Supplemental Figure 1. Single-cell transcriptomic profiles of HSPCs and MNCs from fresh and cryopreserved UCB

(A) Cell viability of $CD34^+$ cells and mononuclear cells from fresh and cryopreserved UCB stored for 1, 5, 10, and 19 years before scRNA-seq. **(B)** UMAP plots displaying distributions of single cells from fresh and cryopreserved UCB stored for 1, 5, 10, and 19 years. **(C)** UMAP plots displaying distributions of single cells from HSPC and MNC. **(D-G)** UMAP plots displaying distributions of single cells from different storage years for (D) HSPCs, (E) T/NK cells, (F) B cells and (G) monocytes.

Supplemental Figure 2. Single-cell transcriptomic profiles of HSPCs and MNCs from fresh and cryopreserved UCB

(A) Bubble plot showing expression levels of signature genes in each cluster of HSPCs. **(B-C)** Cell distributions (B) and signature gene expressions (C) of 8 cell clusters of T/NK cells. **(D-E)** Cell distributions (D) and signature gene expressions (E) of 6 cell clusters of B cells. **(F-G)** Cell distributions (F) and signature gene expressions (G) of 8 cell clusters of monocytes. **(H)** Cellular abundances of 8 cell clusters of T/NK cells from fresh and cryopreserved UCB stored for 1, 5, 10, and 19 years (n = 4 replicates; error bars indicate mean ± SD). **(I)** Cellular abundances of 6 cell clusters of B cells from fresh and cryopreserved UCB stored for 1, 5, 10, and 19 years (n = 4 replicates; error bars indicate mean ± SD). **(J)** Cellular abundances of 8 cell clusters of monocytes from fresh and cryopreserved UCB stored for 1, 5, 10, and 19 years (n = 4 replicates; error bars indicate mean ± SD). **(K)** Cell distribution and cellular abundances of 9 cell

clusters of HSPCs from fresh and cryopreserved UCB collected from Shandong Qilu Stem Cell Engineering Co., Ltd. (n = 4 replicates; error bars indicate mean ± SD). **(L)** Representative flow cytometry plots and frequency of early apoptotic cells ($Annexin^+$ PI^-) and late apoptotic cells ($Annexin^+$ PI^+) in $CD34^+$ population from fresh and cryopreserved UCB (Y10) (n = 2 replicates; error bars indicate mean ± SEM; two-tailed t test).

Supplemental Figure 3. Transcriptomic variations occurred in UCB during cryopreservation

(A) Differences in number of DEGs between any two cryopreservation periods for HSPCs, T/NK cells, B cells, and monocytes. The dotted line represents the median of DEGs in the $Mito^{hi}$ subpopulations. **(B)** Differences in number of DEGs between two consecutive cryopreservation periods for T/NK cells (top), B cells (middle), and monocytes (bottom). **(C)** Bubble plot showing enriched GO terms of upregulated genes in HSPCs from fresh UCB (fresh UCB versus cryopreserved UCB, with UCB samples of different cryopreservation times regarded as a whole). **(D)** Differentiation potential towards MLP, GMP, and MEP lineages of HSCs from fresh and cryopreserved UCB, with UCB samples of different cryopreservation times regarded as a whole. **(E)** The number of DEGs between the HSPCs of fresh and cryopreserved UCB from Shandong Qilu Stem Cell Engineering Co., Ltd.. **(F)** Enriched GO terms of upregulated genes in $Mito^{hi}$ HSC/MPPs from fresh UCB (left) and cryopreserved UCB (right) from Shandong Qilu Stem Cell Engineering Co., Ltd.. **(G)** Percentages of HSC, MPP1,

MPP2, and Mito^{hi} HSC/MPP cells in different cell- cycle phases from fresh and cryopreserved UCB from Shandong Qilu Stem Cell Engineering Co., Ltd. (n = 4 replicates; error bars indicate mean ± SD). For each cluster, the proportions of cells in G2M phase at Y10 was compared with that of fresh UCB using the Chi-squared test (Supporting data values). **(H)** Relative expression of stemness signature genes in HSC, MPP1, MPP2, and Mito^{hi} HSC/MPP cells from fresh and cryopreserved UCB from Shandong Qilu Stem Cell Engineering Co., Ltd.. The stemness signature genes used for the analysis were provided in Data file S2. **(I)** Differentiation potential towards MLP, GMP and MEP lineages of HSCs (left) and Mito^{hi} HSC/MPPs (right) from fresh and cryopreserved UCB from Shandong Qilu Stem Cell Engineering Co., Ltd.

Supplemental Figure 4. Decreased functions of HSPCs after cryopreservation

(A) CFCs per 200 CD34$^+$ cells from fresh and cryopreserved UCB stored for different years with individual UCB (Fresh: 8 replicates; Y1 and Y5: 5 replicates; Y10: 12 replicates; Y19: 6 replicates). **(B)** Representative flow cytometry plots of human cell engraftment in BM of recipients 20 weeks after transplantation. **(C-D)** Percentage of human CD45$^+$ cell engrafted in spleen (SP) of (C) primary and (D) secondary recipients. **(E-F)** Lineage differentiation potential of human hematopoietic cells in (E) BM and (F) SP of primary (top) and secondary (bottom) recipients. B: hCD45$^+$CD19$^+$ B cells, M: hCD45$^+$CD33$^+$ myeloid cells, T: hCD45$^+$CD3$^+$ T cells. NK: hCD45$^+$CD56$^+$ NK cells. **(G)** CFCs per 200 CD34$^+$ cells from fresh and cryopreserved UCB (3 weeks) (5 replicates). **(H)** CFCs per 200 CD34$^+$ cells from fresh and cryopreserved UCB (Y10)

from Shandong Qilu Stem Cell Engineering Co., Ltd. Data from four independent UCB samples (4 replicates). **(I)** Flow chart of patient selection. **(J)** Proportion of engrafted NCG-X mice with successful platelet reconstruction. Successful engraftment was defined as > 0.1% human $CD41a^+$ platelets in recipient PB (4 pooled UCB samples, 4-7 recipient mice per group). **(K)** Number of human platelets in PB (10^3 platelets/μl) of recipients after 10 weeks transplantation. **(L)** Burst-forming unit-erythroid (BFU-E) per 200 $CD34^+$ cells from fresh and cryopreserved UCB (Fresh: 8 replicates; Y1: 5 replicates; Y5: 4 replicates; Y10: 12 replicates; Y19: 6 replicates). **(M)** Percentage of human $CD235a^+$ $CD71a^+$ cells engrafted in the BM of recipients (NCG-X mice). 4 pooled UCB samples; 6-9 recipient mice per group. For transplantation assay, UCB derived $CD34^+$ cells (40,000 /mouse) were injected via the tail vein.

(C-F) 4-6 pooled UCB samples; 15-24 recipient mice per group. **(C-H, K)** Error bars indicate mean ± SEM; two-tailed t test. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Supplemental Figure 5. T and NK cells derived from UCB were resistant to cryopreservation

(A) Relative expression levels of canonical naïve- and cytotoxicity-associated genes (*TCF7, CD27,* and *KLRD1*) in fresh and cryopreserved NK cells and T cells. Wilcoxon test. **, $p \leq 0.01$; ****, $p \leq 0.0001$. **(B)** CD69 expression in $CD3^+$ T cells, with or without human CD3/CD28 activator, from fresh and cryopreserved UCB stored for 10 years (n = 6 replicates, three independent experiments). **(C)** Histograms showing cell division count of $CD3^+$ T cells of fresh and cryopreserved UCB stored for 10 years over

the 8 days (d2-d7) following CD3/CD28 stimulation (n = 2 replicates, three independent experiments). **(D)** Flow cytometry plots depicting distinct subpopulations of $CD3^+CD8^+$ T cells at the 40-hour time point following CD3/CD28 activation. **(E)** Frequency of subpopulations of $CD3^+CD8^+$ T cells (n = 2 replicates, three independent experiments). **(F)** Expression of GZMB and perforin in expanded $CD3^-CD56^+$ cells, with or without PMA and ionomycin treatment, from fresh and cryopreserved UCB stored for 10 years (fresh, n = 4 replicates; Y10, n = 4 replicates. 3 independent experiments).

(B-C, E-F) Error bars indicate mean ± SEM; two-tailed t test. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

Supplemental Figure 6. Abnormal mitochondrial metabolism in cryopreserved HSPCs

(A) Relative expression of oxidative phosphorylation genes, mitochondrial respiratory chain complex assembly and reactive oxygen species biosynthetic process genes in $Mito^{hi}$ $CD4^+$ naive T cells from fresh and cryopreserved UCB. For each cluster: Y1, Y5, Y10, and Y19 samples were respectively compared with the fresh UCB using the Kruskal-Wallis test. **(B)** Representative flow cytometry plots of mitochondrial membrane potential of HSC/MPPs cells from fresh and cryopreserved UCB (Y10), as determined with TMRE staining. **(C)** Relative expression of mitochondria-related genes in fresh and cryopreserved (Y10) HSC/MPPs with low and high TMRE signals. Wilcoxon test. **(D)** Bubble plot showing upregulated gene sets in the HSC/MPP clusters

with low and high TMRE signals. NESs were obtained by GSEA analysis. **(E)** Representative flow cytometry plots of mitochondrial ROS levels of $CD34^+$ cells from fresh and cryopreserved UCB (Y5 and Y10). **(F)** Percentage of $MitoSox^+$ in $CD34^+$ cells from fresh and cryopreserved UCB stored for 3 weeks (2-3 replicates, two independent experiments). **(G)** ECR of $CD34^+$ cells from fresh and cryopreserved UCB (including Y5 and Y10; fresh, 3 pooled UCB, 6 technical replicates; Y5, 4 pooled UCB, 2 technical replicates ; Y10, 4 pooled UCB, 2 technical replicates). **(H)** Mitochondrial morphology, as analyzed by MiNA, of $CD34^+$ cells from fresh and cryopreserved UCB stored for 10 years: mean branches per network (left) and number of individuals (right). Fresh: 90 cells from 3 pooled UCB; Y10: 104 cells from 3 pooled UCB. **(I)** OCR of $CD3^+$ cells, induced by CD3/CD28 stimulation, from fresh and cryopreserved UCB (Y5 and Y10) (fresh, 3 pooled UCB, 4 technical replicates; Y10, 3 pooled UCB, 2 technical replicates).

(F-I) Error bars indicate mean ± SEM; two-tailed t test. *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$.

Supplemental Figure 7. Sulforaphane improved the function of cryopreserved UCB-derived HSPCs

(A) CFCs per 200 $CD34^+$ cells from cryopreserved UCB stored for 10 years cultured with different antioxidants for 40 hours, 4 replicants. Antioxidant concentration, Ctrl: same volume of DMSO; Nac: 0.1 mM; Res: 0.2 μM; SF: 2.5 μM; VitE: 10 mg/ml, 2 mg/ml, 1 mg/ml; CoQ10:10 nM, 5 nM, 1 nM. The red dot plot represents the mean

CFCs of the control group. **(B)** Representative flow cytometry plots of hCD45$^+$ and megakaryocyte engrafted in BM of recipients 12 weeks post-transplantation from UCB CD34$^+$ cells cryopreserved for 10 years (Y10), cultured with (right) or without (left) SF. **(C)** Percentage of human CD45$^+$ cells from UCB cryopreserved for fresh and 10 years (Y10) engrafted in spleen of mouse recipients at 12 weeks after transplantation in SF-untreated and SF-treated groups (2-4 pooled UCB samples; 6-10 recipient mice per group). **(D-E)** Lineage differentiation potential of human hematopoietic cells in (D) BM and (E) SP of recipients in SF-untreated and SF-treated group (B: hCD45$^+$CD19$^+$ B cells, M: hCD45$^+$CD33$^+$ myeloid cells, T: hCD45$^+$CD3$^+$ T cells. 2-4 pooled UCB samples; 6-10 recipient mice per group). **(F)** Number of human platelets in PB (10^2 platelets/μl) of recipients after 12 weeks transplantation. **(G)** Representative image of colony-forming cells of control and SF groups captured by Nikon Ti-E Living Cell Workstation. **(H)** Representative flow cytometry plots of mitochondrial ROS levels of CD34$^+$ cells from blank (MitoSox unstained), control and SF group (left). Percentage of MitoSox$^+$ cells in CD34$^+$ population from control and SF groups (right; 2 biological replicates).

(C-F) Error bars indicate mean ± SEM; two-tailed t test. *, $p \leq 0.05$.

Supplemental Table 1. Characteristics of the study cohort, related to Figure 3H.

Subject co-variates (n = 171)

Year at transplant, n (%)	
2015	41 (24)
2016	22 (13)
2017	22 (13)
2018	26 (15)
2019	28 (16)
2020	32 (19)
Age, years, median (range)	9.6 (0.7–55.4)
Male, n (%)	105 (61)
Leukemia state, n (%)	
CR1	95 (56)
Not CR1	76 (44)
HLA mismatch in GvHD direction, n (%)	
None	8 (5)
1 locus	25 (15)
2 loci	48 (28)
3 loci	48 (28)
4 loci	31 (18)
5 loci	10 (6)
6 loci	1 (1)

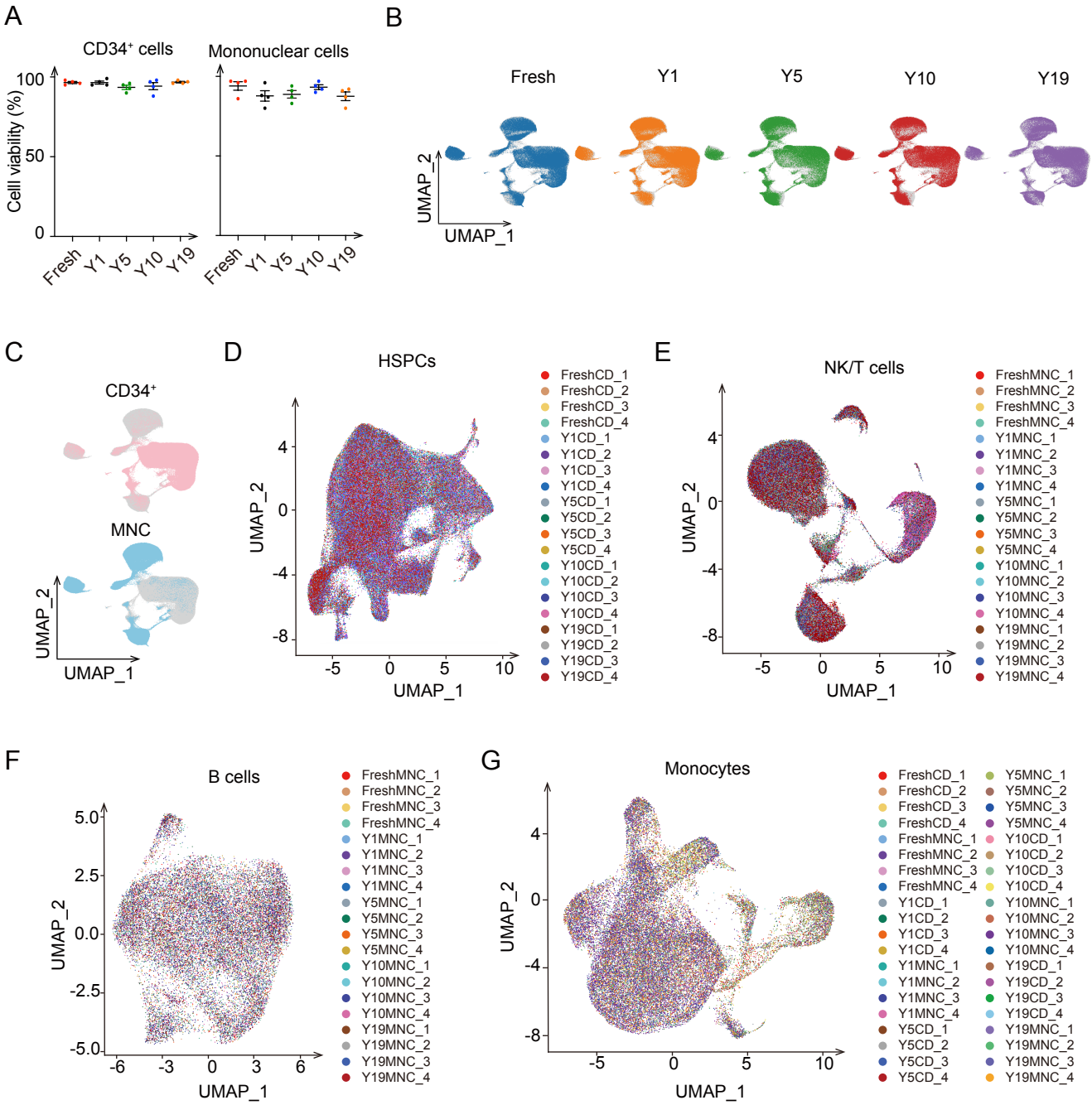
CD34-positive/BW cell dose, 10E+5 cells/kg, median (range)	4.2 (3.0 – 10.9)
Cryopreservation duration, years, median (range)	2.6 (0.8 – 13.6)

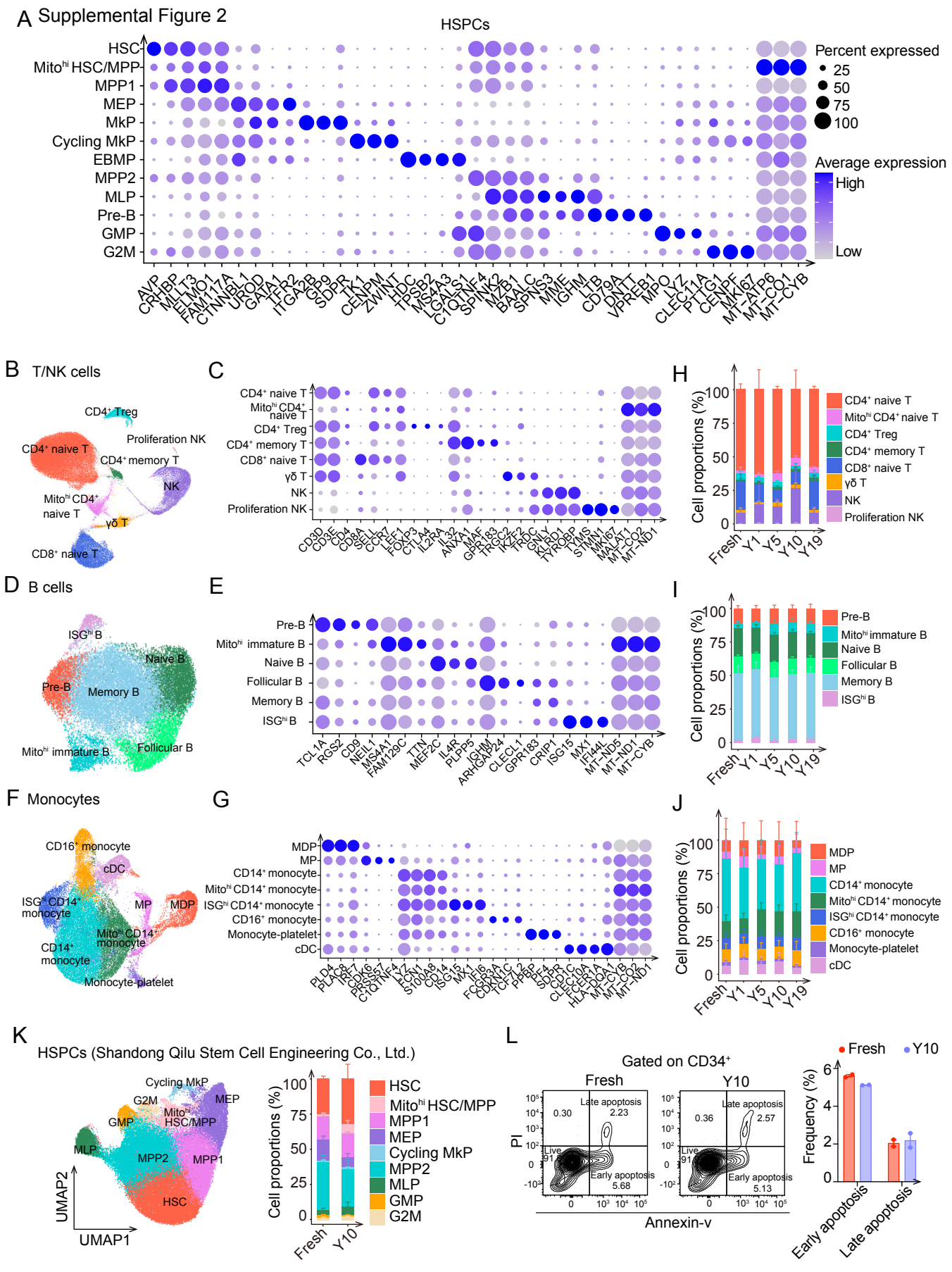
Abbreviations: BW, body weight; CR, complete remission; GvHD, graft-versus-host disease.

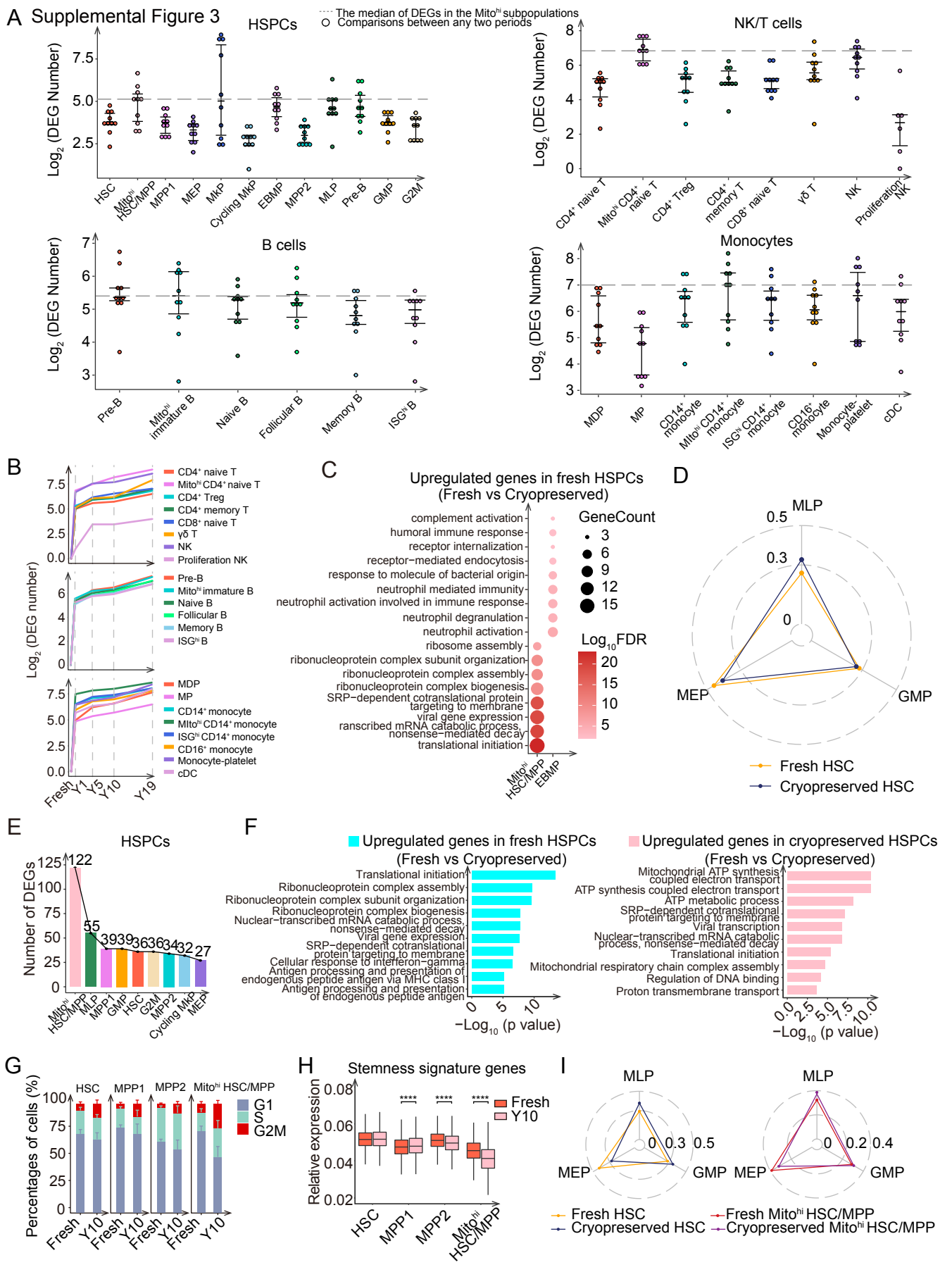
Data file S1. Signature genes of HSPCs, T/NK cells, B cells, and monocytes, related to Figure 1 and Supplemental Figure 1.

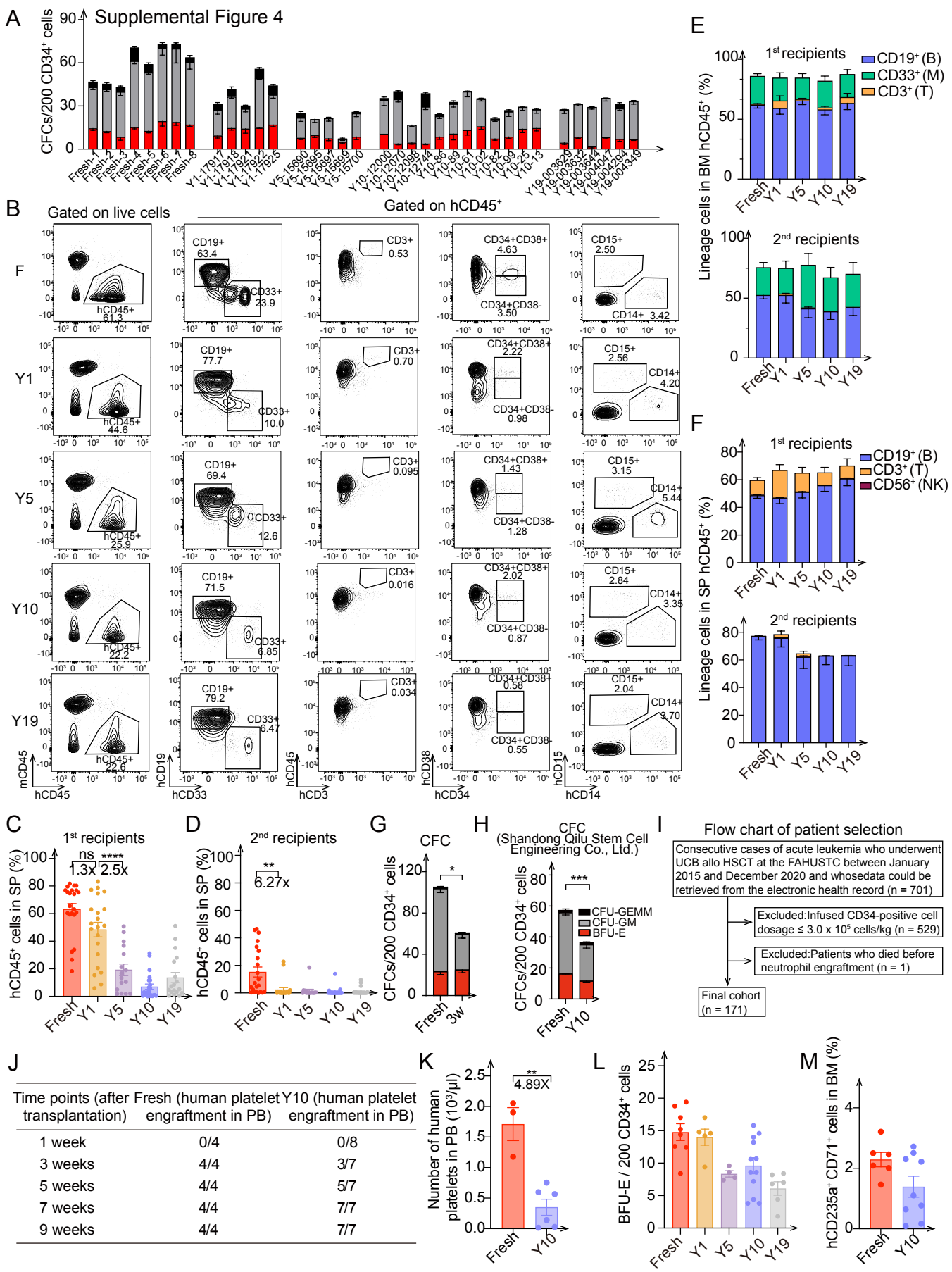
Data file S2. Genes included in stemness signature analysis, related to Figure 2D and Supplemental Figure 3H.

Supplemental Figure 1



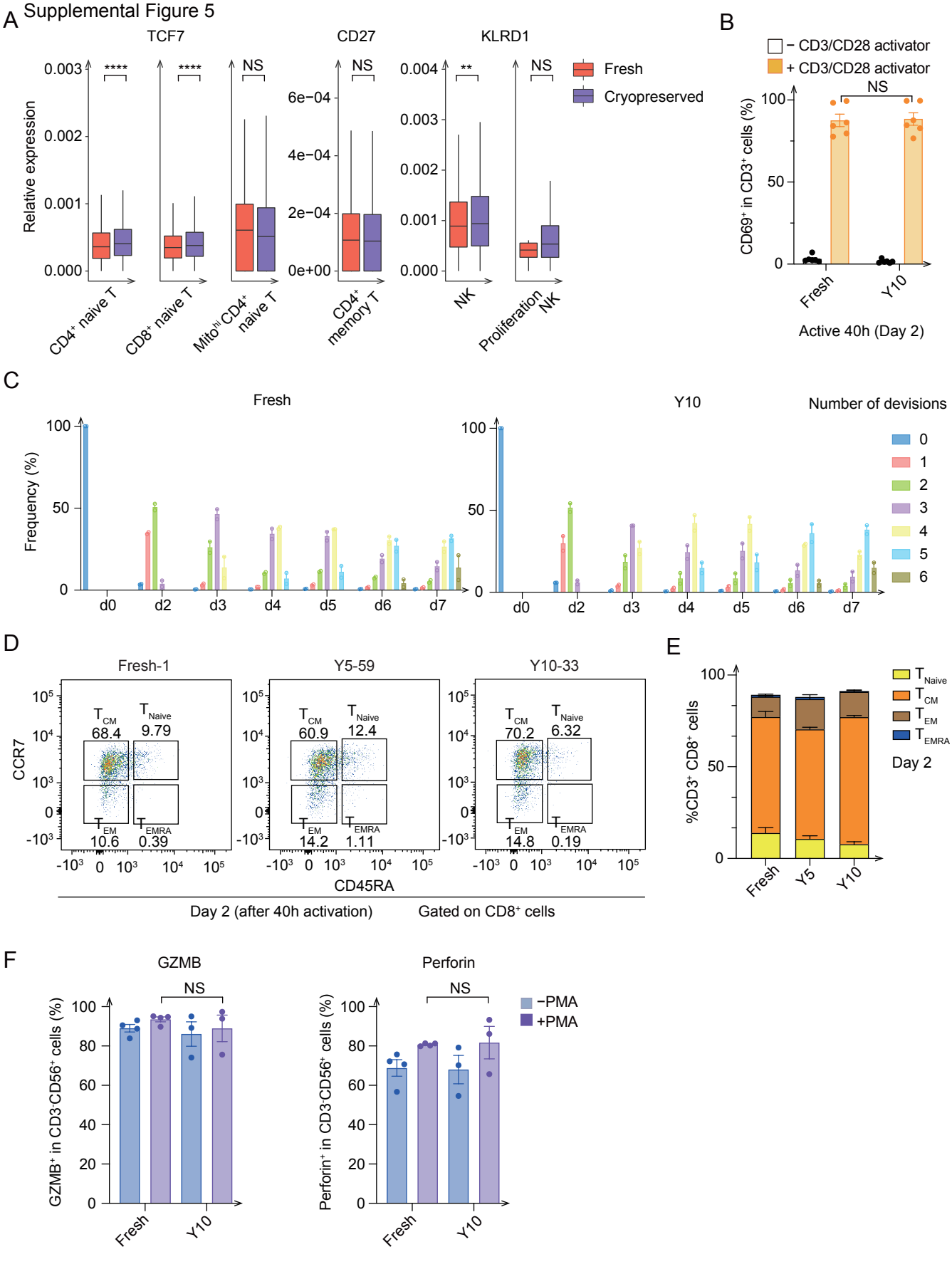

A Supplemental Figure 2
HSPCs
HSC
Mitohi HSC/MPP
MPP1
MEP
MkP
Cycling MkP
EBMP
MPP2
MLP
Pre-B
GMP
G2M
AVP CRHBP MLLT3 MEIS1 FAM117A CTNNBL1 UROD GATA1 TFR2 ITGA2B GP9 SDPR TK1 CENPM ZWINT HDC TPSB2 MS4A3 LGALS1 C1QTNF4 SPINK2 MZB1 BAALC SPNS3 MME IGHM LTB CD79A DNTT VPREB1 MPO LYZ CLEC11A PTTG1 CENPF MKI67 MT-ATP6 MT-CO1 MT-CYB
Percent expressed
25
50
75
100
Average expression
High
Low
B T/NK cells
CD4+ Treg
Proliferation NK
CD4+ naive T
CD4+ memory T
NK
Mitohi CD4+ naive T
γδ T
CD8+ naive T
C
CD4+ naive T
Mitohi CD4+ naive T
CD4+ Treg
CD4+ memory T
CD8+ naive T
γδ T
NK
Proliferation NK
CD3D CD3E CD4 CD8A SELL CCR7 LEF1 FOXP3 CTLA4 IL2RA IL32 ANXA1 MAF GPR183 TRGC2 IKZF2 TRDC GNLY KLRD1 TYROBP LYMS STMN1 MKI67 MALAT1 MT-CO2 MT-ND1
H
Cell proportions (%)
100
75
50
25
0
Fresh Y1 Y5 Y10 Y19
CD4+ naive T
Mitohi CD4+ naive T
CD4+ Treg
CD4+ memory T
CD8+ naive T
γδ T
NK
Proliferation NK
D B cells
ISGhi B
Naive B
Pre-B
Memory B
Mitohi immature B
Follicular B
E
Pre-B
Mitohi immature B
Naive B
Follicular B
Memory B
ISGhi B
TCL1A RGS2 CD9 NEIL1 MS4A1 FAM129C TTN MEF2C IL4R PLPP5 IGHM ARHGAP24 CLEC1L GPR183 CRIP1 ISG15 MX1 IFI44L MT-ND5 MT-ND1 MT-CYB
I
Cell proportions (%)
100
75
50
25
0
Fresh Y1 Y5 Y10 Y19
Pre-B
Mitohi immature B
Naive B
Follicular B
Memory B
ISGhi B
F Monocytes
CD16+ monocyte
cDC
ISGhi CD14+ monocyte
MP
MDP
Mitohi CD14+ monocyte
CD14+ monocyte
Monocyte-platelet
G
MDP
MP
CD14+ monocyte
Mitohi CD14+ monocyte
ISGhi CD14+ monocyte
CD16+ monocyte
Monocyte-platelet
cDC
PLD4 PLAC8 IRF7 CDK6 PRSS57 C1QTNF4 VCAN ECM1 S100A8 CD14 ISG15 MX1 IFI6 FCGR3A CDKN1C TCF7L2 PPBP PF4 SDPR CD1C FCER1A HLA-DQA1 MT-CYB MT-ND1
J
Cell proportions (%)
100
75
50
25
0
Fresh Y1 Y5 Y10 Y19
MDP
MP
CD14+ monocyte
Mitohi CD14+ monocyte
ISGhi CD14+ monocyte
CD16+ monocyte
Monocyte-platelet
cDC
K
HSPCs (Shandong Qilu Stem Cell Engineering Co., Ltd.)
Cycling MkP
G2M
MEP
Mitohi HSC/MPP
GMP
MLP
MPP2
MPP1
HSC
UMAP2
UMAP1
Cell proportions (%)
100
75
50
25
0
Fresh Y10
HSC
Mitohi HSC/MPP
MPP1
MEP
Cycling MkP
MPP2
MLP
GMP
G2M
L
Gated on CD34+
Fresh
Y10
PI
Annexin-v
Late apoptosis
Early apoptosis
Live
0.30
2.23
91
5.68
0.36
2.57
91
5.13
Fresh
Y10
Frequency (%)
6
4
2
0
Early apoptosis
Late apoptosis


A Supplemental Figure 3
HSPCs
The median of DEGs in the Mitohi subpopulations
Comparisons between any two periods
Log2 (DEG Number)
HSC
Mitohi HSC/MPP
MPP1
MEP
MkP
Cycling MkP
EBMP
MPP2
MLP
Pre-B
GMP
G2M
NK/T cells
CD4+ naive T
Mitohi CD4+ naive T
CD4+ Treg
CD4+ memory T
CD8+ naive T
γδ T
NK
Proliferation NK
B cells
Pre-B
Mitohi immature B
Naive B
Follicular B
Memory B
ISGhi B
Monocytes
MDP
MP
CD14+ monocyte
Mitohi CD14+ monocyte
ISGhi CD14+ monocyte
CD16+ monocyte
Monocyte-platelet
cDC
B
Log2 (DEG number)
Fresh
Y1
Y5
Y10
Y19
C
Upregulated genes in fresh HSPCs
(Fresh vs Cryopreserved)
complement activation
humoral immune response
receptor internalization
receptor−mediated endocytosis
response to molecule of bacterial origin
neutrophil mediated immunity
neutrophil activation involved in immune response
neutrophil degranulation
neutrophil activation
ribosome assembly
ribonucleoprotein complex subunit organization
ribonucleoprotein complex assembly
ribonucleoprotein complex biogenesis
SRP−dependent cotranslational protein targeting to membrane
viral gene expression
ranscribed mRNA catabolic process, nonsense−mediated decay
translational initiation
GeneCount
Log10FDR
Mitohi HSC/MPP
EBMP
D
MLP
MEP
GMP
Fresh HSC
Cryopreserved HSC
E
HSPCs
Number of DEGs
122
55
39
39
36
36
34
32
27
Mitohi HSC/MPP
MLP
MPP1
GMP
HSC
G2M
MPP2
Cycling MkP
MEP
F
Upregulated genes in fresh HSPCs
(Fresh vs Cryopreserved)
Translational initiation
Ribonucleoprotein complex assembly
Ribonucleoprotein complex subunit organization
Ribonucleoprotein complex biogenesis
Nuclear−transcribed mRNA catabolic process, nonsense−mediated decay
Viral gene expression
SRP−dependent cotranslational protein targeting to membrane
Cellular response to interferon−gamma
Antigen processing and presentation of endogenous peptide antigen via MHC class I
Antigen processing and presentation of endogenous peptide antigen
−Log10 (p value)
Upregulated genes in cryopreserved HSPCs
(Fresh vs Cryopreserved)
Mitochondrial ATP synthesis coupled electron transport
ATP synthesis coupled electron transport
ATP metabolic process
SRP−dependent cotranslational protein targeting to membrane
Viral transcription
Nuclear−transcribed mRNA catabolic process, nonsense−mediated decay
Translational initiation
Mitochondrial respiratory chain complex assembly
Regulation of DNA binding
Proton transmembrane transport
−Log10 (p value)
G
Percentages of cells (%)
HSC
MPP1
MPP2
Mitohi HSC/MPP
G1
S
G2M
Fresh
Y10
H
Stemness signature genes
Relative expression
****
Fresh
Y10
HSC
MPP1
MPP2
Mitohi HSC/MPP
I
MLP
MEP
GMP
Fresh HSC
Cryopreserved HSC
Fresh Mitohi HSC/MPP
Cryopreserved Mitohi HSC/MPP

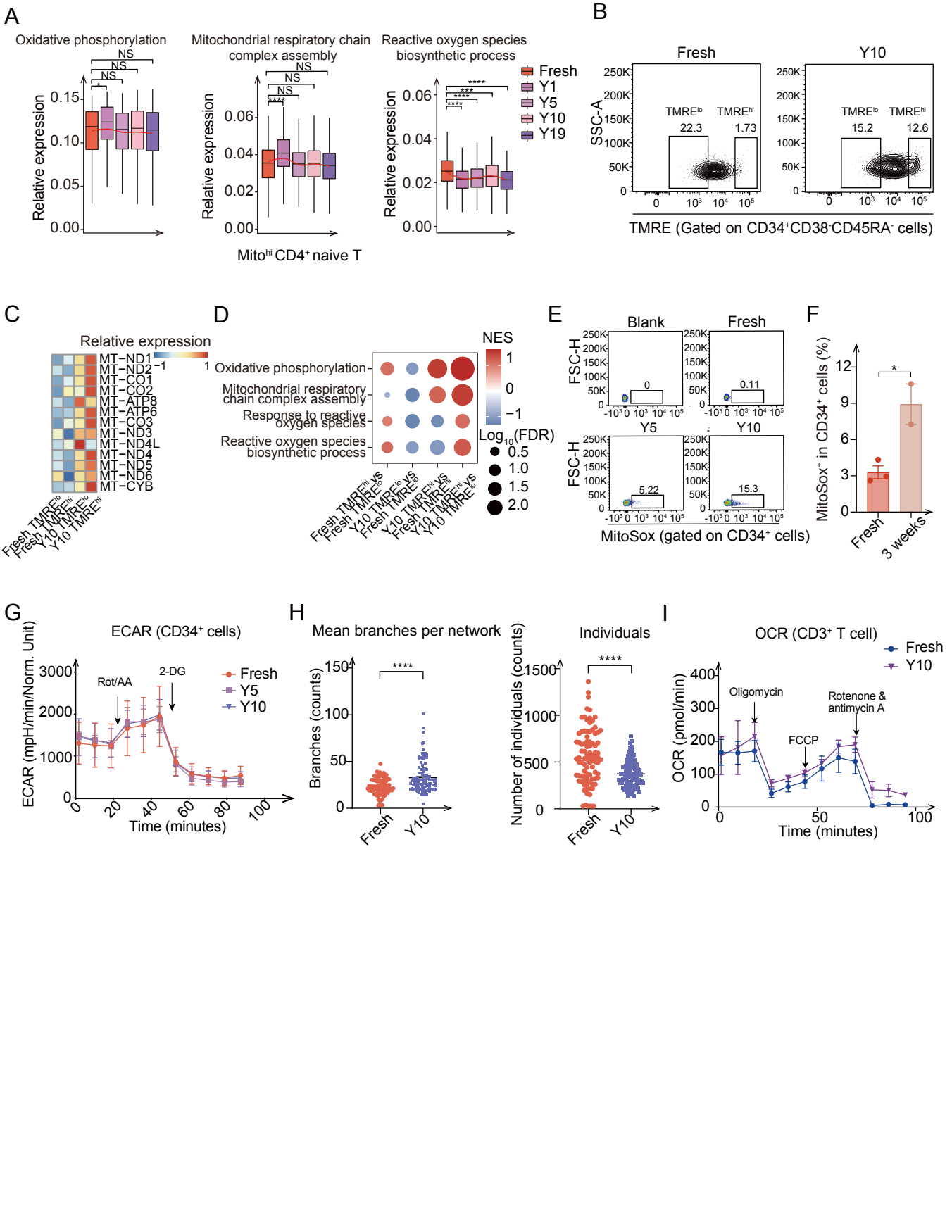


Time points (after transplantation)	Fresh (human platelet engraftment in PB)	Y10 (human platelet engraftment in PB)
1 week	0/4	0/8
3 weeks	4/4	3/7
5 weeks	4/4	5/7
7 weeks	4/4	7/7
9 weeks	4/4	7/7

Supplemental Figure 5



Supplemental Figure 6



Supplemental Figure 7

