Supplemental Information

Title: *Ex-vivo* Human Hematopoietic Stem Cell Expansion Requires Coordination of Cellular Reprogramming with Mitochondrial Remodeling and P53 Activation

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Figure Legend:

Figure S1. Treatment with VPA leads to the preferential expansion of long-term HSCs

(A) Schematic representation of the strategy for *ex-vivo* expansion of HSCs utilizing VPA and a cytokine combination. (B) Representative flow cytometry plots of total nucleated cells stained with Annexin V and 7-AAD after 6 days of incubation with VPA. Numbers represent percentage of apoptotic cells determined by Annexin V and dead cells stained with 7-AAD (n=3). (C) Gating strategy (CD34-APC, CD90-FITC). (D, E) Percentage and the absolute numbers of phenotypically defined long-term HSCs ($CD34^+CD90^+CD49f^+$) expanded from UCB-$CD34^+$ cells cultured with cytokines alone or VPA during a seven-day culture period and determined by flow cytometry (n=22). PC denotes uncultured $CD34^+CD90^+CD49f^+$ cells present in UCB. (F) Percentage of different subsets of $CD34^+$ cells generated throughout 2 days of incubation with VPA (n=11). Error bars with SEM, ****p≤0.0001 were determined by Beta models for D and negative-binomial models for E, 2-way ANOVA for F. n: number of biological replicates.

Figure S2. VPA alters the global transcriptome of *ex-vivo* expanded $CD34^+$ cells.

(A) Volcano plot representing the differentially expressed genes identified by the bulk RNA-seq performed in $CD34^+$ cells isolated from cultures expanded for 2 days with VPA. X-axis represents fold change (log_2) of gene expression in $CD34^+$ cells treated with VPA relative to those treated with cytokines alone (n=3). (B) List of significantly up-regulated genes identified by the bulk RNA-seq performed in $CD34^+$ cells after 4 days of incubation with VPA compared to $CD34^+$ cells cultured with cytokines alone. (C) Down-regulation of CDKs' gene expression and up-regulation of CDK inhibitors' gene expression determined as fold change by RNA-seq in $CD34^+$ cells expanded with VPA compared

to $CD34^+$ cells present in cultures treated with cytokines alone for 4 days. NES: normalized enrichment score. (D) Enrichment analysis (GSEA) of Ivanova geneset on the up-regulated genes identified by the bulk RNA-seq in $CD34^+$ cells after 4 days of incubation with cytokines with VPA relative to $CD34^+$ cells cultured with cytokines alone. n: number of biological replicates.

Figure S3. Alterations of the mitochondrial profile induced by VPA occur simultaneously with acquisition of the HSC phenotype

(A, B) Mitochondrial mass, membrane potential and superoxide anion (ROS) production in different subsets of $CD34^+$ cells after 2 days (A) and 6 days (B) of incubation with VPA as assessed by flow cytometry utilizing MitoTracker Green FM, TMRM and MitoSOX™ Red, respectively. Graph represents the fold change of median fluorescence intensity of each of the respective dyes used to quantify mitochondrial mass, membrane potential or ROS relative to the corresponding values of the same $CD34^+$ subsets of cells generated in cultures treated with cytokines alone (gray line) (n=10). (C) Representative flow cytometry histogram of mitochondrial mass in $CD34^+CD90^+$ cells cultured for 24 hrs with either cytokines alone or VPA in the presence of Verapimil. (D) Median fluorescence intensity of $CD34^+CD90^+$ cells stained with MitoTracker Green FM and treated as in (C) (n=3). (E) Electron microscopy images of $CD34^+CD90^+CD49f^+$cells expanded for 4 days with VPA. Images were captured under 1000 (left) and 100000 (right) magnification. (F) Up-regulation of MCT1 and MCT4 gene expression levels determined by RNA-seq in $CD34^+$ cells expanded for 4 days with VPA and compared to those treated with cytokines alone. (G) Representative flow cytometry plots of cells cultured as indicated for 6 days and stained with Annexin V and 7-AAD. Numbers represent percentage of apoptotic cells determined by Annexin V and dead cells stained with 7-AAD (n=3). Error bars with SEM, ****$p \leq 0.0001$; ***$p \leq 0.001$; ns, not significant were determined by 2-way ANOVA for A, B and t-test for D. n: number of biological replicates.

Figure S4. Inhibition of p53 impairs the HSC expansion without affecting their viability.

(A) Enrichment of p53 pathway analyzed by GSEA on the up-regulated genes identified by the bulk RNA-seq in $CD34^+$ cells cultured for 4 days with VPA relative of $CD34^+$ cells cultured with cytokines alone. NES: normalized enrichment score. (B) Transcript levels of p53 in total nucleated cells expanded for 4 days. mRNA levels were represented as fold change relative to mRNA levels of cells cultured with VPA within each experiment (n=5). (C, D) Effect of p53 siRNA on absolute numbers and percentage of HSCs expanded as indicated for 4 days (n=4). (E) Effect of pifithrin α (pα) on percentage of HSCs expanded for 2 days (n=7). (F) Effect of p53 siRNA on percentage of viable cells in cultures expanded for 4 days (n=6). (G) Effect of pifithrin α treatment on percentage of viable total nucleated cells as determined by acridine orange/propidium iodide staining (n=10). (H) Representative flow cytometry plots of total nucleated cells cultured for 6 days and stained with Annexin V and 7-AAD. Numbers represent percentage of apoptotic cells determined by Annexin V and dead cells stained with 7-AAD (n=3). Experiments in figures (F-H) were performed in triplicates. (I, J) Effect of p53 siRNA on mitochondrial mass (I) and ROS levels (J) in $CD34^+CD90^+$ cells expanded for 4 days. Graphs represent fold change of median fluorescence *MitoTracker* Green (I) and intensity of *MitoSOX*™ (J) in $CD34^+CD90^+$ cells relative to VPA cultures within each experiment (n=6). (K) ATP, basal (BR) and max (MR) oxygen respiration measured by seahorse analyzer on $CD34^+$ cells isolated for cultures expanded for 4 days (n=4). Each experiment was performed in triplicates and graphs represent fold change normalized within each experiment. (L) p53 protein levels in total nucleated cells expanded for 4 days and examined by Western Blotting. GAPDH was used as an internal loading control. Error bars with SEM, *p≤0.05; ns, not significant were determined by multiple t-test for B, C, I. 2-way ANOVA for D, I, J and negative binomial for E. n: number of biological replicates.

Figure S5. MnSOD inhibition via p53 limits the VPA-induced HSC expansion without affecting their viability.

(A) Effect of pifithrin α on the MnSOD transcript levels of the total nucleated cells expanded for 4 days with VPA. (B) Effect of p53 siRNA on the sestrin 2 transcript levels of the total nucleated cells expanded for 4 days with VPA. Transcript levels were assessed by qRT PCR and mRNA levels and presented as fold induction relative to mRNA levels in cultures of $CD34^+$ cells incubated with VPA within each experiment (n=3). (C) Effect of MnSOD siRNA on MnSOD transcript levels of the total nucleated cells generated in cultures of $CD34^+$cells incubated for 4 days with VPA (n=3). (D) Effect MnSOD siRNA treatment on percentage of viable nucleated cells measured by acridine orange/propidium iodide staining after 4 days of incubation of $CD34^+$ cells with VPA (n=6). Each experiment presented in Figures A-D was performed in triplicates. (E, F) Effect of the second MnSOD siRNA treatment on percentage (E) and absolute numbers (F) of $CD34^+CD90^+$ expanded for 4 days in cultures treated as indicated (n=3). Error bars with SEM, ***p≤0.0001; **p≤0.01; *p≤0.05 were determined by unpaired t-test. n: number of biological replicates.

Methods

***Ex-vivo* culture.** UCB collections were purchased from the Placental Blood Program at the New York Blood Center. Mononuclear cells were isolated by Ficoll-Hypaque (GE Healthcare) density centrifugation, and $CD34^+$ cells were purified by immunomagnetic sorting using the CD34 Microbead kit (Miltenyi) and the AutoMACS Pro Separator (Miltenyi). Highly purified (90%–98%) PCs were seeded at a density of $2.5x10^4$ cells/mL in SF Stemline II (Sigma-Aldrich) culture medium containing 1% penicillin-streptomycin (Life Technologies) supplemented with 150 ng/ml human stem cell factor (SCF), 100 ng/ml human fms-like tyrosine kinase receptor 3 (FLT3 ligand), 100 ng/ml human thrombopoietin (TPO), and 50 ng/ml human interleukin 3 (IL-3) (R&D Systems). Following priming for 16 hours with cytokines, cells were exposed to 1mM VPA (Sigma-Aldrich).

Seahorse analysis. Oxygen consumption rate was measured using a 24-well Seahorse Bioanalyzer XF24 according to the manufacturer's instructions (Agilent Technologies). Sorted $CD34^+$ cells from expanded cultures were plated ($2x10^5$ cells/well) into 24-well plates pre-coated for 12 hrs with poly-lysine (Sigma-Aldrich, P4707) and incubated overnight. The following day, cells were washed and media was replaced with 500 µl of temperature/CO2 pre-adjusted Seahorse XF Base medium. Cells were analyzed using the XFcell Mito-stress kit following the manufacturer instruction and using ETC inhibitors such as 1 µM oligomycin, 2 µM FCCP,0.5 µM Rotenone and 0.5 µM Antimycine.

Mitochondrial DNA quantification. Isolated $2x10^5$ $CD34^+$ cells were lysed in lysis buffer (10 mM Trid-HCl pH7.5, 50 mM NaCl, 0.045% NP40, 0.45% Tween20) supplemented with proteinase K

(1mg/ml) for 2 hours at 56°C and 15 min at 95°C. 5 ng of genomic of DNA was amplified by qPCR using SYBR green (Applied Biosystem) and the NovaQuant Human mitochondrial to nuclear DNA ratio kit (Millipore). Relative mtDNA:nDNA ratio was calculated using the ΔΔCt method upon targeting of nuclear-encoded genes BECN1, NEB and mitochondrial-encoded gene ND1, ND6.

Cell cycle analysis. The percentage of quiescent cells was measured by incubating cells in culture media supplemented with 10 µg/ml Hoechst 33342 (Invitrogen) for 45 min at 37°C followed by incubation with 0.5 µg/ml Pyronin Y (Sigma-Aldrich). Cells were stained with CD34-APC and CD90-FITC and analyzed by flow cytometry.

Cell counting. 20 µl of cell suspension was mixed with 20 µl of acridine orange (AO)/propidium iodide (PI) (Nexcelom) in PBS solution. Viable nucleated cells stained by AO and the nucleated dead cells stained by PI stain were counted using the Cellometer Auto 2000 Cell Viability Counter (Nexcelom). The viability was calculated as the ratio of PI positive cells over TNCs.

Detection of mitochondrial superoxide anion production, membrane potential and mass. Mitochondrial superoxide anion generation and mitochondrial membrane potential were measured by flow cytometry using superoxide specific fluorescent probes *MitoSOX™ Red* and Tetramethylrhodamine, methyl ester (TMRM) (Invitrogen). Briefly, live cells were incubated for 20 min at 37°C with 5µM *MitoSOX™ Red* or 200nM TMRM. Following washes, the cells were incubated with CD34-APC (1:100 BDBioscience) and CD90-FITC (1:100 BioLegend) in PBS containing 7.5% BSA and 0.5% EDTA for 20 min at room temperature. Cells were washed and analyzed immediately by flow cytometry.

The mitochondrial mass was measured by staining the cells with 200 nM *MitoTracker Green FM* (Invitrogen) for 20 min at 37°C followed by washes and incubation with CD34-APC (1:100 BDBioscience) and CD90-PE (1:100 BioLegend) for 20 min and flow cytometry analysis. Data were analyzed by using Flow Jo software (Version 9.6.2, Tree Star Inc).

Cell division and CFSE staining assay. Freshly isolated UCB-CD34$^+$ cells were labeled with 2.5 μM CellTrace™ carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) and incubated for 20 min at 37°C in the dark. Staining was stopped by washing with 10 volumes of PBS containing 5% FBS. Next, the cells were washed with PBS alone and incubated with cytokines alone overnight. The subpopulation of cells that was highly and similarly stained with CFSE dye was sorted by FACS and cultured with cytokines alone or the combination of cytokines and VPA for 7 days. Cells were stained with CD34-APC and CD90-PE antibodies and analyzed by flow cytometry.

Western Blotting. Cells were lysed in NP-40 lysis buffer (Boston BioProducts) supplemented with protease inhibitors and the whole cell extracts were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Detection of acetylated proteins was performed by lysing the cells with lysis buffer supplemented with (1mM) TSA and (5 mM) nicotinamide. After incubation with 5% nonfat milk in TBST (10 mMTris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membranes were incubated with antibodies (Cell Signaling Technologies) against p53 (1:100), acetylated p53 (K-382), MnSOD (1:1000), GAPDH (1:1000) at 4°C overnight. Following washes, membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies for 1 hr (1:3000 Jackson Immunoresearch). Blots were washed with TBST and developed with the ECL system (Amersham Biosciences) according to the manufacturer's instructions.

Confocal and electron microscopy. $CD34^+$ cells cultured with either VPA and cytokines or cytokines alone were sorted by FACS and seeded at a density of $1x10^5$ cell/mL on Poly-L-Lysine coated chamber slides (Nunc™ Lab-Tek™) and allowed to adhere overnight. Next, cells were stained with 100 nM MitoTracker Orange (Invitrogen) dye for 25 min at 37°C followed by washes with PBS and fixed with 4% formaldehyde (Sigma Aldrich). Cells were mounted and nuclei were stained by DAPI using ProLong™ Gold antifade reagent (Invitrogen). Images were taken on a confocal microscope Leica TCS SPS II. For electron microscopy, isolated $CD34^+$ cells from cultures incubated with either cytokines alone or cytokines with VPA were fixed at 4°C overnight in 2.5% glutaraldehyde and 4.0% paraformaldehyde in 0.1 M phosphate buffer and were processed by the microscopy facility at ISMMS.

Media Removal. Media containing cytokines alone or cytokines and VPA was removed and cells were washed and centrifuged. Cells previously treated with cytokines alone were cultured back on their own media. Cells previously treated with cytokines and VPA were cultured in conditioned media containing cytokines alone. To avoid addition of fresh cytokines, the conditional media was obtained by culturing additional cells derived from the same UCB unit with cytokines alone for the same period of time as cells cultured with VPA and cytokines.

Single-cell RNA-seq. Viable cell suspensions of VPA treated hematopoietic stem cells at various time points were isolated and characterized using standard DAPI staining to determine viability. The bulk cellular suspensions were then characterized to determine cell density using a C-Chip DHC-N01 disposable hemocytometer. A mixture of 5µl of 0.04% trypan blue (BioRad) and 5µl of the single cell suspension was loaded unto the hemocytometer and examined at 10X magnification, using the Invitrogen Evos FL Cell Imaging System-digital inverted microscope. GEM Drop-seq was performed as described

(10x Genomics, Pleasanton, CA)(Zheng et al., 2017), following the Single Cell 3' Reagents Kits V1 User Guide. The final cell concentration was set at 1000 cells/uL density and the 10X chip (Chromium Single Cell 3' Chip kit v1) was loaded to target 6,000 cells final. Reverse-transcription was performed in the emulsion and cDNA was amplified before library construction. Each library was tagged with a different index for multiplexing. Quality control and Quantification of the constructed libraries was evaluated using Qubit dsDNA HS Assay Kit (Thermo Fisher), Agilent cDNA High Sensitivity Kit, and Kapa DNA Quantification Kit for Illumina platforms, following the manufacturers' instructions. Sequencing was carried out with Illumina Hiseq 2500 according to V1 chemistry with the number of cycles for each read as follows: Read 1: 98 cycles, i7 index: 14 cycles, i5 index: 8 cycles and Read 2: 10 cycles.

Single-cell RNA-seq data analysis. sc RNA-seq data generated about 166 Million reads (1528-4921cells) per sample. The data were processed using the Cell Ranger pipeline v1.3 (Zheng et al., 2017). The Cell Ranger pipeline aligned 70-80% of the reads uniquely to the transcriptome. Differentially expressed genes were analyzed using the sSeq method (Yu et al., 2013a), as implemented in the R package cellrangerRkit v1.1.

The t-SNE analysis (Amir el et al., 2013, van der Maaten and Hinton, 2008) was performed using the implementation from the Seurat package 2.0 (Macosko et al., 2015). Data can be accessed at GEO accession https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110974 with token:krqhegqudbmtdmh.

Bulk RNA-seq data analysis. The RNA-seq data generated about 44-81 million single-ended 1x100 reads per sample. The RNA-seq reads were aligned using STAR (Dobin et al., 2013) v2.5.1b with the human genome (GRCh38.p5 assembly) and gene annotations (release 24, Ensembl version 83 and 84) downloaded from the https://www.gencodegenes.org/ web site. 86-90% of the reads were uniquely mapped to the human transcriptome. The matrix counts of gene expression for all 21 samples were computed by featureCounts v1.5.0-p1 (Liao et al., 2014). Differentially expressed genes (5% FDR, at least 2 log2 fold change and at least 4.0 of the average gene expression in terms of log2 transformed counts per million) were identified using the voom method (Law et al., 2014) in the Bioconductor (Gentleman et al., 2004) package Limma (Ritchie et al., 2015).

Cell transfection. Freshly isolated UCB-$CD34^+$ cells were transfected using the AMAXA system nucleofection (Lonza) and the Human CD34 Cell Nucleofector® Kit (Lonza) according to the manufacturer's instructions. $1x10^6$ cells are nucleofected with 30 nM p53 siRNA, 30 nM MnSOD siRNA, or 30 nM Ambion® *Silencer*® Negative Control #1 siRNA (Thermofisher).

RT-PC qPCR. RNA was extracted using the QIAGEN RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. 1ug of RNA was transcribed into cDNA by using the SuperScript® IV Reverse Transcriptase kit (Invitrogen). Gene expression levels were quantified by using the Power SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Final concentration of primers used for qPCR varied between 100 nM and 300 nM. qPCR was performed using the CFX96 Touch™ Real-Time PCR Detection System (Bio-RAD). Cycling conditions were 95°C for 10 min then 40 cycles of 95°C for 15 sec and 56°C for 1 min, followed by one Melt-curve step. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Data were analyzed using the $2^{-\Delta\Delta ct}$ method.

Table of RNA primers and siRNA

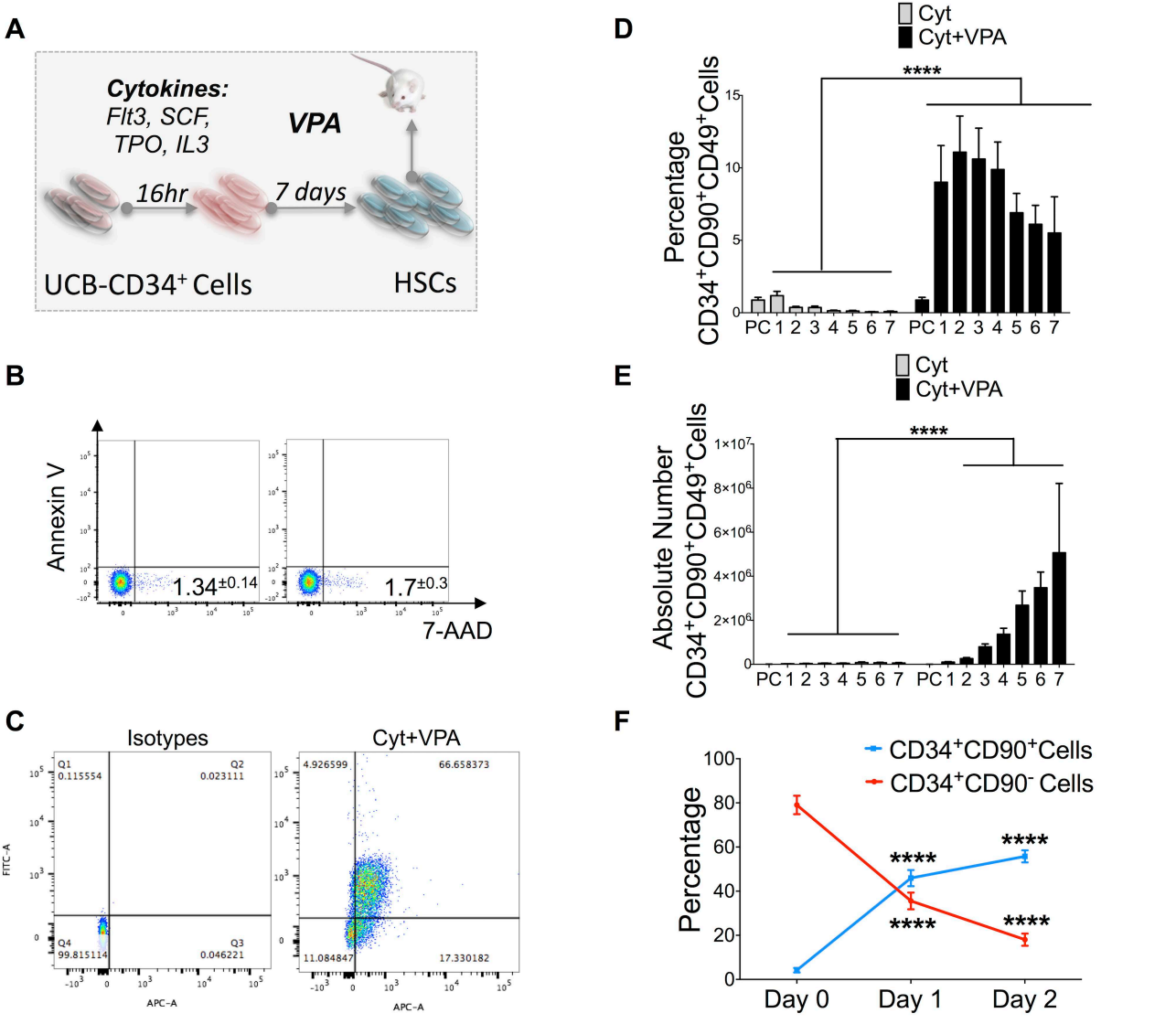
Oligonucleotides		
RT² qPCR Primer Assay for Human SESN2	Qiagen	Cat# PPH07580A-200
RT² qPCR Primer Assay for Human GAPDH	Qiagen	Cat# PPH00150F-200
RT² qPCR Primer Assay for Human POU5F1 (OCT4)	Qiagen	Cat# PPH02394E-200
RT² qPCR Primer Assay for Human Nanog	Qiagen	Cat# PPH17032E-200
RT² qPCR Primer Assay for Human SOX2	Qiagen	Cat# PPH02471A-200
RT² qPCR Primer Assay for Human Mnsod	Qiagen	Cat# PPH01716B-200
Primer for human P53 F: CATCCTCACCATCATCACAC	Genelink	N/A
Primer for human P53 R: TGGACTTCAGGTGGCTGGAG	Genelink	N/A
p53 siRNA	Thermo Fisher Scientific	Cat # AM16708; assay ID : 106141
MnSOD siRNA	Thermo Fisher Scientific	Cat # 4390824; Assay ID : s13268 Cat # AM16708 Assay ID: 260737
Silencer® Negative Control #1 siRNA	Thermo Fisher Scientific	Cat# AM4635

***In-vivo* marrow repopulating potential of *ex-vivo* expanded UCB-CD34$^+$ cells.** Female NSG mice (The Jackson Laboratory-stock number 005557) were maintained in a pathogen-free environment and monitored at the Research Animal Resource Center of ISMMS. All animals were randomly assigned to experimental groups. Mice were sublethally irradiated with 300 cGy 12 hours prior to transplantation. The progeny of cells derived from $0.5x10^5$ UCB-CD34$^+$ cells isolated from 4 different donors and expanded with VPA for 4 days were transplanted through tail vein injection. Mice were sacrificed 16 weeks after transplantation. Bone marrows cells (BM) were isolated by flushing femur bones with PBS containing 2% FBS and 1% Penicillin/Streptomycin (GIBCO). Whole BM cells were lysed on ice with red blood cell lysis solution (Invitrogen), and

washed in PBS with 2% FBS. BM cells from each mouse were analyzed for the presence of cells expressing human CD45-FITC. The presence of at least 0.1% human $CD45^+$ cells in the marrow of each recipient mouse was considered indicative of donor human hematopoietic cell engraftment.

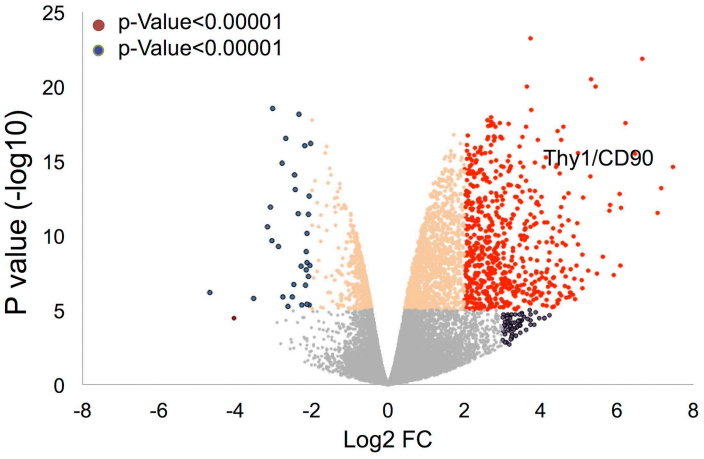
Statistical Analysis. Values are shown as the mean SEM. Statistical analysis were performed by descriptive statistics such as median and minimum-maximum values. Multilevel analysis for Beta models was performed to test the change in HSC percentages. On the other hand, to test the difference in absolute HSC counts, negative-binomial models were fitted. P values of less than 0.05 were considered to indicate statistical significance. All other statistical analyses were performed using GraphPad Prism 7.0 software. Student's t test was used for comparisons between two groups, whereas two-way ANOVA were used for comparisons among multiple groups and P values.

Study approval. All animal experiments were reviewed and approved by the IACUC of ISMMS (IACUC-2016-0092)



Supplemental Figure 1

A



B

Up-regulated Genes

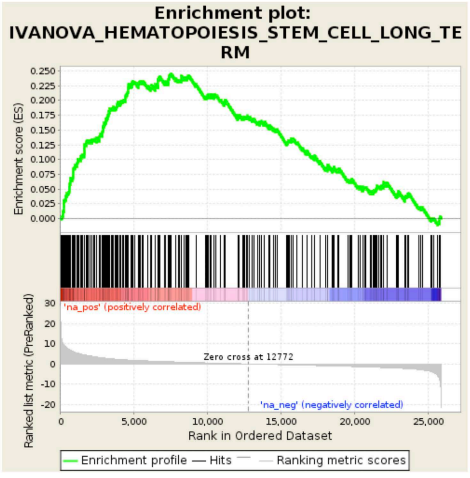
Transcript	Log2 Fold Change	p Value
THY1	4.973	0.000005268
ACE	3.11	8.591E-07
PROCR	2.984	0.0000252
TEK	2.647	0.000007966
ITGA6	2.586	0.000003361
ALDH1A1	2.564	0.00007609
PBX1	2.399	0.000003339
HES1	2.041	0.0001015
FOXO1	1.575	0.0002271
MEIS1	1.459	0.00007525
GATA2	1.401	0.0001403
PROM1	0.8968	0.00958

C

Differentially Expressed Genes

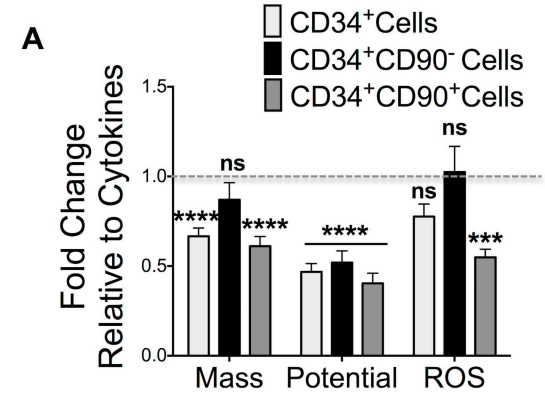
Name of Genes	Log2 Fold Change	p value
CDK3	-2.008	0.002269
CDK6	-0.5478	0.04027
p27	2.04	0.3248
p21	1.572	8.32E-05
p57	0.1874	5.06E-05

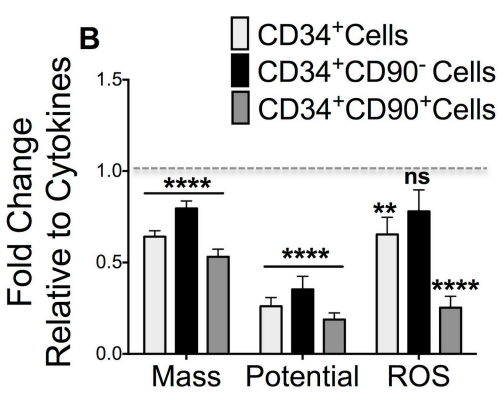
D

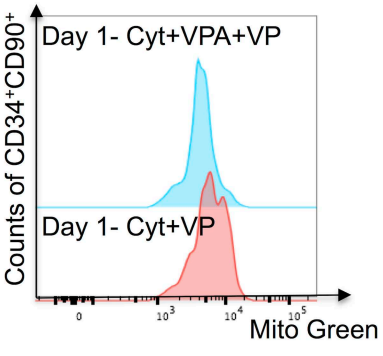


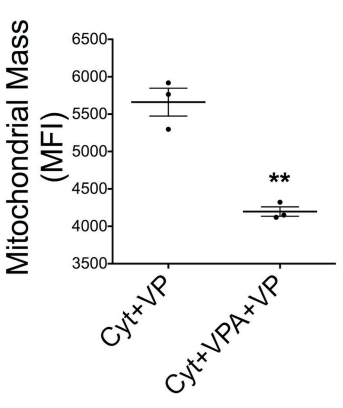
NES	4.568
Nominal p-Value	<0.0001
FDR q-Value	<0.0001

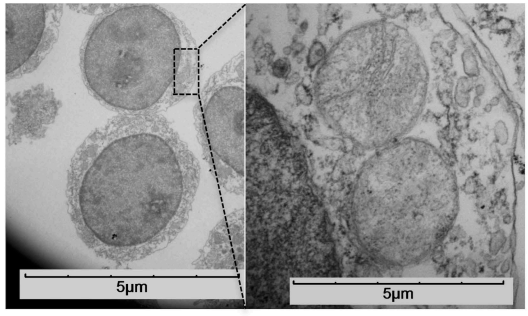
Supplemental Figure 2





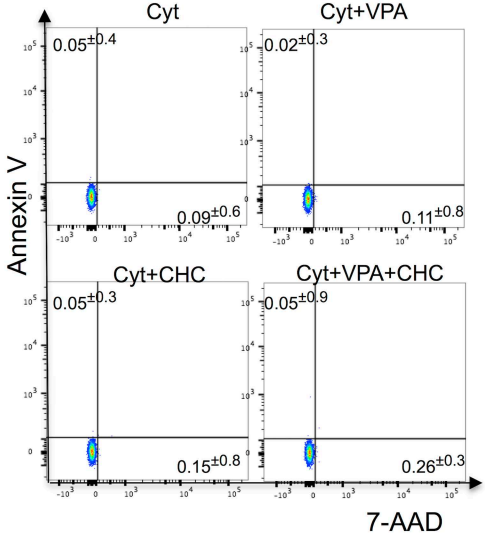




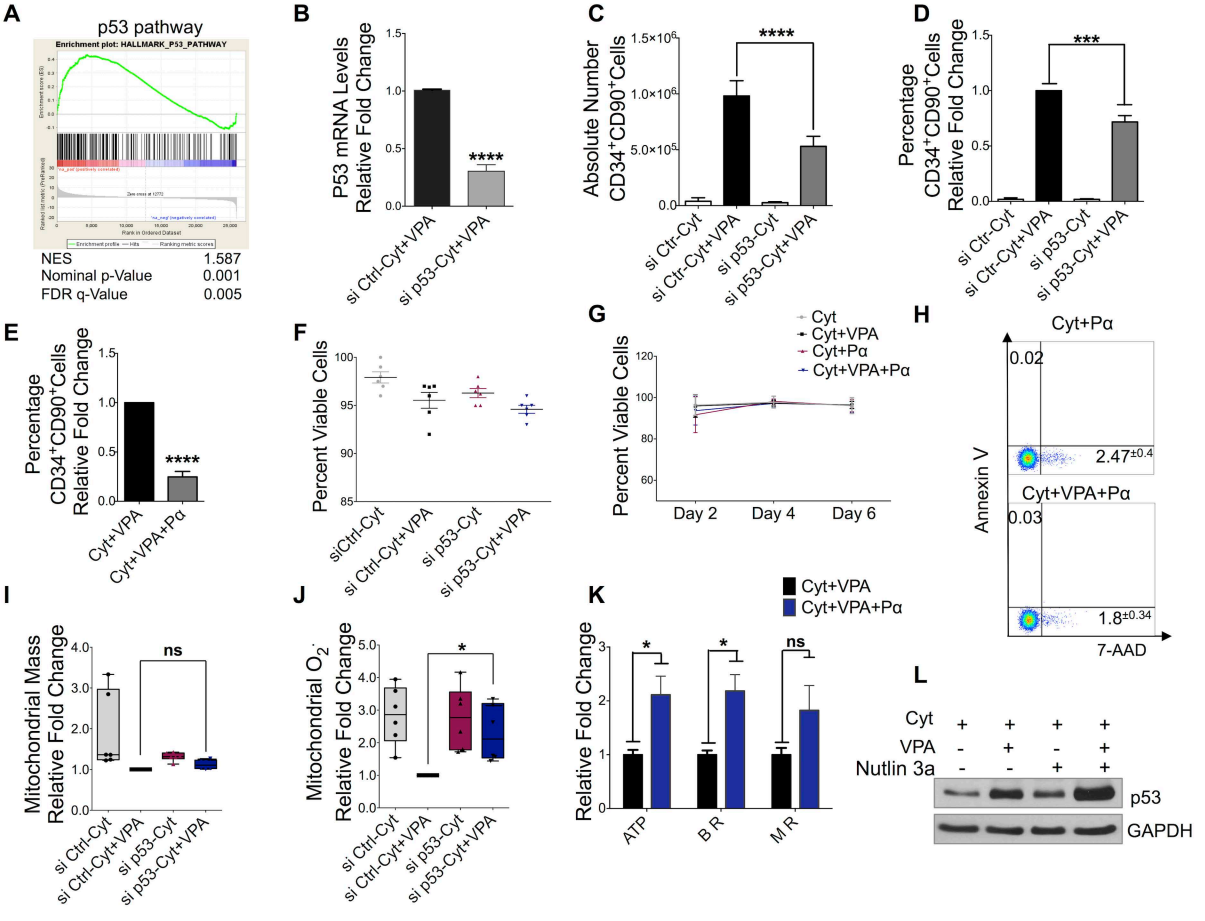


F

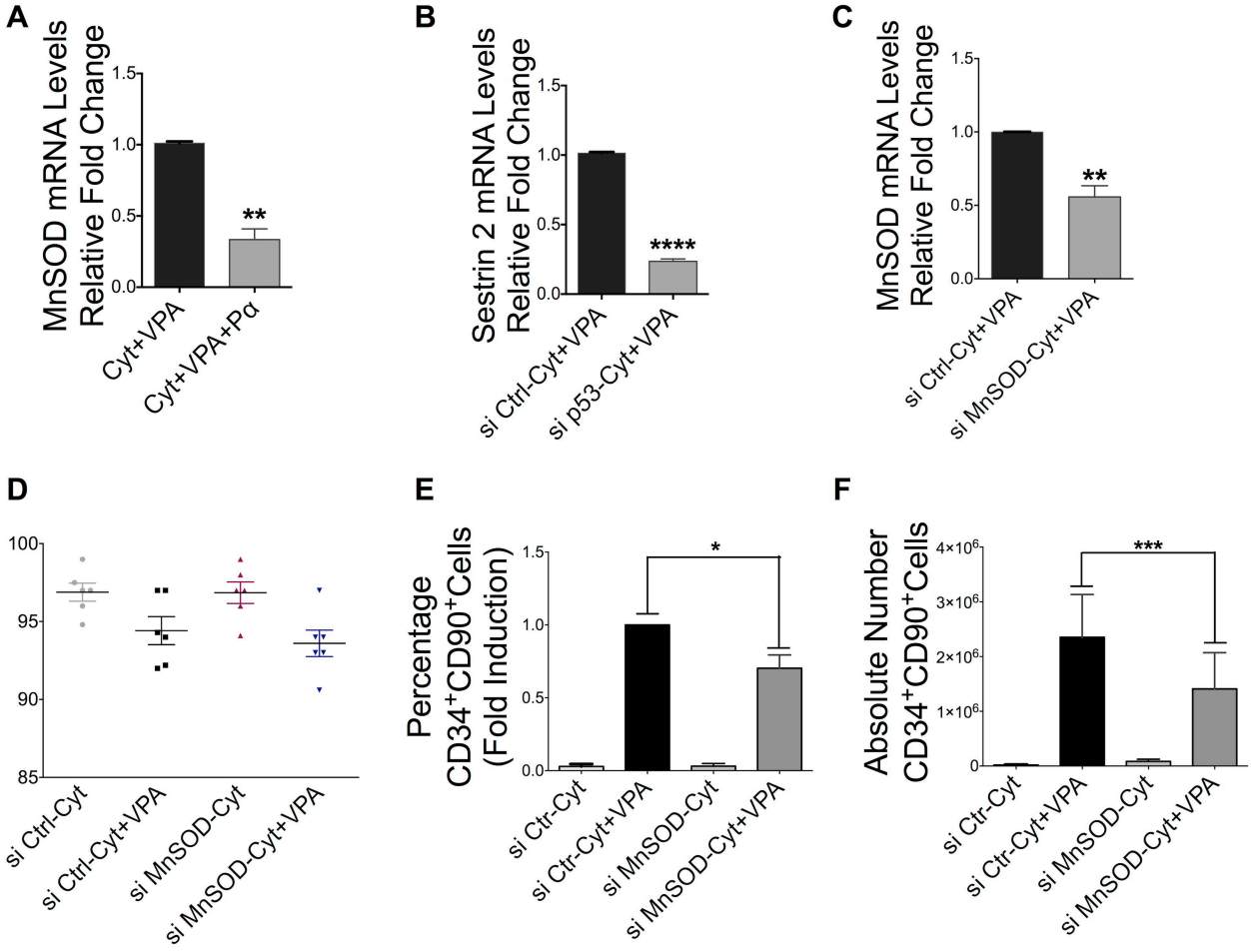
Gene Name	Fold Increase	p value
MCT1 (SLC16A1)	1.9	0.0027
MCT4 (SLC16A3)	3.88	3.092×10^{-8}



Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5