | ☐ | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☐ | ☒ | Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated |
| ☐ | ☒ | Clearly defined error bars<br>*State explicitly what error bars represent (e.g. SD, SE, CI)* |

*Our web collection on statistics for biologists may be useful.*

## Software and code

Policy information about availability of computer code

| | |
|---|---|
| Data collection | FACS raw data were collected withFACS DIVA Software version 7 (BD).<br>qPCR raw data to determine VCN were collected with Quanta software (BIORAD), or ViiA™7 Software version 1.1 (Applied Biosystem)<br>BLI analysis raw data were collected with IVIS SpectrumCT System (Perkin Elmer).<br>Sequencing reads were processed by a dedicated bioinformatics pipeline (VISPA2) as previously described in Spinozzi et al, BMC Bioinformatics, 2017.<br>Additional specification are reported in the Supplementary Information file. All scripts are available to editors and reviewers for consultation and testing upon request. |
| Data analysis | Graphical output and statistical analysis of transfusion volume was generated with SAS version 9.4 (SAS Institute) and R version 3.4.4 (R Foundationfor Statistical Computing).<br>Graphical output was generated thoughtGraph Pad Prism version 5.<br>BLI analysis were performed with IVIS SpectrumCT System (Perkin Elmer).<br>FACS raw data were analyzed withFCS Express version 5 and version 6 (DeNovo Software). |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

## Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data corresponding to the primary or secondary end-points generated and/or analyzed during this study are included in this article with a data cut-off of December 2017. Additional raw data files will be made available by the corresponding authors upon reasonable request.

# Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

# Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| | |
|---|---|
| Sample size | The study will enrol 10 patients. Sample size is based on feasibility. In addition, considering a mixed Poisson refence process with an assumed pre-gene therapy rate of 16 transfusions/person year of observation, a sample size of 10 patients with 1.75 years of follow-up would be sufficient to show a rate reduction of at least 50%.<br>We collected all analyzed in vivo biological material during years 2015-2017 (after informed consent from patients or their parents). |
| Data exclusions | Technically validated results were always included to the analyses and we did not apply any exclusion criteria for outliers.<br>Out of a planned ten treated patients, ten patients were enrolled and nine patients treated (six males, three females) between September 2015 and December 2017. Patient 2 did not meet inclusion criteria. |
| Replication | For VCN evaluation, qPCR was validated for reproducibility. We run each sample in triplicate and values are reported as mean of the three triplicates. All attempts at replication were successful. |
| Randomization | The experimental design did not include allocation of samples to randomised experimental group. |
| Blinding | The experimental design did not include allocation to groups nor to blinding. |

# Reporting for specific materials, systems and methods

### Materials & experimental systems

| n/a | Involved in the study |
|---|---|
| ☐ | ☒ Unique biological materials |
| ☐ | ☒ Antibodies |
| ☒ | ☐ Eukaryotic cell lines |
| ☒ | ☐ Palaeontology |
| ☐ | ☒ Animals and other organisms |
| ☐ | ☒ Human research participants |

### Methods

| n/a | Involved in the study |
|---|---|
| ☒ | ☐ ChIP-seq |
| ☐ | ☒ Flow cytometry |
| ☒ | ☐ MRI-based neuroimaging |

## Unique biological materials

Policy information about availability of materials

| | |
|---|---|
| Obtaining unique materials | All material was obtained from commercial sources except for anti-Band3 antibody (kindly provided by Xiuli An) |

## Antibodies

Antibodies used

For each antibodies we described --> fluorochrome/marker/clone/manufacturer/cat.number:
FITC/ Band3 (kindly provided by Xiuli An)
PE/GpA/clone JC159/Dako/R7078
APC/a4 integrin/ REA545/Miltenyi Biotech/130-108-231
VioBlue/CD45/clone 5B1/Miltenyi Biotec/130-092-880
FITC/CD45.1/clone A20/BD Pharmingen/561871
PE/CD45.2/clone 104/BD Pharmingen/558702

Validation

All the antibodies but one were purchased from BD Pharmingen or Miltenyi Biotech and they are well characterized and validated by providers.Representative flow cytometric data is included in all data sheet.
Band3 was kindly provided by Xiuli An and characterized and validated and as described in Hu et al, Blood, 2013

## Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

C57BL/6 and C57BL/6-CD45.1 (B/6.SJLCD45a-Pep3b) mice (Charles River),8-12 weeks old, male
All animal experiments were performed in accordance with approved protocols of the Institutional Animal Care and Use Committees of the Fondazione San Raffaele del Monte Tabor Committee (Protocol no. 537) and were communicated to the Ministry of Health and local authorities according to Italian law

Wild animals

*Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.*

Field-collected samples

*For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.*

## Human research participants

Policy information about studies involving human research participants

Population characteristics

Eligible participants were transfusion dependent thalassemia patients aged ≥ 3 years of any genotype and underwent LV GT (Clinicaltrials.gov NCT02453477). Since April 2015, 3 adults were enrolled; in March 2016, following evidence of preliminary safety and efficacy in adults, the independent data safety monitoring board approved enrollment of the second group (3 participants aged 8-17 years) and, in September 2016, approved the inclusion of the youngest participants (4 participants aged 3-7 years).
Participants characteristics are reported in Table 1

Recruitment

Participants were referred by thalassemia centers, by patient societies or self-referred. All subjects interested in the trial received trial specific information and were screeed for inclusion and exclusion criteria. Subjects meeting all inclusion criteria and without all exclusion criteria were enrolled sequentially in the specific age cohort until completion of enrolment. Written informed consent was obtained from patients and/or parents.

## Flow Cytometry

### Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☐ A numerical value for number of cells or percentage (with statistics) is provided.

### Methodology

Sample preparation

Bone marrow derived MNCs were incubated with CD45 microbeads (Miltenyi Biotec) for negative selection according to the manufacturer's instructions.
1x 105 CD45- cells are suspended in 25ul of buffer and stained with FITC-conjugated Band3, PE- conjugated GpA and APC-conjugated a4 integrin and VioBlue- conjugated CD45

Donor/host chimerism was evaluated on mouse peripheral blood from transplanted mice by staining peripheral blood using FITC-conjugated antibody specific against mouse CD45.1 (A20) and APC-conjugated antibody specific against mouse CD45.2 (104) (BD Biosciences) and FACS analysis at 2, 4, 7 months after BMT. Cells were pretreated with a rat anti-mouse CD16/CD32 (BD Pharmingen) to prevent unspecific immunoglobulin binding

| | |
|---|---|
| Instrument | BD Canto II |
| Software | FACS DIVA Software version 7 (BD), FCS Express version 4, 5 |
| Cell population abundance | Cell subsets were not sorted, but evaluated on total CD45- cells. In healthy donor, the physiologic progression of normal human terminal erythroid differentiation follows a ratio of Pro: Early Baso: Late Baso:Poly:Ortho, of 1:2:4:8:16 (as from Hu et al. Blood 2013)<br>Engraftment in transplanted mice was calculated as %CD45.1pos cells/(CD45.1pos+CD45.2pos) cells. |
| Gating strategy | Cells were analysed based on phisical parameters and selected for CD45- expression. Erythroid cells at different maturation stages are reported based on BAND3 and Integrin a4 expression as described in Hu et al. Blood 2013. |

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.