Plerixafor and G-CSF combination mobilizes hematopoietic stem and progenitors cells with a distinct transcriptional profile and a reduced *in vivo* homing capacity compared to plerixafor alone

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SUPPLEMENTARY FIGURES

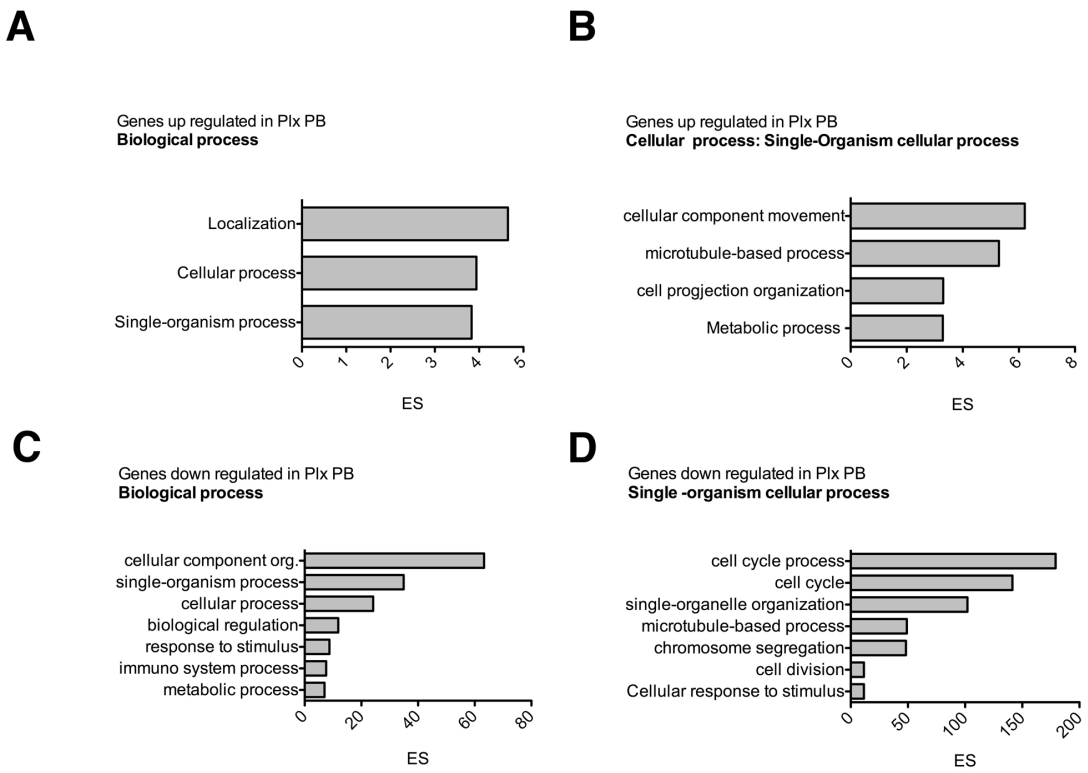

Fig. S1. Gene Ontology analysis of differentially expressed genes in Plx PB. Functional annotation by Partek software showed that genes could be grouped into a limited number of biological categories. Most of probesets upregulated in Plerixafor mobilized cells were enriched in cellular organization and metabolic process (p value <0.05) **(A-B),** while most of the probesets down regulated in Plx PB are involved in cell cycle process (p value <0.001) **(C-D).** One-way ANOVA statistical analysis was performed. Enrichment score (ES) for each process is reported.

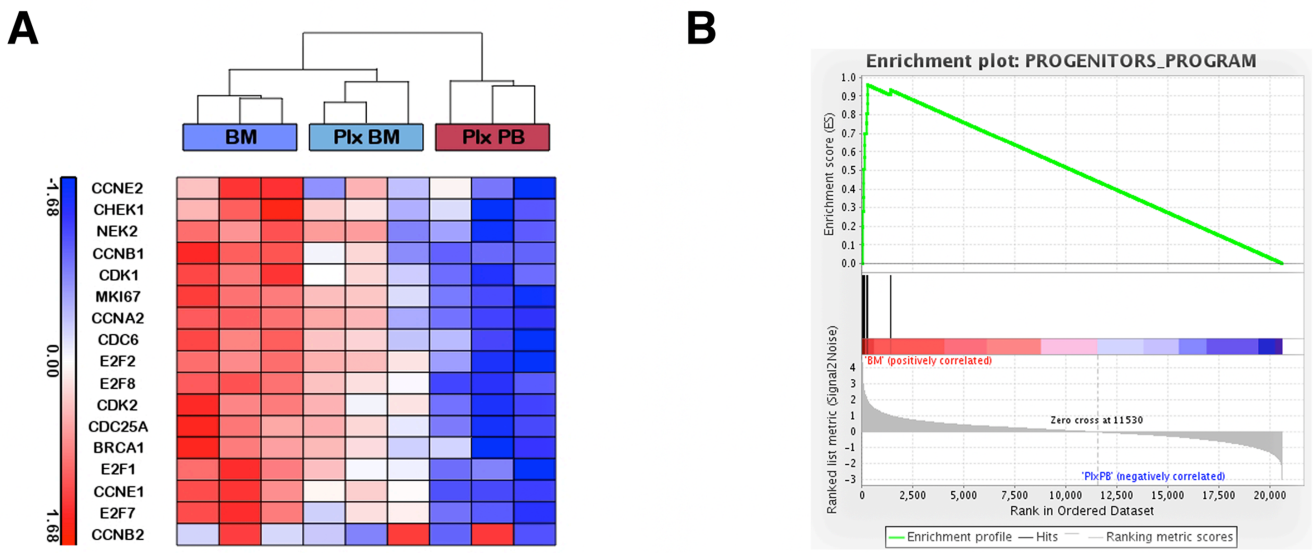

Fig. S2. HSPCs from BM are transcriptionally enriched for genes associated with PROGENITORS_PROGRAM.

A. Heat map of regulators of cell cycle and DNA repair, showing core enriched in “Progenitor associated program” in BM expression profile. In this image, the normalized expression levels of genes are presented according to a colored gradient from the highest (red) to lowest (blue, see colored scale).

B. List of genes reported in panel (A) was used to create the gene set PROGENITOR_PROGRAM. GSEA plot enrichment of PROGENITOR_PROGRAM in BM (n=3) vs Plx PB (n=3) expression profile ($p<0.01$).

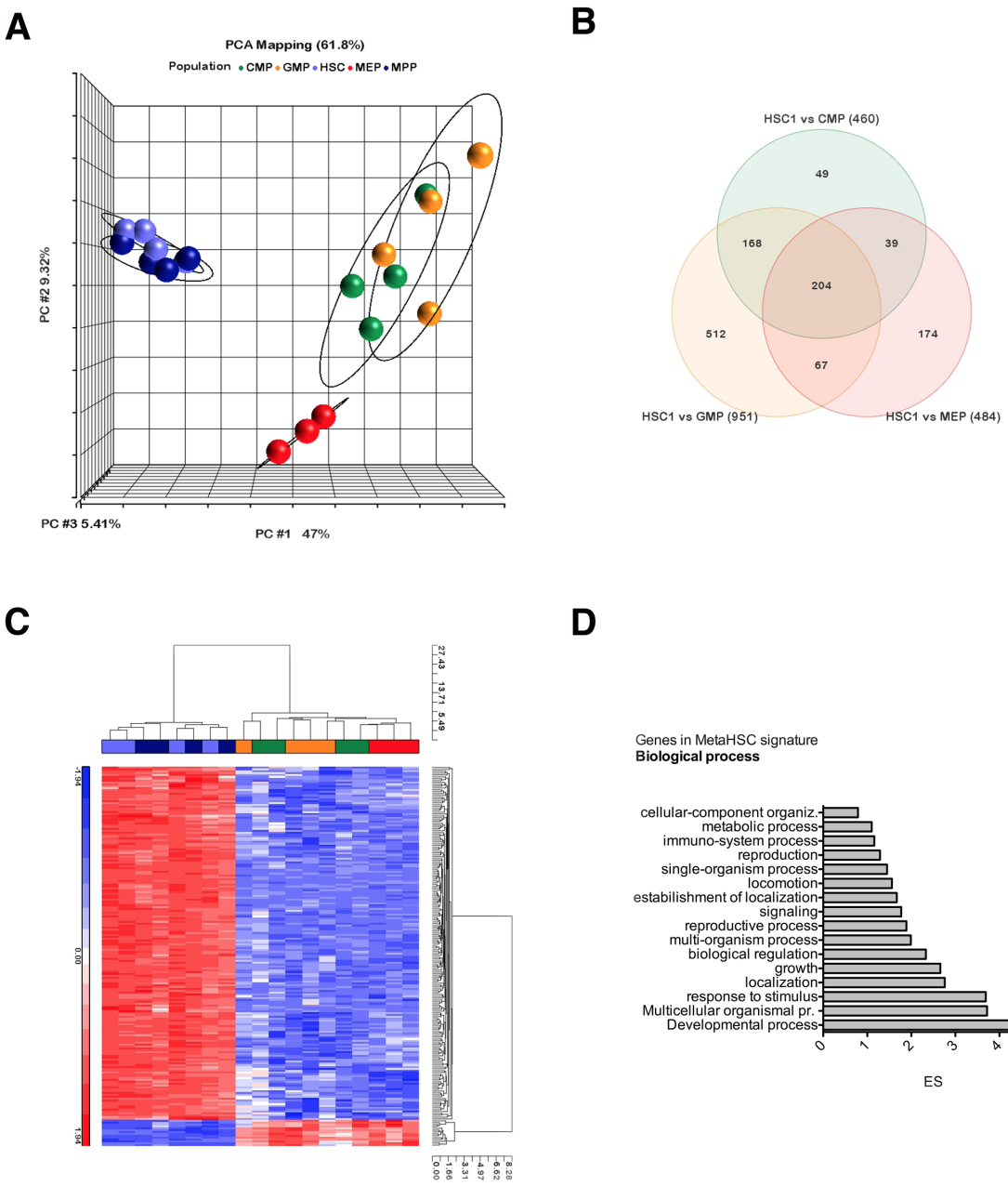

Fig. S3 Meta-Analysis of gene expression data identifies a molecular signature characteristics for HSCs. Results of the *in silico* identification of the molecular signature of HSCs. Meta-analysis on raw gene expression data available in Gene Expression Commons data base (https://gexc.stanford.edu/population/). CEL files were downloaded. The quality of the data sets was checked using a

series of QC metrics. Gene expression data were reannotated according to the Entrez genome annotation using CDF files. GC-RMA (robust multiarray averaging) normalization was applied. Differentially expressed genes were identified with Partek using a 10-fold change as a threshold, an adjusted FDR <0.001, and the ‘‘AND’’ operator to identify consistently upregulated or downregulated genes. **A.** Population –distance analysis of BM-derived HSPC subsets including HSCs, MPPs and oligopotent progenitors, i.e. CMP, GMP, MEP (n=3). **B.** Venn diagram illustrating the number of genes that show altered expression in HSC1 compared with CMP GMP, and MEP cells. We refer collectively to HSC and MMP as HSC1. Values indicate the number of genes significantly upregulated or downregulated. **C.** Heatmap of genes enriched in HSC and MPP populations than in those no detectable HSC in three different sorted bone marrow populations (CMP, GMP, MEP). In this image, the normalized expression levels of genes are presented according to a colored gradient from the highest (red) to lowest (blue, see colored scale). **D**. Gene Ontology analysis of genes from MetaHSc signature.

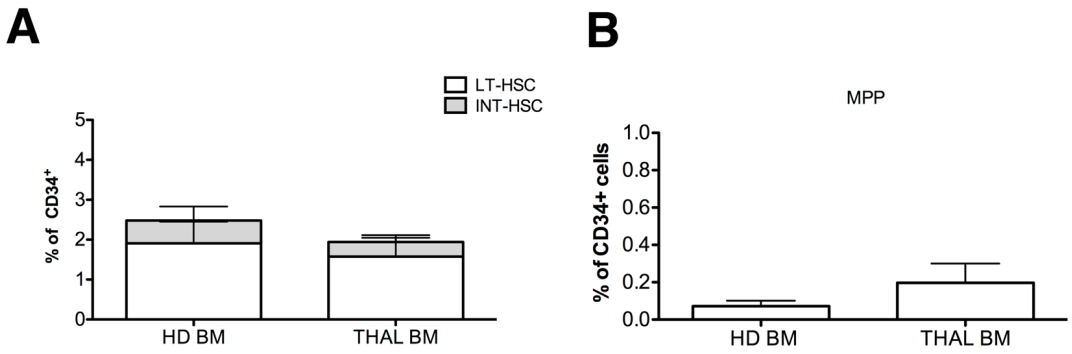

Fig. S4 Comparison of immunophenotypic analyses from thalassemic (THAL) and healthy donor (HD) BM CD34$^+$ cells. A. Analysis LT-HSC and INT-HSC frequency of HD BM (*n* = 9) and THAL BM samples on CD34$^+$ cells (*n* = 3). **B.** Immunophenotypic analysis of MPP in HD BM (*n* = 9) and THAL BM samples on CD34$^+$ cells (*n* = 3). Data are represented as mean±SEM

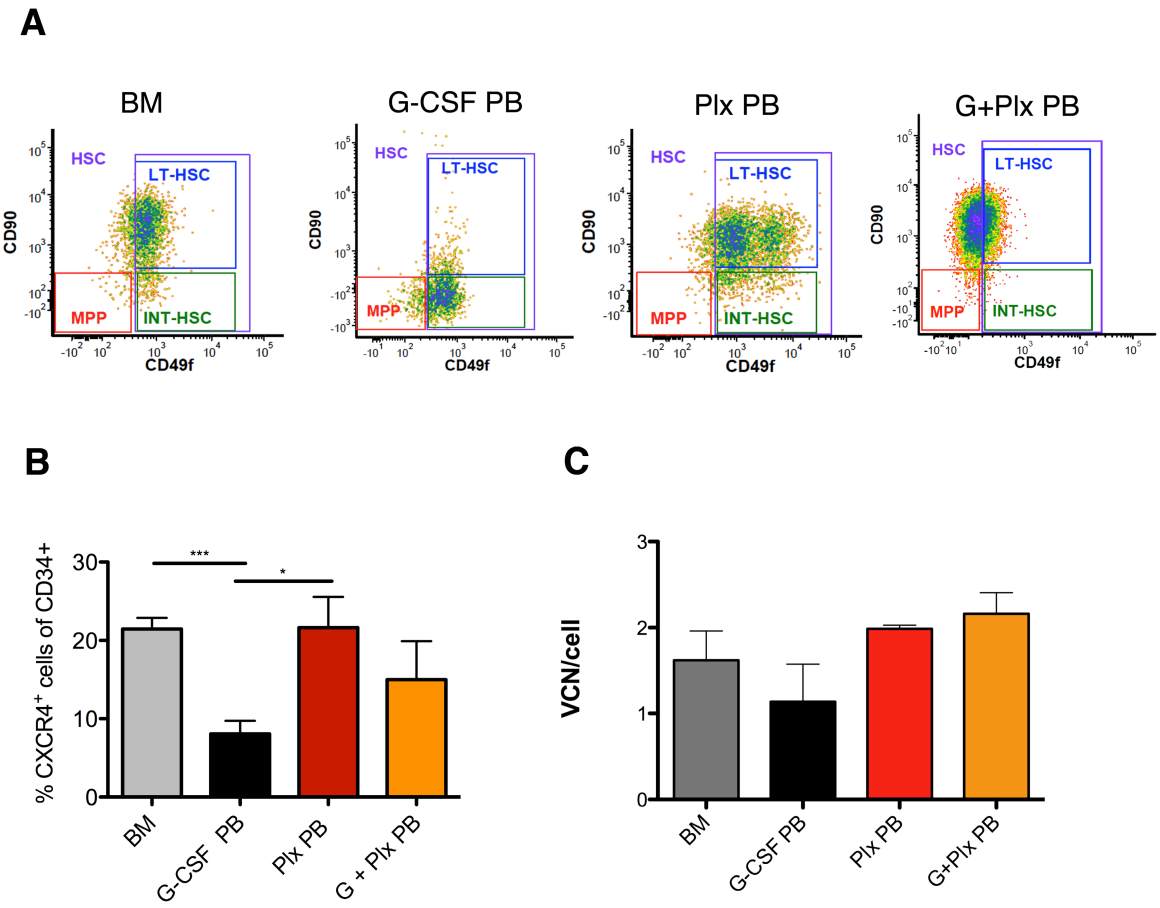

Fig. S5. A. Representative flow cytmetry gated used to isolate the stem and progenitors populations. The expression of CD38, CD90, CD45RA and CD49f cell surface markers is considered the "gold standard" for predicting primitive HSCs [20] [21] Using these markers three main subpopulations of $CD34^+$ cells were identified: HSCs ($CD34^+$ $CD38^{-/low}$ $CD90^{+/-}$ $CD45RA^-$ $CD49f^+$); LT-HSCs ($CD34^+$ $CD38^{-/low}$ $CD90^+$ $CD45RA^-$ $CD49f^+$); MPPs ($CD34^+$ $CD38^{-/low}$ $CD90^-$ $CD45RA^-$ $CD49f^-$).

B. Summary of CXCR4 cell surface marker expression on $CD34^+$ cells from BM (n=5), G-CSF (n=4), Plx (n=3) or G+Plx (n=2) mobilized cells. Percentage of $CXCR4^+$ was significant lower in G-CSF mobilized $CD34^+$ cells compared to BM and Plerixafor mobilized $CD34^+$ cells (***$p<0.001$, * $p<0.05$). Data are represented as mean±SEM.

C. Analysis of VCN in the bone marrow of mice transplanted with BM, G-CSF PB, Plx PB and G+Plx PB $CD34^{+}$ $CD38^{-/low}$ transduced by LV-PGK/Luc vector.

Data are represented as mean±SEM.

Suppl. Table 1. Transcriptome of HSPCs.

For each different stem cell sources, differential expression gene lists were generated, using Partek software, of transcripts > 2-fold differentially expressed ANOVA. Number of identified probesets, genes and p value are indicated for each contrast.

Contrast	p value	n° **probesets**	**n° genes**	**Fold change**
BM vs Plx PB	FDR 0.05	829	567	>2
BM vs Plx BM	p<0.01	306	213	
Plx BM vs Plx PB	p<0.01	505	363	

Suppl Table 2 Selected genes under-expressed in Plx PB *versus* BM

Probeset ID	Entrez Gene	Gene Symbol	Fold-Change	p-value
211851_x_at	672	BRCA1	-2.13671	0.000428339
204531_s_at	672	BRCA1	-3.29405	0.000531836
203418_at	890	CCNA2	-9.45447	3.22E-06
214710_s_at	891	CCNB1	-3.32846	1.91E-05
202705_at	9133	CCNB2	-7.15253	1.62E-06
213523_at	898	CCNE1	-2.38877	5.12E-05
211814_s_at	9134	CCNE2	-2.17766	0.00120547
1555772_a_at	993	CDC25A	-6.68769	2.55E-06
204695_at	993	CDC25A	-3.29747	1.02E-05
203968_s_at	990	CDC6	-7.30528	9.78E-07
203967_at	990	CDC6	-7.0528	7.76E-06
203213_at	983	CDK1	-10.8246	3.71E-09
210559_s_at	983	CDK1	-4.54162	3.30E-07
203214_x_at	983	CDK1	-2.55404	7.70E-06
231534_at	983	CDK1	-6.02867	1.08E-05
211804_s_at	1017	CDK2	-2.12117	1.88E-06
205394_at	1111	CHEK1	-3.2863	7.06E-06
238075_at	1111	CHEK1	-9.77372	0.000215113
204947_at	1869	E2F1	-3.78991	2.70E-07
2028_s_at	1869	E2F1	-2.45467	2.17E-05
228361_at	1870	E2F2	-12.5314	2.98E-05
207042_at	1870	E2F2	-6.18138	0.000280652
228033_at	144455	E2F7	-10.5439	1.84E-06
241725_at	144455	E2F7	-2.34462	0.00102819
219990_at	79733	E2F8	-8.15568	3.17E-07
212023_s_at	4288	MKI67	-12.4961	8.91E-08
212022_s_at	4288	MKI67	-13.0685	3.17E-07
212020_s_at	4288	MKI67	-15.3332	2.96E-07
212021_s_at	4288	MKI67	-5.87754	9.29E-06
204641_at	4751	NEK2	-4.34153	9.86E-05
211080_s_at	4751	NEK2	-7.35375	2.67E-07

Suppl Table 3. Genes over-expressed in Plx PB *versus* BM

Probeset ID	Entrez Gene	Gene Symbol	Fold-Change	p-value
209993_at	5243	ABCB1	2.76108	0.00122862
204753_s_at	3131	HLF	3.54048	0.00589902
204754_at	3131	HLF	3.0511	0.0157823
204755_x_at	3131	HLF	4.83873	0.0127038
208557_at	3203	HOXA6	2.17172	0.00022513
208414_s_at	3213	HOXB3	2.17433	0.000206938
228904_at	3213	HOXB3	2.05225	0.00419518
231049_at	4005	LMO2	2.23371	0.000432283
204249_s_at	4005	LMO2	2.01981	0.000972646
215851_at	2122	MECOM	2.61226	0.00266186
1559477_s_at	4211	MEIS1	2.46408	0.000928568
204069_at	4211	MEIS1	2.58789	0.000509454
225221_at	7586	ZKSCAN1	2.9368	0.000302157

Suppl. Table 4. Gene expression comparison between HD and THAL BM

Differentially expressed genes **HD BM vs THAL BM**			
	p value	Nr of genes	Fold change
$CD34^+$ sample	FDR 0.05	0	>2

Suppl. Table 5. SRCs frequency in HSPCs cell sources

CD34+Stem cell Source	Transplanted Cell dose	Engrafted Mice /transplated mice	Stem Cell Frequency (95% Confidence Interval)
Plx PB	2.5×10^4	0/4	**1/47875 (28222-79523)**
	5×10^4	4/4	
	1×10^5	12/14	
	2×10^5	4/4	
	3×10^5	16/16	
	5×10^5	13/13	
	7×10^5	11/11	
G-CSF PB	1×10^5	1/5	**1/141203 (71330-279518)**
	3×10^5	5/5	
	5×10^5	5/5	
	7×10^5	5/5	
G+Plx PB	2.5×10^4	0/5	**1/201803 (114990-354157)**
	5×10^4	0/10	
	1×10^5	4/11	
	3×10^5	8/8	
BM	5×10^4	1/4	**1/56020 (26945-116469)**
	1×10^5	4/4	
	2×10^5	5/5	
	3×10^5	4/4	

Suppl. Table 6. Statistic analysis of SRCs frequency

Contrast		P value
Plx PB	BM	0.74
Plx PB	G-CSF	0.0185
Plx PB	G+Plx	0.00015
G+Plx PB	G-CSF PB	0.454
G+Plx PB	BM	0.00855
G-CSF PB	BM	0.0884

METHODS

Human subjects

Four adult subjects affected by transfusion dependent beta-thalassemia were enrolled in a clinical protocol of $CD34^+$ cells mobilization (EudraCT 2011-000973-30) and treated with Plerixafor (Mozobil or AMD3100). All the patients signed informed consent and the experiments performed were approved by San Raffaele Scientific Institute Ethical Committee.

Two more thalassemic patients were treated with G-CSF and Plerixafor for the purpose of mobilization of peripheral blood stem cells as back-up prior to allogeneic stem cell transplantation and signed an informed consent for off label use of Plerixafor and for research use of biological material.

Peripheral blood stem cell mobilization protocols

A clinical protocol exploring the use of Plerixafor as single agent (EudraCT 2011-000973-30) was conducted at San Raffaele Hospital in Milan, Italy. Four adult transfusion dependent beta-thalassemia patients were enrolled and treated with Plerixafor subcutaneously at the dose of 0.24 mg/kg at time 0. $CD34^+$ cell amount was monitored in the peripheral blood using cytofluorimetric analysis. If this was found to be less than 20 $CD34^+$ cells/ μl (threshold used to predict poor mobilizer patients) no apheresis was done. On day +1, immunophenotype for CD34 expression was repeated and, unless > $5x10^6$ $CD34^+$/kg had been collected on day 0, a second dose of Plerixafor (0.40 mg/Kg) was administered and a second apheresis performed. No severe adverse events occurred.

Two adult transfusion dependent beta-thalassemia patients were subjected to $CD34^+$ cells mobilization with G-CSF and Plerixafor for the purpose of

transplantation back-up harvest. G-CSF was administered at a daily dose of 5-10 µg/kg sub-cutaneously, divided in 2 doses, for 2-3 days, followed by a single administration of Plerixafor at the dose of 0.24 mg/kg sub-cutaneously.

Isolation and immunophenotypic characterization of CD34$^+$ cells

Healthy donors **(**HD) BM-derived and G-CSF mobilized CD34$^+$ cells were purchased from Lonza (Basel, Switzerland) and ALLCELLs (Alameda, CA, USA) respectively. The mobilized leukoapheretic products from patients were enriched for CD34$^+$ cells using CliniMACS (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

Mononuclear cells from BM aspirates, Plerixafor alone or with G-CSF leukoapheretic products were isolated by Ficoll density separation. CD34$^+$ cells were selected using anti-CD34 microbeads (Miltenyi Biotech, Auburn, CA, USA). CD34$^+$ cells labelled with PECy7-coniugated antibody (clone 8G12) (Becton Dickinson, San Jose, CA, USA) were analyzed for the expression of other markers using the following antibodies: CD45RA (FITC, clone HI100), CD90 (APC, clone 5E10), CD49f (PE, clone GoH3), CD38 (PerCPCy5.5, clone HIT2) and CXCR4 (PE, clone 12G5) (BD Pharmingen, San Jose, CA, USA).

Subpopulation composition of the committed progenitors (CMP, GMP, MEP and CLP) were analyzed by the using following antibodies: CD34 (PECy7, clone 8G12) (Becton Dickinson, San Jose, CA, USA), CD45RA (FITC, clone HI100), CD38 (PerCPCy5.5, clone HIT2), CD45RA (FITC, clone HI100), CD10 (PE, clone HI10a) (Becton Dickinson, San Jose, CA, USA), CD7 (BV421, clone M-T701) (BD

Horizon, San Jose, CA, USA), CD135 (APC, clone BV10A4H2) BioLegend, San Diego, CA, USA).

To sort $CD34^+$ $CD38^-$ cells, $CD34^+$ cells were stained with PE-conjugated anti human CD34 (Becton Dickinson, San Jose, CA, USA) and APC-conjugated anti human CD38 (Becton Dickinson, San Jose, CA, USA).

Cells were acquired using a FACS Canto II (BD Biosciences, San Jose, CA, USA) and analysed with FCS Express Software (De Novo Software, Los Angeles, CA, USA).

Lentiviral Vector

To generate the LV-PGK/Luc vector, Luciferase transgene was cloned into the *EcoRV-SalI* sites of the pCCLppt.PGK.GFP.WPRE-18 SIN-LV plasmid [18] VSV-G pseudotyped viral stocks were produced by transient transfection in 293-T cells, collected and concentrated as previously described [19]

Cell culture and transduction

Following sorting, $CD34^+$ $CD38^{-/low}$ cells were seeded on non-tissue culture Retronectin coated plates (30 μg/ml, TaKaRa Bio, Shiga, Japan) at 1×10^6 cells/ml in serum-free CellGro SCGM Medium (Cell Genix Technologies, Friburg, Germany) in the presence of cells SCF (300 ng/ml), FLT3-L (300 ng/ml), Thrombopoietin (100 ng/ml), and IL-3 (60ng/ml) (all from Peprotech, Rocky Hill, NY). Following 4h of prestimulation, cells were transduced with LV-PGK/Luc at multiplicity of infection (MOI) 100 o/n. At the end of transduction, cells were transplanted in sublethally irradiated NSG mice.

Xenotransplantation assay

NOD/ShiLtSz-*scid*/IL2Rγ^{null} (NSG) mice (Jackson Laboratory) were maintained in a specific pathogen-free animal facility. Procedures were performed according to protocols approved by the Committee for Animal Care and Use of San Raffaele Scientific Institute (Committee Protocol no. 375). 9-10 wks old female NSG mice were sublethally irradiated and i.v. injected with CD34$^+$ cells in a volume of 200 μl phosphate-buffered saline.

Transplanted mice were sacrificed 3-4 months after transplant. Mice were analyzed for the presence of human CD45$^+$ cells (clone HI30, BD Pharmingen, San Jose, CA, USA). The bone marrow and peripheral blood were analyzed for human cell engraftment.

***In vivo* homing assay**

9-10 wks old NSG mice were sublethally irradiated and 3 x 10^4 LV-PGK/Luc transduced CD34$^+$ CD38$^-$ cells were intravenously injected along with 2 x 10^5 not transduced CD34$^+$ CD38$^+$ cells, in order to avoid trapping of primitive cells in filter organs.

24 hrs after injection mice were analyzed at IVIS instrument SpectrumCT System (Perkin Elmer). Four different cohorts of mice were generated by transplantation of BM (CD34$^+$ cells form 5 donors), G-CSF PB (3 donors), Plx PB (3 donors) and G+Plx PB (2 donors).

***In vivo* bioluminescence imaging**

In vivo bioluminescence imaging (BLI) was performed by using the IVIS SpectrumCT System (Perkin Elmer). This system is equipped with a back-thinned, back-illuminated CCD camera cooled at -90°C with a quantum efficiency in the visible range above 85%.

Each animal received an intra-peritoneal injection of 150 mg luciferin/kg body weight 10 minutes before performing BLI. During BLI acquisition, the animals were kept at 37°C and under gaseous anesthesia (2–3% isoflurane and 1 l/min oxygen).

A set of images was acquired every 2 minutes from 10 to 20 minutes after luciferin injection in order to detect the highest BLI signal. The images were obtained using the following settings: exposure time=auto, binning=8, f=1 and a field of view equal to 13 cm (field C). Dark images were acquired before and then subtracted to bioluminescence images, no emission filters were used during BLI acquisitions.

BLI image analysis

BLI image analysis was performed by placing region of interests (ROI) over the legs of the animals as shown in figure. The total flux (photons/seconds) was measured in order to quantify light emission in each ROI.

Images were acquired and analyzed using the Living Image 4.5 software (Perkin Elmer).

Microarray and Bioinformatic Analysis.

The quality of total RNA was first assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA).

Bioin-labeled cDNA targets were synthesized starting from 5-30 ng of total RNA. Double stranded cDNA synthesis and related cRNA was performed with Ovation®

Pico WTA Systems V2 (NuGEN Technologies, Inc). With the Encore® Biotin Module (NuGEN Technologies, Inc) was synthesized the fragmented and labeled cDNA. All steps of the labeling protocol were performed according to the manufacturer's recommended protocols.

Hybridization was performed using the Affymetrix GeneChip® Hybridization, Wash and Stain Kit, according to the Encore® Biotin Module (NuGEN Technologies, Inc) recommended protocol for Affymetrix GeneChip® Human Genome U133 Plus 2.0 Arrays.

It contains mix for target dilution, DMSO at a final concentration of 10% and pre-mixed biotin-labeled control oligo B2 and bioB, bioC, bioD and cre controls (Affymetrix cat# 900299) at a final concentration of 50 pM, 1.5 pM, 5 pM, 25 pM and 100 pM respectively. Targets were diluted in hybridization buffer at a concentration of 23 ng/ul, denatured at 99 °C for 2 minutes, incubated at 45 °C for 5 minutes and centrifuged at maximum speed for 1 minute prior to introduction into the GeneChip cartridge. A single GeneChip® Human Genome U133 Plus 2.0 was then hybridized with each biotin-labeled target.

Hybridizations were performed for 18 hours at 45°C in a rotisserie oven. GeneChip® cartridges were washed and stained in the Affymetrix Fluidics Station 450 following the FS450_0004 standard protocol, including the following steps: (1) (wash) 10 cycles of 2 mixes/cycle with Wash Buffer A at 25 °C; (2) (wash) 4 cycles of 15 mixes/cycle with Wash Buffer B at 50 °C; (3) stain of the probe array for 10 min in SAPE solution at 25 °C; (4) (wash) 10 cycles of 4 mixes/cycle with Wash Buffer A at 30 °C; (5) stain of the probe array for 10 min in antibody solution at 25 °C; (6) stain of the probe array for 10 min in SAPE solution at 25 °C; (7) (final

wash) 15 cycles of 4 mixes/cycle with Wash Buffer A at 30 °C; (8) fill the probe array with Array Holding buffer.

GeneChip arrays were scanned using an Affymetrix GeneChip® Scanner3000 7G using default parameters. Affymetrix GeneChip® Command Console software (AGCC) was used to acquire GeneChip® images and generate .DAT and .CEL files, which were used for subsequent analysis with proprietary software.

Partek Genomics Suite v 6.4 was used for analysis of microarray data.

Statistics

Comparison of two means was performed by unpaired Student's *t* test and one way-anova.

For microarray analysis ANOVA was applied considering unjusted p value or FDR, as reported in the figure legends.

STUDY APPROVAL

All the patients signed informed consent and the experiments performed were approved by San Raffaele Scientific Institute Ethical Committee. Animals procedures were performed according to protocols approved by the Committee for Animal Care and Use of San Raffaele Scientific Institute (Committee Protocol no. 375).