SUPPLEMENTARY METHODS

Patient enrollment

This clinical study is a pilot trial to assess the feasibility and safety of utilizing G-CSF to mobilize 8 x 10^6 CD34+ cells/kg or more in subjects with severe β-thalassemia 18 years or older. Subjects were screened for the inclusion criteria as specified on the Institutional Review Board approved protocol and eligible subjects signed a consent form. All volunteers were evaluated with a medical history, physical examination. Baseline laboratory tests, including complete blood count and differential, reticulocyte count, iron, TIBC, transferrin saturation, ferritin, coagulation profile, blood chemistry studies (electrolytes and renal function tests), ABO blood typing, and testing for infectious disease markers were performed. All female volunteers of childbearing age underwent a serum pregnancy test. The trial was approved by the Memorial Sloan-Kettering Cancer Center Institutional Review Board. Informed consent was obtained from eligible patients in accordance with the Declaration of Helsinki. Adverse events during and after therapy were assessed according to the National Institutes of Health Common Terminology Criteria for Adverse Events Version 4.0.

Mobilization and apheresis

The entire procedure of mobilization and apheresis was performed in the outpatient clinic. Subjects received G-CSF at a dose of 10 μg/kg once daily subcutaneously for six days. Patients underwent a history and physical examination prior to enrollment as well as specific monitoring of side effects and adverse events and complete blood counts on days 1, 3, 5 and 6 of mobilization. Apheresis was performed via two peripheral veins when available (n=4) or via a central venous catheter when intravenous access was insufficient to allow for the insertion of large bore intravenous access catheter (n=1). Each subject underwent leukapheresis with a collection goal of 8 x 10^6 $CD34^+$ cells/kg. Leukaphereses were performed on the mornings of the 5^{th} and 6^{th} day of G-CSF treatment on a continuous flow cell separator according to institutional standards utilizing acid citrate dextrose for anti-coagulation and calcium prophylaxis to prevent citrate toxicity. The volume of blood processed per leukapheresis session was approximately 3 times the total blood volume as tolerated by the patient. $CD34^+$ cells were positively selected using an ISOLEX TM 300i system (Patients 1-3), or a CliniMacs system (Patients 4-5) as per Standard Operating Procedures from the Cytotherapy Laboratory at MSKCC. The final number of collected $CD34^+$HPC per patient was measured by fluorescence-activated cell sorting (FACS) analysis.

Transduction

Selected CD34+ HPC were released to MSKCC Cell Therapy and Cell Engineering Facility where they were either cryopreserved in a controlled rate freezer, or transduced immediately or after storage overnight at 4ºC. Frozen cells were thawed rapidly at 37^{o}C and washed in media. Prior to prestimulation, a fraction of the cells was also tested for colony formation potential in methylcellulose colony formation unit (CFU) assays. On Day 0, selected CD34+ HPC were prestimulated for 18-24hrs in serum-free X-VIVO 10 supplemented with 100 ng/ml human stem cell factor (SCF), human Flt-3 ligand (Flt-3L), human thrombopoietin (TPO) and 20 ng/ml human IL-3 (IL-3). CD34+ HPC were transduced two rounds of transduction 18-24 hrs apart using either GLP or GMP TNS9.3.55 vector stocks. Cells were subsequently cultured in serum-free X-VIVO 10 with the same cytokines. On day 0 and at the end of the cultures on day 3, CD34+ HPC were inoculated into methylcellulose for colony formation. As part of the validation runs, transduced CD34+ HPC were cryopreserved on day 3. An aliquot was differentiated to the erythroid lineage. Genomic DNA samples were taken from cultures at day 14-16 for determination of the average vector copy number (VCN) in liquid erythroid cultures (EC) as well as from individual CFU colonies by Q-PCR.

Erythroid differentiation and colony forming unit assay

CD34+ cell were seeded at 0.5- 1x10^6/mL in a 6 well plate containing Alpha MEM (Sigma), supplemented with 10% FBS (Stem Cell Technologies), 2mM of Glutamin (Gibco), 1% deionized BSA (Sigma), 1U/mL human erythropoietin (Amgen), 10^{-5}M □-mercaptoethanol (Gibco), 10^{-6}M dexamethasone (American Regent Laboratories), 0.3mg/ml holo-transferrin (American Regent Laboratories), and 10ng/mL of human recombinant SCF (Sigma). Cells were counted and fed from day 4 to day 10 with the same EC differentiation medium. This differentiation process was used as *in vitro* modeling of erythropoiesis and was monitored by flow cytometry analysis for CD34 (BD Pharmagen), and Glycophorin A (Invitrogen) expression. For CD34+ cells pre- and post-transduction, CD34+ cells were plated at 500 cells per plate in MethoCultH4435 Enriched (StemCell Technology).

Vector copy number quantification

DNA from cells in EC culture was extracted using the Gentra Puregene kit (QIAGEN, Valencia, CA) as per manufacturers' directions. 100 ng of genomic DNA were used for real time PCR reaction. For genomic DNA of CFU colonies, well-isolated colonies were aspirated with a pipette tip under microscope and the cells were suspended into 25 ul of proteinase K (Roche) containing lysis buffer (14mM Tris HCl, pH=8.3; 2.5 mM $CaICl_2$; 10^5mM KCl; 0.3 mg/mL Gelatin; 0.45% Tween-20; 0.45% NP40; and 0.2 mg/mL proteinase K) in wells of a U-bottom 96 well plate. Plates were incubated at 55°C for 60 min, 95°C for 15min to end the reaction and kept at 4°C until use within 48 hrs. Similarly to Charrier et al.[1], as we extracted genomic DNA from individual CFUs with proteinase K, we found that the frequency of vector in CFUs is underestimated when compared to liquid EC cultures from which genomic DNA is extracted with Puregene kit. Quantification of the TNS9.3.55 lentiviral vector copy number was performed using the Applied Biosystems 7500 real-time PCR system. Average TNS9.3.55 vector copy number per cell was calculated by normalizing to the endogenous *ALB* gene. Analysis was performed using the 7500 System SDS software v.1.2.3.

TNS9.3.55 vector stock titration on Hela cells

Stocks of the TNS9.3.55 lentiviral vector [2,3] were manufactured under cGMP conditions by the Center for Biomedicine and Genetics (CBG, Duarte), CA. TNS9.3.55 vector stocks were titrated on a quality controlled cell bank of HeLa cells at various dilutions (in the range of 1/5 to 1/12500) of the vector. Titers were calculated using consecutive dilutions that yield linear data. The titer of the GMP TNS9.3.55 vector stocks was 3.5 E+08 TU/ml. The titer of the GLP TNS9.3.55 vector stocks was 6.6E+08 TU/ml.

Analysis of human cells engrafted in NSG mice

NOD.Cg-*Prkdc*^{scid} IL2R null (NOD scid gamma, NSG) mice were purchased from the Jackson laboratories. Mice were conditioned with busulfan (Sigma) and received a single dose of 35 mg/kg[4]. Mice were injected IP 24h prior to receiving TNS9.3.55-transduced CD34+ HPC. One mouse per group was kept untransplanted as control for flow cytometry analysis. All murine studies were done in the context of a Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee approved protocol (IACUC #98-08-029). Bone marrow was analyzed 3 ½ months to 7 months post-transplantation. Fow cytometry analysis was performed to evaluate multi-lineage engraftment. FACS for CD45 (lymphocytes) and CD34 (hematopoietic progenitor and stem cell); CD41, CD61 (megakaryocytes); CD3, CD4, CD8 (T cell); CD14 (monocytes); CD16 (NK cell); and CD19, CD10 (B cell) . The monoclonal antibodies to CD45, CD34, CD14, CD are from BD Biosciences; the monoclonal antibodies for CD3, CD8, CD16, CD33, CD41, CD61 are from Invitrogen; the monoclonal antibody for CD4 is from eBiosciences; and the monoclonal antibody for CD10 is from Becton Dickson. BM cell colony formation assay

was performed using Methocult H4230 containing 60ng/ml Human SCF, 20ng/ml IL-3, 10ng/ml GM-CSF and 6U/ml EPO. Colonies were picked after 2 weeks in culture. Genomic DNA was extracted from bone marrow and from colonies and Q-PCR was performed as described above.

HPLC analysis

Expression of the lentiviral vector encoded transgene (ß-globin) was analyzed by HPLC (MSKCC Analytical Pharmacology Core) as previously described [5]. Frozen blood and cell samples were thawed and diluted to be within the calibration standard curve range (10 to 400ug/ml). The calibration curves were determined for the alpha & beta globin chains to permit conversion of peak areas to individual sub-chain amounts against the external reference standards.

RNA Analysis

Total RNA was isolated using Ambion Trizol reagent kit (Life Technology, CA, USA) from peripheral blood. BFU-Es were generated from CD34+ hematopoietic cells, or from the bone marrow of mice engrafted with human CD34+ hematopoietic cell transduced with TNS9.3.55. RNA concentration and purity were determined by NanoDrop 2000 Spectrophotometer (Thermo Scientific, MA, USA). DNA contamination in the RNA sample was eliminated by DNase treatment (Qiagen, CA, USA). Multiplex one-step real-time RT-qPCR for quantification of α-and β-globin mRNA was performed in 15μL volume containing 25ng of RNA, 115nM of each primer, 50nM of each probe and reagents supplied by Taqman ® one-step RT-PCR master mix reagents kit (Applied Biosystems, CA, USA). The sequences of primers and probes used in this study have been described previously[5]. Normal donor RNA was used as the caliber for normalizing the β/α globin mRNA ratio according to the $2^{-\Delta\Delta CT}$ method. Real time RT-PCR amplifications were performed in duplicate using Applied Biosystems 7500 as following: 30 min at 48°C for reverse transcription; 10 min at 94°C for inactivation of reverse transcriptase and AmpliTaq Gold activation; and 35 cycles of PCR with 15 s at 94°C and 1 min at 58°C.

Supplemental references

1. Charrier S, Ferrand M, Zerbato M, et al. Quantification of lentiviral vector copy numbers in individual hematopoietic colony-forming cells shows vector dose-dependent effects on the frequency and level of transduction. Gene Ther. 2011;18:479-487.
2. May C, Rivella S, Callegari J, et al. Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. Nature. 2000;406:82-86.
3. Lisowski L, Sadelain M. Locus control region elements HS1 and HS4 enhance the therapeutic efficacy of globin gene transfer in beta-thalassemic mice. Blood. 2007;110:4175-4178.
4. Hayakawa J, Hsieh MM, Uchida N, Phang O, Tisdale JF. Busulfan produces efficient human cell engraftment in NOD/LtSz-Scid IL2Rgamma(null) mice. Stem Cells. 2009;27:175-182.
5. Papapetrou EP, Lee G, Malani N, et al. Genomic safe harbors permit high beta-globin transgene expression in thalassemia induced pluripotent stem cells. Nat Biotechnol. 2011;29:73-78.

Table S1. Characteristics of Patients with β–Thalassemia

Age (years)	Gender	Mutation	Genotype	Chelation	Ferritin	Spleen	Hepatitis C
23	Female	IVS1, 6 IVS1, 110	β+/+	Desferrioxamine	1,733	Not palpable	Negative
29	Female	IVS1, 110 IVS1, 110	β+/+	Desferrioxamine Deferiprone (held)	1,500	Not palpable	Serology positive RNA negative
24	Female	c□□□□ 39 IVS2, 745	β0/+	Deferasirox	1,257	Splenectomy	Negative
28	Male	IVS1, 1 IVS1, 6	β0/+	Deferasirox	1,758	Splenectomy	Negative
23	Female	IVS1, 1 IVS1, 110	β0/+	Deferiprone (held)	1,355	Splenectomy	Negative

None of the patients had previously received hydroxurea or erythropoietin.

Table S2: Blood counts and hematological parameters during G-CSF mobilization

	Days/ Cell counts	+1	+2	+3	+4	+5	+6	Total CD34/kg
Pt 1	WBC	9.6	42.8	56.9	NA	61.4	*65.2*	
	ANC	6.9	38.7	52.3	NA	52.7	55.4	
	NRBC %	*<1*	*<1*	*<1*	*NA*	*1*	*1*	
	HGB	*10.3*	*9.5*	*9.9*	*NA*	*9.8*	*9.2*	
	Plts	*320*	*290*	*330*		*292*	*206*	
	%CD34 blood					*0.2%*	*NA*	
	CD34/kg					**3.0 x 10^6**	**5.0 x 10^6**	**8.0 x 10^6**
Pt 2	WBC	3.5	18.8	19.7	24.9	44.6	*46.5*	
	ANC	1.8	13.7	17.2	20.9	40.1	43.3	
	NRBC %	*<1*	*0*	*<1*	*<1*	*0*	*<1*	
	HGB	*11.3*	*11.6*	*11.0*	*11.1*	*10.8*	*10.3*	
	Plts	*182*	*173*	*174*	*192*	*218*	*94*	
	%CD34 blood					*0.2%*	*0.1%*	
	CD34/kg					**6.4 x 10^6**	**5.6 x 10^6**	**12 x 10^6**
Pt 3	WBC	10.1	60.1	69.4	66.0	*74.6*	73.8	
	ANC	5.9	53.5	65.0	57.8	59.8	56.7	
	NRBC %	*<1*	*0*	*<1*	*<1*	*5*	*3*	
	HGB	*11.9*	*11.3*	*11.1*	*11.0*	*11.3*	*10.6*	
	Plts	*361*	*350*	*338*	*375*	*405*	*215*	
	%CD34 blood					*0.1%*	*0.1%*	
	CD34/kg					**4.0 x 10^6**	**4.5 x 10^6**	**8.5 x 10^6**
Pt 4	WBC	12.6	79.3	*80.4*	71.2	62.4	65.3	
	ANC	9.2	68.9	68.4	49.2	48.2	52.7	
	NRBC % (Pre 178)	*160*	*35*	*57*	*88*	*133*	*149*	
	HGB	*10.9*	*10.4*	*10.4*	*10.3*	*10.1*	*9.7*	
	Plts	*542*	*462*	*470*	*484*	*431*	*216*	
	%CD34 blood					*0.4%*	*0.3%*	
	CD34/kg					**6.2 x 10^6**	**2.7 x 10^6**	**8.9 x 10^6**
Pt 5	WBC	9.6		72.3	*92.6**	78.6	57.9	
	ANC	4.1		65.7	84.4	68.8	51.6	
	NRBC %	*<1*		*<1*	*<1*	*<1*	*<1*	
	HGB	*10.8*		*9.9*	*10.6*	*10.1*		
	Plts	*461*		*357*	*338*	*375*		
	%CD34 blood					*0.2%*	*NA*	
	CD34/kg					**3.0 x 10^6**	*NA*	***NA***

All but one patient underwent two leukaphereses on days 5 and 6. The G-CSF dosage (10 µg/kg/day daily for 6 days) was reduced once on day 4 in Pt 5 because of high WBC. Pt 5 decided not to proceed with the second collection because of anxiety ; NA: not available; WBC: White blood cell count; ANC: absolute neutrophil counts; HBG: hemoglobin; Plts: Platelets; <1% NRBC means not measured because automated differential was normal.

Table S3: Adverse events during Mobilization protocol – Grading (in parenthesis) as per the National Institute of Health Common Terminology Criteria for adverse events (CTCAE) version 4.0

	Day 1	Day 2	Day 3	Day4	Day 5	Day 6
Patient 1	Fatigue (1) Myalgia (1) Headache (1)	Myalgia (1) Headache (1)	None	Fatigue (1) Anemia (2)	Fatigue (1) Spleen tip	Fatigue (1) Spleen tip
Patient 2	None	Fatigue (1)	Fatigue (1)	Fatigue (1) Bone pain (1)	None	Thrombocytopenia (1)
Patient 3	None	Headache (1) Bone pain (1) Pain injection site	Fatigue (1) Arthralgia (1) Bone pain(1)	Fatigue (1) Arthralgia (1) Headache (1)	Fatigue (1) Arthralgia (1) Headache (1)	Fatigue (1) Arthralgia (1) Headache (1)
Patient 4	Headache (1) Arthralgia (1) Nausea (1)	None	None	None	None	None
Patient 5	Headache (1) Arthralgia (1) Bone pain (1)	None	Headache (1)	None	Pain at apheresis sites Anxiety	Anxiety

The side effects lasted 2-3 days and resolved spontaneously; Patient 2 developed a mild thrombocytopenia (94,000/mm^3) that completely recovered within one week.

Table S4: Enumeration of TNS9.3.55-transduced and untransduced CFU colonies in Validation runs

Validation #	L-BFU-E BFU-E	CFU-G, CFU-M & CFU-GM	Total Colonies	% CD34+	% colonies derived from CD34+
1 (T, d3)	40	52.5	92.5	99.8	18.5
1 (UT, d3)	49.5	56.5	106	99.6	21.3
2 (UT, d0)	44.5	38.5	83	95	17.5
2 (UT, d3)	93	16	109	97.9	22.3
2 (T, d3)	71.5	14	85.5	98.0	17.4
3 (UT, d0)	82	88	170	92.9	31.6
3 (UT, d3)	77	41	118	98.6	23.9
3 (T, d3)	69.5	43.5	113	99.0	22.4

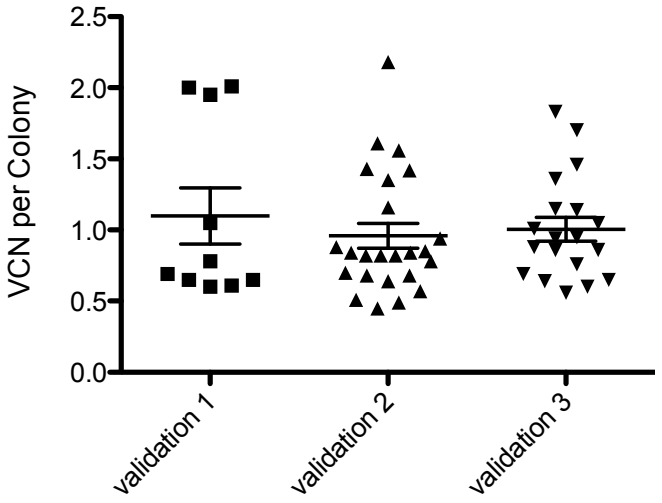
Five hundred cells were seeded per plate. Average of 2 or 3 plates; T: transduced with TNS9.3.55; UT: untransduced

Table S5. Multilineage engraftment of human cells in the bone marrow of NSG mice

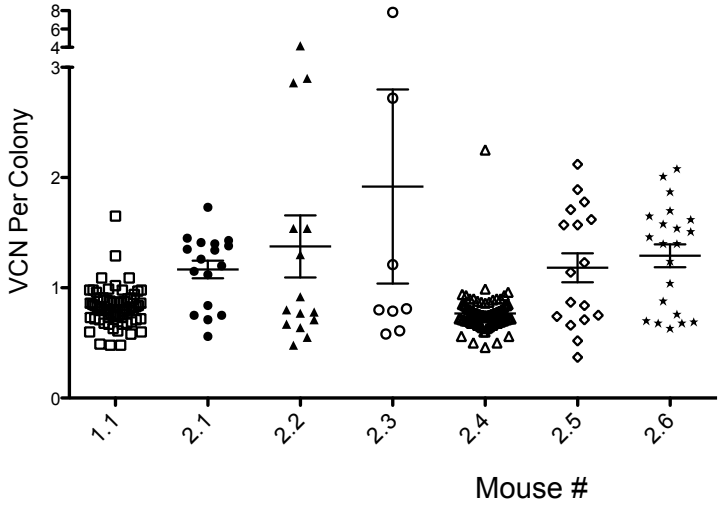
Human cell subsets	BM (3 1/2 to 7 months)				
	Exp 1 (n=1)	Exp 2 (n=6)	Exp 3 (n=3)	Exp 4 (n=3)	Exp 5 (n=4)
Total Leukocytes: CD45%	54	13.6 ±9.8	1.0 ±0.3	2.5 ±2.6	3.2 ±4.6
B cell lineage: % of CD45+					
CD19+	76.2	13.7 ±13.6	2.7 ±0.5	72.8±10.3	51.1 ±8.5
CD19+CD10+ (immature B cells)	60.2	11.2 ±11.4	ND	16.0±11.2	ND
T cell lineage: % of CD45+					
CD3+	0.09	15.4 ±13.3	0.5 ±0.3	0.04 ±0.07	11.5 ±6.5
CD3+CD4+	0.04	13.7 ±13.6	0.2 ±0.1	0	ND
CD3+CD8+	0.01	4 ±3.4	0.1 ±0.1	0	ND
Myeloid/Granulocytic/Neutrophils: % of CD45+					
CD33+	9.1	49.7 ±10.2	ND	ND	ND
CD14+	2.8	15.6 ±4.3	3.9 ±0.6	2.6 ±0.9	26 ±11
CD14+ CD16+	0.02	0.9 ±1.3	0.1 ±0.05	ND	ND
CD19- CD10+	10.5	4.6 ±3.6	ND	2.9±4.1	ND
Hematopoietic progenitors: % of CD45+					
CD34+	7.6	3.4 ±2.0	ND	2.3 ±1.8	11.6 ±6.8
Megakaryocyte/Platelets: % of CD45					
CD41+	0.05	1.9 ±1.4	0.6 ±0.7	3.6 ±2.7	2.1 ±1.1
CD61+	1.9	9.4 ±6.1	0.2 ±0.2	9.6±5.8	11.1 ±6.2
Other					
CD3-CD4+	9.2	46.5 ±19.8	77.7 ±3.6	16.0 ±7.5	ND
CD3-CD8+	0.03	0.3 ±0.2	0.9 ±0.1	1.4±1.0	ND
CD36	ND	ND	ND	3.6 ±0.4	7.5 ±4.0

Figure S1. Vector copy number in individual CFUs derived from post-transduction CD34+ cells from the validation runs (A) and from the BM of long-term transplanted mice (B). Percentage of colonies harboring the indicated vector copy number (VCN) per cell in CFUs derived from post-transduction CD34+ cells from the validation runs (C) and from the BM of long-term transplanted mice (D) Approximately 80 colonies per validation run and 40 to 190 colonies per mouse in the bone marrow were screened. All colonies with ≥ 0.45 copies per cell are reported.

A



B



C

VCN/cell	Validation 1	Validation 2	Validation 3
≥ 0.5, <1.5	70%	86.4%	89.5%
≥1.5, <2.5	30%	13.6%	10.5%

D

VCN/cell	1.1	2.1	2.2	2.3	2.4	2.5	2.6
≥ 0.5, <1.5	98.4%	94.1%	64.3%	75%	99	56.2%	57.1%
≥1.5, <2.5	1.6%	5.9%	14.3%	0.0%	1.0%	43.7%	42.9%
≥2.5, <3.5	0%	0%	14.3%	12.5%	0%	0%	0%
≥3.5	0%	0%	7.1%	12.5%	0%	0%	0%