SUPPLEMENTARY APPENDIX

Repopulating hematopoietic stem cells from steady-state blood before and after *ex vivo* culture are enriched in the CD34$^+$CD133$^+$CXCR4^{low} fraction

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 doi:10.3324/haematol.2017.183962

Received: November 3, 2017.

Accepted: May 24, 2018.

Pre-published: June 1, 2018.

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Supplemental Methods:

Human steady-state peripheral blood cells:
The LDFs were obtained after leukocyte-depletion of the normal donor blood units, collected during the afternoon sessions on the territory of the Nouvelle Aquitaine region. The cells were extracted the next morning after their transport to the Bordeaux transfusion center.

Ex vivo expansion of LDF-recovered CD34+ cells:
When necessary, thawed cells from several cryotubes were pooled, and the same cell pool was used for tests performed at Day-0 and Day-7.

$CD34^+$ cell detection, immunophenotypical analysis and selection of cell subfractions:
Anti-CD34 (FITC), anti-CD184 clone 12G5 (APC), anti-CD9 (PE), anti-CD11a (PE), anti-CD49d (PE), anti-CD49e (PE), anti-CD49f (PE), anti-CD90 (PE), anti-CD45RA (PE), anti-CD26 (PE) (all from BD Pharmingen, BD Biosciences, Le Pont de Claix, France) and anti-CD133/2 (PE) (Miltenyi Biotec, Paris, France). All sorting gates were established on $CD34^+$ cells and delimitated so as to avoid overlapping between each other. The purity of each subpopulation was verified during and after cell sorting. At least 10^5 $CD34^+$ events were collected for analysis, assuming 0.1% false positives in control samples. With respect to the expression of CD34, CD184 and CD133, the cells were sorted on a FACS AriaTM III Sorter (Becton Dickinson, San Jose, CA) to isolate $CXCR4^{neg}$, $CXCR4^{low}$, $CXCR4^{bright}$ subfractions, and $CXCR4^{neg}CD133^-$, $CXCR4^{neg}CD133^+$, $CXCR4^{low}CD133^-$, $CXCR4^{low}CD133^+$, $CXCR4^{bright}CD133^-$, and $CXCR4^{bright}CD133^+$ subfractions.

Detection of stem cells by their in vivo repopulating capacity (SRC)
i) Short-term HSCs (ST-HSC). ST-HSC activity
Limiting dilution analysis: 1.5×10^3, 6.25×10^3, 1.25×10^4, 2.5×10^4, 5×10^4 or 1×10^5 Day 0 (before expansion) cells and 2.5×10^3, 6.25×10^3, 1.25×10^4, 2.5×10^4, 5×10^4, 1×10^5 or 2×10^5 of Day 7 (expanded) $CD34^+CXCR4^{low}CD133^+$ cells were injected per mouse (8 to 12 mice per cell dose). A transplanted mouse was scored as positive as explained in the main text of Methods. The frequency of SRC in the $CD34^+CXCR4^{low}CD133^+$ was calculated as referenced (reference 30, main text). The individual SRC proliferative capacity was evaluated only taking into consideration the cell dose presenting less than 37% of positive mice. In this way, every positive mouse is supposed to be reconstituted by one only SRC.
ii) Long-term HSCs (LT-HSC).
The bone marrow from both femurs of primary recipients was flushed out in 1 mL of medium (RPMI). Apart from a 65µL aliquot of cell suspension that was used for cell counting and detection of human $CD45^+$ cells by flow cytometry, the total cellular content from both femurs of each primary recipient was centrifuged, resuspended in 20 µL of RPMI medium, DNase 10% (2500 U/mL, Pulmozyme®, Roche, Boulogne-Billancourt, France), and injected intra-femorally (ref 6 and 25, main text) to the secondary recipient NSG mice (Figure 1, main text). The mice were sacrificed 7-8 weeks later and the same analyses as the ones for primary recipients were performed on the bone marrow cells of injected femurs. To detect LT-HSCs before expansion, 2×10^5 Day-0 cells were injected per mouse; to obtain the same information after expansion, 1.6×10^6 of Day-7 cells were injected per mouse.

Supplemental Table 1

	Total cells	Total $CD34^+$	Total $CXCR4^+$	$CD34^+$ $CXCR4^-$	$CD34^+$ $CXCR4^+$	$CD34^-$ $CXCR4^-$	$CD34^-$ $CXCR4^+$
Day 0 % total cells	/	93,8 ± 2,5	16,9 ± 3,1	81,6 ± 3,0	12,0 ± 3,6	1,3 ± 0,7	5,1 ± 1,9
Day 7 % total cells	/	39,8 ± 10,6	67,0 ± 5,4	10,2 ± 2,1	28,7 ± 10,3	22,0 ± 9,4	37,3 ± 5,4
Fold expansion	25,1 ± 9,9	10,9 ± 4,8	85,6 ± 37,1	3,9 ± 1,6	38,9 ± 17,7	1399 ± 906,3	305,6 ± 155,7

Table S1. Fold expansion of CXCR4 expressing cells among $CD34^-$ and $CD34^+$ SS-PB cells after Day-7 culture expansion. FITC-anti-CD34 and APC-anti-CXCR4 antibodies were used for SS-PB cell labelling and analysis. All values are mean ± SD after cell sorting for Day-0, and Day-7 cells (15 and 12 samples, respectively).

Supplemental Table 2

Cell source	Cell dose x 10^3	No of positive mice	No of transplanted mice	Percentage of positive mice	Mean chimerism in positive mice
Day 0	1,56	0	6	0	
	6,25	2	9	22,2	0,2
	12,5	4	12	33,3	0,2
	25	6	12	50	0,86
	50	7	8	87,5	1,13
	100	16	16	100	18,2
	200	13	13	100	49,6
Day 7	2,5	0	4	0	
	6,25	0	6	0	
	12,5	2	10	20	1,1
	25	1	9	11	0,3
	50	3	11	27,3	1
	100	5	8	62,5	3
	200	9	9	100	2
	400	15	15	100	9,2
	1600	10	10	100	19,4

Table S2. Number and proportion of "positive" mice with respect to the $CD34^+CD133^+CXCR4^{low}$ cell dose injected per one mouse and the mean human CD45 chimerism per cell dose before and after *ex vivo* expansion.

Supplemental Figure 1

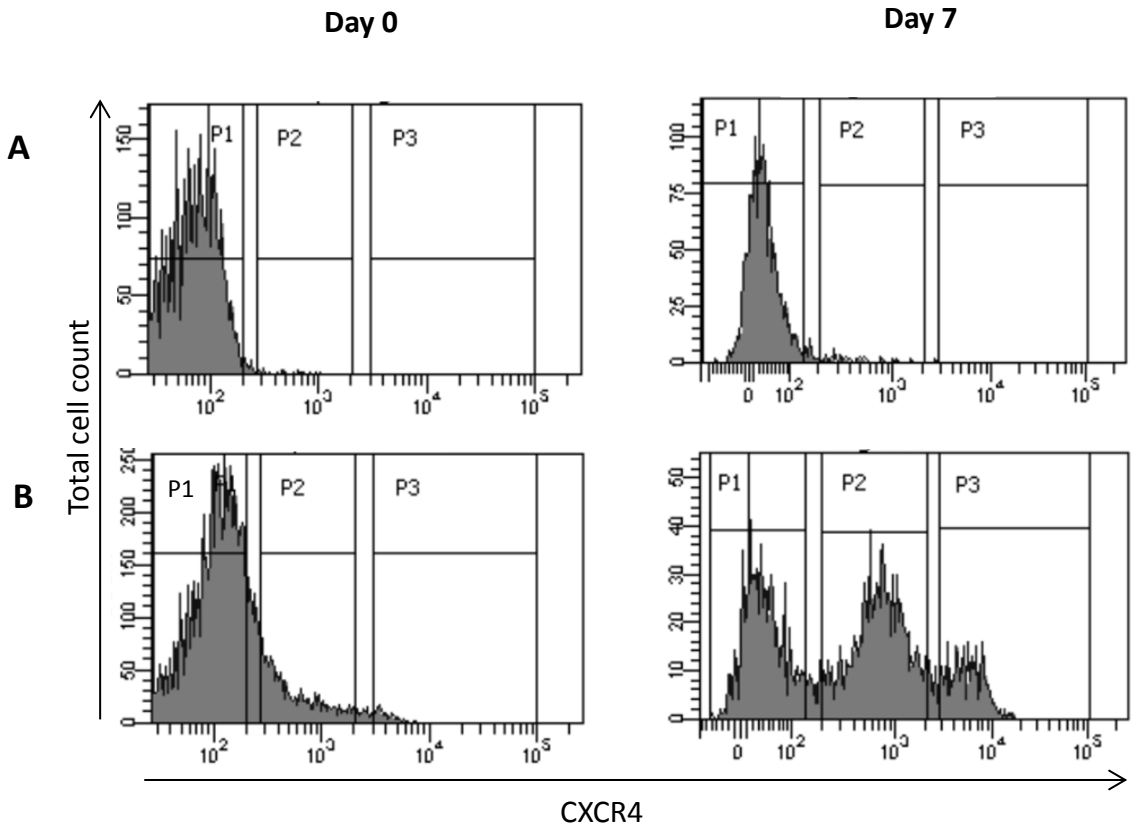
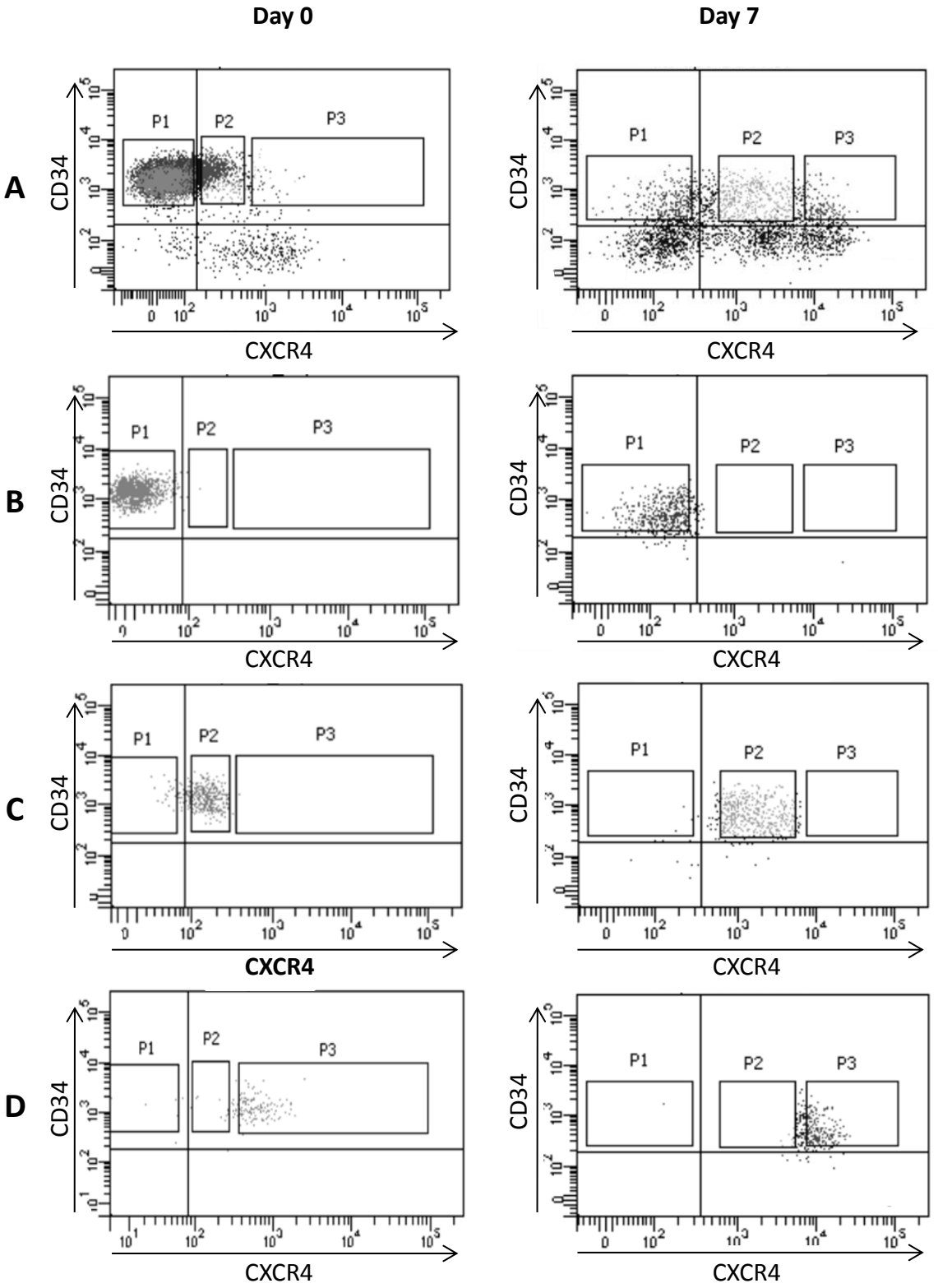
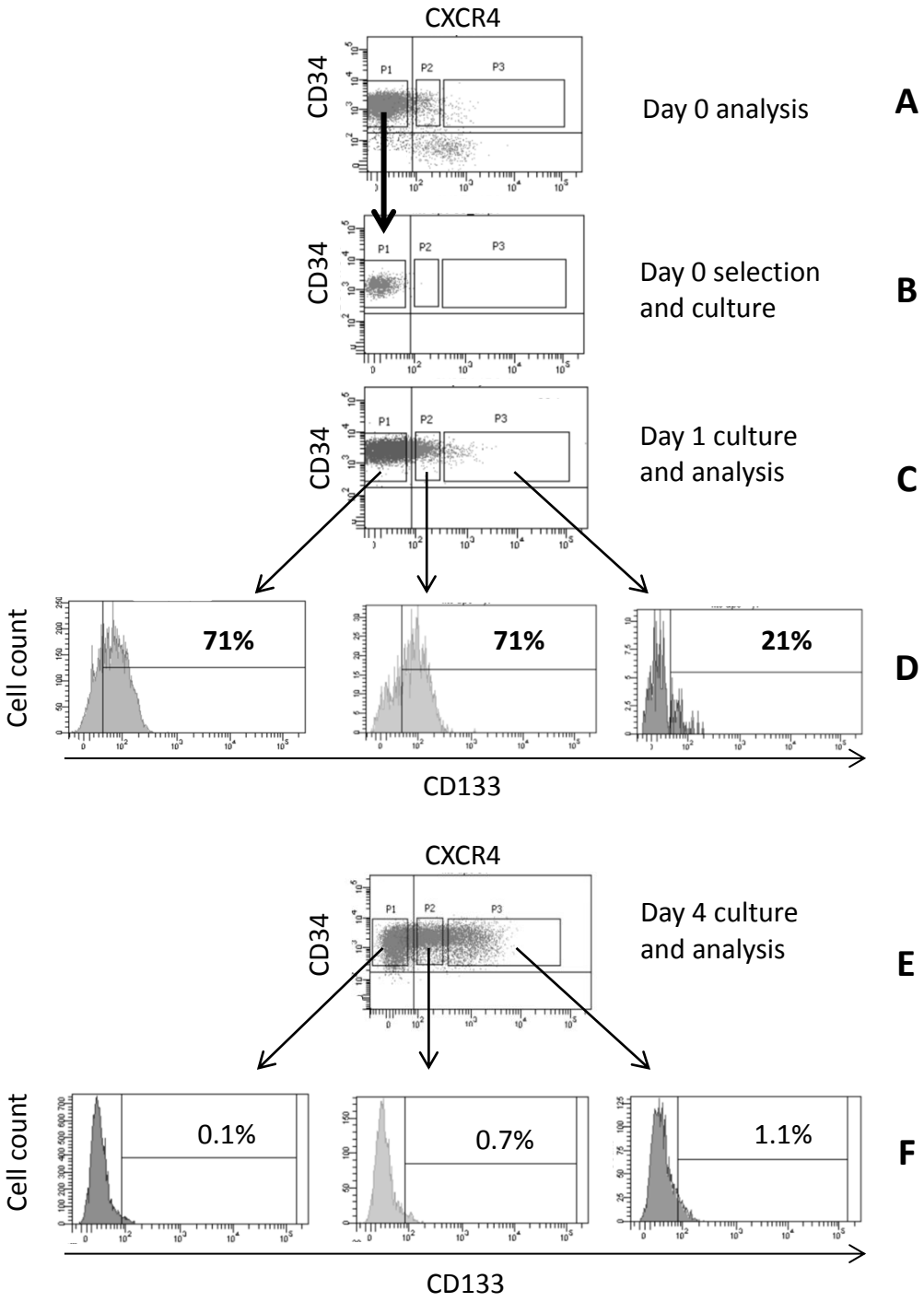


Figure S1. Analysis by flow cytometry of distinct CXCR4 expressing populations among the total SS-PB cells before (Day-0) and after (Day-7) expansion. The cell population of interest was selected based on FSC vs SSC, then width and height parameters for FSC and SSC gates were used to exclude cell doubles, as Area Scaling with FACS AriaTM III Sorter. (A) Isotypic control (IgG-APC conjugated); (B) APC-anti-CXCR4 (clone 12G5) positive analysis of total SS-PB cells. Three cell subpopulations are defined: P1 - $CXCR4^{neg}$; P2 - $CXCR4^{low}$; P3 - $CXCR4^{bright}$. At Day-0, total $CD34^{+}$ SS-PB cells were incubated for 7 days in HP01 clinical-grade medium supplemented with G-CSF, SCF (100 ng/mL each), TPO (20 ng/mL) and IL-3 (0.5 ng/mL).

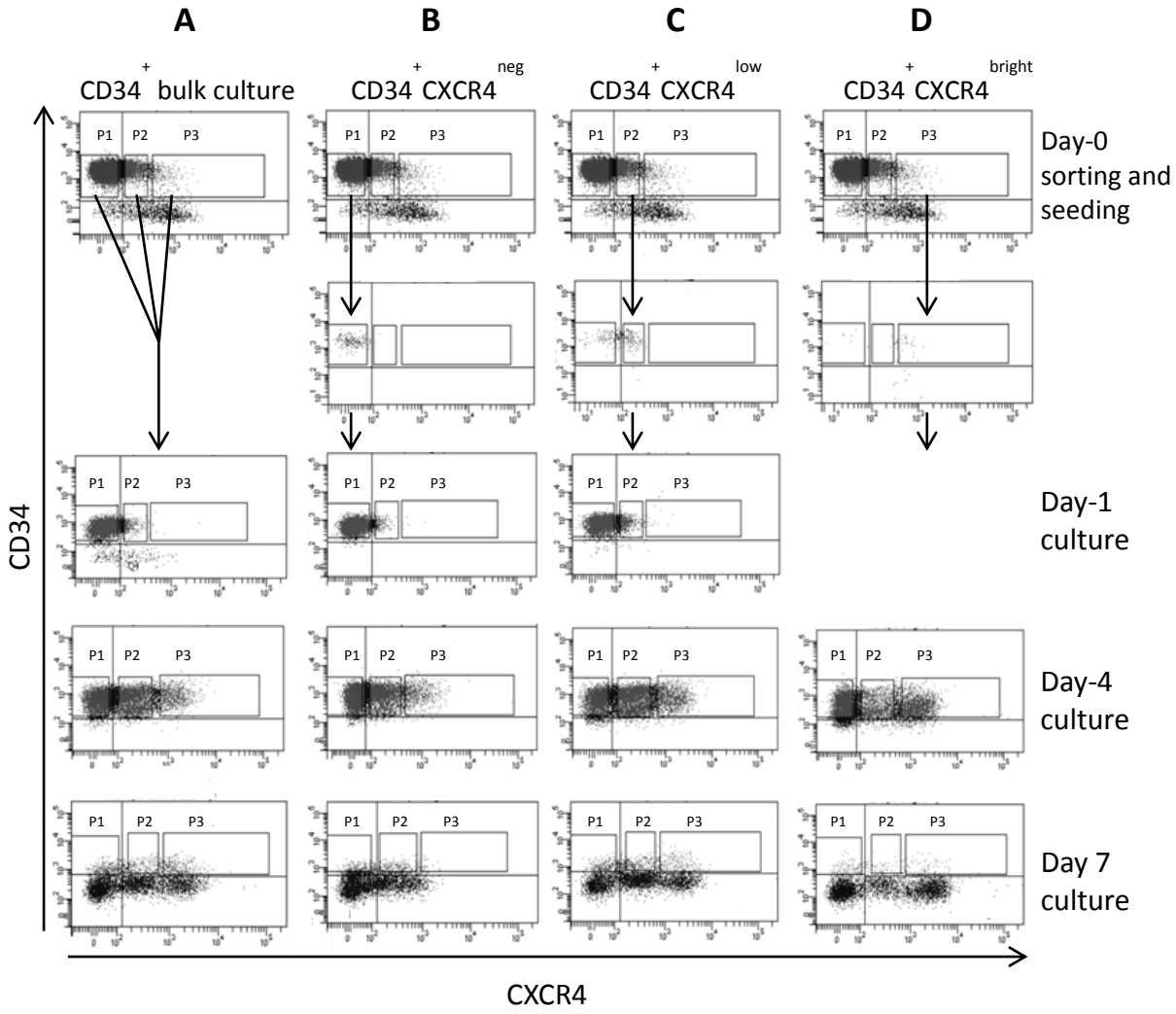
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Supplemental figure legends

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Figure S2. Gating and sorting of three $CD34^+$ cell populations of SS-PB with respect to their CXCR4 expression level at Day-0 and after Day-7 *ex vivo* expansion. (A) Based on CXCR4 expression, cell populations defined as P1, P2 and P3 were further delimited and sorted on the basis of $CD34^+$ expression before and after *ex vivo* expansion as the following subpopulations: P1 - $CD34^+CXCR4^{neg}$; P2 - $CD34^+CXCR4^{low}$; P3 -$CD34^+CXCR4^{bright}$. (B) P1 sorting gate purity is verified during and at the end of the sorting procedure. (C) P2 sorting gate purity is verified during and at the end of the sorting procedure; (D) P3 sorting gate purity is verified during and at the end of the sorting procedure. Day-0 total $CD34^+$ SS-PB cells were incubated for 7 days in HP01clinical-grade medium supplemented with G-CSF, SCF (100 ng/mL each), TPO (20 ng/mL) and IL-3 (0.5 ng/mL).

Figure S3. Phenotypic evolution of the sorted Day-0 $CD34^+CXCR4^{neg}$ cell subpopulation when incubated overnight (Day-1) and during short *ex vivo* expansion (Day-4). Three cell gates were delimited: P1 - $CD34^+CXCR4^{neg}$; P2 - $CD34^+CXCR4^{low}$; P3 - $CD34^+CXCR4^{bright}$ subpopulation (A). At Day-0, $CD34^+CXCR4^{neg}$ SS-PB cells were sorted (B) and incubated overnight (C) or for 4 days (E) in HP01clinical-grade medium supplemented with G-CSF, SCF (100 ng/mL each), TPO (20 ng/mL) and IL-3 (0.5 ng/mL) and analyzed for their expression of CD34 and CXCR4. At Day-1 (D) and at Day-4 (F) of *ex vivo* culture, P1, P2 and P3 subpopulations were analyzed for their CD133 expression and the percentage of $CD133^+$ cells is given for each subpopulation.

Figure S4. Evolution of CXCR4 expression on $CD34^+$ cells. Three cell gates were delimited: P1 - $CD34^+CXCR4^{neg}$; P2 - $CD34^+CXCR4^{low}$; P3 - $CD34^+CXCR4^{bright}$ subpopulation. (A) Culture started with total $CD34^+$ cells; (B) Culture started with $CD34^+CXCR4^{neg}$ cells. (C) Culture started with $CD34^+CXCR4^{low}$ cells. (D) Culture started with $CD34^+CXCR4^{bright}$ cells. Note that a substantial number of $CXCR4^{neg}$ cells become positive during the culture (B) in the same manner as when the culture started from the bulk $CD34^+$ population (A; supplemental Figure 3). ND: not determined.