ARTICLE - Hematopoietic Stem Cell

The glycolytic enzyme PFKFB3 determines bone marrow endothelial progenitor cell damage after chemotherapy and irradiation

Zhong-Shi Lyu,[1,2] Shu-Qian Tang,[1] Tong Xing,[1,2] Yang Zhou,[1] Meng Lv,[1] Hai-Xia Fu,[1] Yu Wang,[1] Lan-Ping Xu,[1] Xiao-Hui Zhang,[1] Hsiang-Ying Lee,[2,3] Yuan Kong[1] and Xiao-Jun Huang[1,2]

[1]Peking University People's Hospital, Peking University Institute of Hematology, National Clinical Research Center for Hematologic Disease, Beijing Key Laboratory of Hematopoietic Stem Cell Transplantation, Collaborative Innovation Center of Hematology, Peking University; [2]Peking-Tsinghua Center for Life Sciences, Academy for Advanced Interdisciplinary Studies, Peking University and [3]School of Life Sciences, Peking University, Beijing, China

Correspondence: XJ. Huag
huangxiaojun@bjmu.edu.cn
Y. Kong
yuankong@bjmu.edu.cn

Received: August 2, 2021.
Accepted: March 24, 2022.
Prepublished: MArch 31, 2022.

https://doi.org/10.3324/haematol.2021.279756

Supplementary Materials

Glycolytic enzyme PFKFB3 determines bone marrow endothelial progenitor cell damage post chemotherapy and irradiation

Authors: Zhong-Shi Lyu, Shu-Qian Tang, Tong Xing, Yang Zhou, Meng Lv, Hai-Xia Fu, Yu Wang, Lan-Ping Xu, Xiao-Hui Zhang, Hsiang-Ying Lee, Yuan Kong[#], Xiao-Jun Huang[#]

[#]XJH and YK are co-correspondence authors.

Supplementary methods

PFKFB3 expression analysis in primary bone marrow(BM) endothelial progenitor cells(EPCs)

To evaluate PFKFB3 level in BM EPCs, BM mononuclear cells(BMMNCs) were isolated from poor graft function(PGF), good graft function(GGF) and healthy donors(HDs) and incubated with BM EPC markers(CD34, CD45, vascular endothelial growth factor receptor 2(CD309) and CD133)[1, 2] at 4°C for 30 minutes and then fixed, permeabilized, and incubated with an antibody against PFKFB3(Abcam, Cambridge, MA). PFKFB3 level was analyzed using LSRFortessa software(Becton Dickinson) and presented as the mean fluorescence intensity(MFI)(mean±SEM). Aliquots of isotype-identical antibodies served as negative controls.

Cultivation, characterization and functional analyses of primary BM EPCs

BMMNCs from PGF, GGF and HDs were cultured in fibronectin(Sigma, St. Louis,

MO) precoated culture plates with EGM-2-MV-SingleQuots(Lonza, Walkersville, MD) and 10% fetal bovine serum(Gibco, Rockville, MD) at 37°C in a humidified incubator with 5% CO_2 for 7 days and characterized according to the above EPC markers using an LSRFortessa(Becton Dickinson).

The adherent human BM EPCs at day 7 of cultivation were gently detached with trypsin with 0.25% EDTA(Gibco) and stained with trypan blue(Solarbio, Beijing, China). The number of living cells per well was counted by independent blinded investigators.

As described previously[1-3], to detect the levels of apoptosis, the cultivated human BM EPCs with indicated treatment were incubated with the EPC markers(CD34, CD45, vascular endothelial growth factor receptor 2(CD309) and CD133), and then stained with the Annexin-V and 7-amino-actinomycin D Apoptosis Detection Kit(Becton Dickinson) and analyzed by flow cytometry.

As described previously[1-3], to detect the levels of reactive oxygen species (ROS), the cultivated human BM EPCs with indicated treatment, were stained with the above EPC markers, and then incubated with 10 mM 2',7'-dichlorofluorescence diacetate(Beyotime Biotechnology, China) at 37°C for 15 minutes. The mean fluorescence intensity(MFI) of intracellular ROS was analyzed by flow cytometry,

Cell migration of BM EPCs was followed by the protocols described previously[1-3]. Briefly, Cell migration assay was performed via a transwell chamber(Corning, NY, USA). The cells(5×10^4 cells per well) in culture medium without fetal bovine serum were seeded to the upper chambers, while 500 μL of complete medium

with 10% fetal bovine serum was added to the lower chambers. After 24 hours, the migrated cells were fixed with paraformaldehyde for 30 minutes, and then stained with crystal violet for 20 minutes. The migrated cells were counted in 6 random fields/sample manually with a phase-contrast microscope(Olympus).

As described previously[1-3], to detect the levels of tube formation, the cultivated human BM EPCs with indicated treatment(5×10^4 cells per well) were transferred to the Matrigel(Corning, NY)-precoated plates for 48 hours at 37°C in 5% CO_2. Tube formation was then analyzed via an inverted light microscope and Image Proplus.

As described previously[1-3], to perform DiI-AcLDL and FITC-UEA-1 double-staining assay, cultivated BM EPCs were washed 3 times with phosphate-buffered saline(1×PBS) and then incubated with 10 mg/mL DiI-Acetylated Low Density Lipoprotein(DiI-AcLDL; Life Technologies) at 37°C for 4 hours, the cells were then washed and fixed in 4% paraformaldehyde for another 10 minutes. The fixed cells were then washed and incubated for 1 hour with 10 mg/mL fluorescein isothiocyanate–labeled Ulex Europaeus Agglutinin-1(FITC-UEA-1, Sigma) at room temperature. The numbers of DiI-AcLDL and FITC-UEA-1 double positive staining cells per well was then analyzed via a fluorescence microscope(Olympus, Tokyo, Japan).

Coculture of BM $CD34^+$ cells with BM EPCs for colony-forming unit(CFU) assays

As described previously[2-4], BM $CD34^+$ cells were isolated from BMMNCs from HDs via a CD34 MicroBead Kit(Miltenyi Biotec, Bergisch Gladbach, Germany)

and then cocultured with pretreated BM EPCs for 4 days in StemSpan™ SFEM(Stem Cell Technologies, Vancouver, BC, Canada).

CFU assays were performed with MethoCult™ H4434 Classic(Stem Cell Technologies). Cocultured $CD34^+$ cells were collected and re-plated in 24-well plates and cultured in MethoCult™ H4434 Classic for another 14 days. Colony-forming unit erythroid(CFU-E), burst-forming unit erythroid(BFU-E), colony-forming unit-granulocyte/macrophage(CFU-GM), and colony-forming unit-granulocyte, erythroid, macrophage and megakaryocyte(CFU-GEMM) values were determined by viewing the cultures using an inverted light microscope. Cultures were assayed in triplicate, and the results are expressed as mean ± SEM.

Immunofluorescence analysis of the BM microenvironment

BM trephine biopsies(BMBs) were obtained from the posterior superior iliac spine and then fixed with 4% paraformaldehyde, decalcified, embedded with Optical Cutting Temperature(OCT) Compound(SAKURA, Tissue-Tek) and sectioned for immunofluorescence staining. Sections were air-dried, washed, and blocked with 20% goat serum at room temperature(RT) for 1 h. Incubation with mouse anti-human CD34(Becton Dickinson Biosciences, San Jose, CA) and rabbit anti-human PFKFB3(Abcam, Cambridge, MA) was performed at 4°C overnight. After washing 3 times with 1x phosphate buffered saline(PBS), the sections were then incubated with goat anti-rabbit 488 and donkey anti-mouse 555(Invitrogen, Eugene, OR) and 49,6-diamidino-2-phenylindole(DAPI). Fluorescence images were collected and analyzed using a LeicaTCS SP8

microscope(Leica Microsystems, Wetzlar, Germany).

Analysis of glucose consumption and lactic acid production

The levels of glucose and lactic acid in culture medium were measured by a glucose assay kit and a lactic acid assay kit(Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In brief, the cells were counted, and the media were collected for the detection of glucose and lactic acid concentrations. The results were normalized to cell numbers.

Transfection of primary BM EPCs from HDs

BM EPCs from HDs were plated at 2×10^5 cells/60-mm dish 24h before transfection. Lipofectamine 3000(Invitrogen, Carlsbad, CA, USA) was used to transfect PFKFB3 overexpression plasmids or control plasmids or small interfering RNAs(siRNAs)(200 nM) targeting *PFKFB3* or small interfering RNAs(siRNAs)(100 nM) targeting *FOXO3A* or non-targeting siRNA controls into cells. The transfection efficiency was confirmed by western blot assay.

siRNAs target sequence

The siRNAs target sequence of human *PFKFB3* :5′-ACCCGCTCATGAGACGCAATA-3′. The siRNAs target sequence of human *FOXO3A*: 5′- GAGCTCTTGGTGGATCATC-3′. These siRNAs were purchased from Guangzhou Ruibo. Biotech Co., Ltd. (Guangzhou, China).

RNA sequencing(RNA-seq) and data analysis

Based on fluorescence-activated cell sorting analysis, BM EPCs($CD34^+$ $CD309^+CD133^+$)[1, 2] were sorted from BMMNCs of PGF(N=5) and their matched

GGF(N=5) patients by Aria III(Becton Dickinson). The sorted BM EPCs were then subjected to RNA-seq analyses as reported previously[5]. Differential gene expression between BM EPCs of PGF(PGF EPCs) and GGF EPCs was analyzed by the DESeq2 package in R(1.16.1). Statistical overrepresentation testing of differentially expressed genes was performed via the protein analysis through evolutionary relationships(PANTHER)Classification System[6]. Kyoto Encyclopedia of Genes and Genomes(KEGG) metabolism pathway enrichment analysis was performed using the DAVID Bioinformatics Resources 6.7[7] and the ggplot228 packages implemented in the omicsbean workbench. A *P*-value<0.05 was set as the cutoff for the above statistical analysis.

Real-time quantitative polymerase chain reaction(qRT-PCR)

For qRT-PCR, RNA was extracted using the RNeasy Mini kit(QIAGEN, Germany). One microgram of RNA was reverse transcribed into cDNA by the RT reagent Kit with gDNA Eraser(TaKaRa, Japan). The mRNA levels of *VCAM1*, *ICAM1*, *SELE* were detected by the SYBR-Green qRT-PCR kit(Thermo Fisher Scientific, USA) and normalized relative to the 18S mRNA levels.

The relative mRNA levels of *VCAM1*(forward primer: 5′-CAGGCTGGAGATAGACTTACTG-3′; reverse primer: 5′-CCTCAATGACAGGAGTAAAGGT-3′), *ICAM1*(forward primer: 5′-TGCAAGAAGATAGCCAACCAAT-3′; reverse primer: 5′-GTACACGGTGAGGAAGGTTTTA-3′), *SELE*(forward primer: 5′-TGGAACACAACCTGTACATTTG -3′; reverse primer: 5′-AATTCCCAGATGAGGTACACTG -3′), *CXCL12*(forward primer: 5′-

CTCCAAACTGTGCCCTTCAGA -3′; reverse primer: 5′-GCCCTTCCCTAACACTGGTT -3′) and *KITLG*(forward primer: 5′-GTGGCAAATCTTCCAAAAGACT-3′; reverse primer: 5′-CCATCTCGCTTATCCAACAATG-3′) in the cultivated BM EPCs transfected with a control vector or a vector expressing PFKFB3 and with or without 5FU treatment(N=6) were analyzed. Normalized levels of the *VCAM1, ICAM1* and *SELE* ratios in the qRT-PCR assays were evaluated through comparisons with the *18S* levels(forward primer: 5′- GTAACCCGTTGAACCCCATT-3′; reverse primer: 5′-CCATCCAATCGGTAGTAGCG-3′).

Western blot analysis

Proteins from BM EPCs were extracted and immunoblot analyses were performed with the antibodies against PFKFB3(Abcam), FOXO3A(CST), p21(CST), p27(CST), NF-κB p65(CST), β-actin(CST), phospho-NF-κB p65(CST), FAS(Proteintech, Wuhan, China), E-selectin(Proteintech) and α-tubulin(Sigma). The detailed antibody information could be found in Supplementary Table 2.

Establishment of a BM EC-specific PFKFB3 overexpression murine model via AAV-mediated gene delivery system and intraosseous injection

To generate a BM EC-specific PFKFB3 overexpression murine model, AAV-mediated gene delivery system, a well-established tool for *in vivo* gene transfer safely and effectively[8], was used. Adult C57BL/6J female mice(8-10 weeks) received a single dose of a recombinant AAV-V_{EC}(an optimized AAV variant for EC transduction[9], Hanbio Biotechnology Co., Ltd. Shanghai, China) encoding

PFKFB3 and Zsgreen gene under the control of EC-specific Tie promoter[10](intraosseous injection[11] with a dose of 1×10^{13}vg/mL, 30 μL per femur), control mice(age- and sex-matched) received AAV-V_{EC} that encoded Zsgreen gene under the control of Tie promoter.

To evaluate the effects of BM EC-specific PFKFB3 overexpression and pharmacological inhibition of PFKFB3 on BM EC damage, mice were treated with 5FU(250 mg/kg) at day 0 and then with DMSO or 3PO(25 mg/kg; Sigma) at days 3, 5, and 7. The kinetics of peripheral blood(PB) was analyzed. BM from mouse femurs and tibias was stained with the markers of hematopoietic stem-cell(HSC(lineage$^-$cKIT$^+$SCA1$^+$CD150$^+$CD48$^-$)), Hematopoietic stem and progenitor cell(HSPC(lineage$^-$cKIT$^+$SCA1$^+$)), myeloid progenitor (lineage$^-$cKIT$^+$SCA1$^-$)[12], myeloid cell, T, B cell and EC(CD45$^-$Ter119$^-$CD31$^+$VE-Cadherin$^+$) using the antibodies listed in supplementary methods. Intracellular levels of FOXO3A(Cell Signaling Technology, Danvers, MA), NF-κB p65(CST) and PFKFB3(Abcam) in BM ECs of mice were analyzed by flow cytometry.

Hematoxylin and eosin(H&E) and immunohistochemistry(IHC) staining of EC marker Endomucin(Emcn) were used for histopathological analysis of BM in all mice.

All mouse experiments were approved by the Ethics Committee of Peking University People's Hospital.

HSC, HSPC, myeloid cell, T, B cell and BM EC surface markers in mice

HSC, HSPC, myeloid progenitor, myeloid cell, T, B cell and BM EC surface markers were analyzed using the following antibodies: HSC/HSPC: cKIT

(Biolegend), SCA1(Biolegend), CD150(Biolegend), CD48(Biolegend) and lineage(Biolegend). Myeloid progenitor: cKIT(Biolegend), SCA1(Biolegend) and lineage(Biolegend). Myeloid cell:CD45(Biolegend),CD11B(Biolegend), GR1(Biolegend). T, B cell: CD45(Biolegend), B220(Biolegend), CD3(Biolegend). BM EC: CD45(Biolegend), TER119(Biolegend), CD31(Biolegend), VE-Cadherin(Becton Dickinson).

References

1. Kong Y, Cao XN, Zhang XH, et al. Atorvastatin enhances bone marrow endothelial cell function in corticosteroid-resistant immune thrombocytopenia patients. Blood. 2018;131(11):1219-1233.

2. Shi MM, Kong Y, Song Y, et al. Atorvastatin enhances endothelial cell function in posttransplant poor graft function. Blood. 2016;128(25):2988-2999.

3. Lyu ZS, Cao XN, Wen Q, et al. Autophagy in endothelial cells regulates their haematopoiesis-supporting ability. EBioMedicine. 2020;53(102677.

4. Zhao HY, Zhang YY, Xing T, et al. M2 macrophages, but not M1 macrophages, support megakaryopoiesis by upregulating PI3K-AKT pathway activity. Signal Transduct Target Ther. 2021;6(1):234.

5. Chen J, Suo S, Tam PP, Han JJ, Peng G, Jing N. Spatial transcriptomic analysis of cryosectioned tissue samples with Geo-seq. Nat Protoc. 2017;12(3):566-580.

6. Mi H, Muruganujan A, Huang X, et al. Protocol Update for large-scale genome and gene function analysis with the PANTHER classification system (v.14.0). Nat Protoc. 2019;14(3):703-721.

7. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 2009;37(1):1-13.

8. Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. Nat Rev Drug Discov. 2019;18(5):358-378.

9. Zhang L, Rossi A, Lange L, et al. Capsid Engineering Overcomes Barriers Toward Adeno-Associated Virus Vector-Mediated Transduction of Endothelial Cells. Hum Gene Ther. 2019;30(10):1284-1296.

10. Gustafsson E, Brakebusch C, Hietanen K, Fassler R. Tie-1-directed expression of Cre recombinase in endothelial cells of embryoid bodies and transgenic mice. J Cell Sci. 2001;114(Pt 4):671-676.

11. Selenica ML, Reid P, Pena G, et al. Adeno associated viral-mediated intraosseous labeling of bone marrow derived cells for CNS tracking. J Immunol Methods. 2016;432(51-56.

12. Spevak CC, Elias HK, Kannan L, et al. Hematopoietic Stem and Progenitor Cells Exhibit Stage-Specific Translational Programs via mTOR- and CDK1-Dependent Mechanisms. Cell Stem Cell. 2020;26(5):755-765 e757.

Supplementary Table 1. Characteristics of allo-HSCT patients with PGF and GGF

Characteristics	PGF* (N=15)	GGF* (N=30)	*P*-Value**
BM evaluated time (post-HSCT days)	90(30-240)	90(30-240)	0.87
Blood cell count			
Median WBC ($\times10^9$/L) (range)	2.27(1.20-5.18)	4.33(2.3-16.07)	<0.0001
Median ANC ($\times10^9$/L) (range)	1.50(0.00-4.14)	2.65(0.89-14.61)	0.002
Median Hb (g/L) (range)	81(66-123)	100(74-134)	0.001
Median PLT ($\times10^9$/L) (range)	24(10-60)	106(35-214)	<0.0001
Age at HSCT (years, median, range)	45(11-65)	42(12-62)	0.70
Gender (male/female)	6/9	20/10	0.09
Underlying disease			1.00
AML	8	16	
ALL	3	6	
MDS	4	8	
Status at HSCT			1.00
Standard-risk	15	30	
High-risk	0	0	
Source of stem cell			0.21
PB	6	18	
BM and PB	9	12	
Transplanted total nucleated cell dose ($\times10^8$/ kg, median, range)	8.62(6.71-11.07)	8.06(4.99-13.53)	0.15
Transplanted CD34$^+$ cell dose ($\times10^6$/ kg, median, range)	2.37(1.13-6.14)	2.64(1.06-5.85)	0.29
Donor match			0.28
HLA-identical sibling donor	4	3	
HLA-partially matched related donor	10	26	
HLA-identical unrelated donor	1	1	
Sex mismatch			0.44
No	6	16	
Female to male	5	5	
Male to female	4	9	
ABO mismatch			0.67
No	8	12	
Minor	3	9	
Major	4	9	
Pre-HSCT cycles of chemotherapy	4(0-6)	3(0-6)	0.40
Conditioning			1.00
BU/CY+ATG	15	30	

History of CMV reactivation	7	13	0.90
Onset of CMV reactivation (days, median, range)	18(12-46)	15(3-41)	0.80
History of aGvHD	6	10	0.76
Onset of aGvHD (days, median, range)	20(7-43)	15(4-50)	0.65

***** Group matching criteria included age at HSCT (±5years), pre-HSCT cycles of chemotherapy (±1cycle), disease status at HSCT and BM evaluated time after HSCT (±5 days). For each case, two GGF control was randomly selected from the same cohort at which the PGF occurred ("risk-set sampling").

*** *** The continuous variables were compared using the Mann-Whitney U-test, and the differences in frequency between the 2 groups were compared using the chi-square test. The criterion for statistical significance was $P<0.05$.

Abbreviations: allo-HSCT indicates allogeneic hematopoietic stem cell transplantation; aGvHD, acute graft-versus-host disease; PGF, poor graft function; GGF, good graft function; BM, bone marrow; PB, peripheral blood; WBC, white blood cell; ANC, absolute neutrophil cell; Hb, hemoglobin; PLT, platelet; AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia; MDS, myelodysplastic syndrome; HLA, human leukocyte antigen; BU/CY, busulfan, cyclophosphamide and cytarabine; CMV, cytomegalovirus.

Supplementary Table 2. Characteristics of patients with acute leukemia between pre- and post- chemotherapy

Characteristics	Pre-chemotherapy (N=15)	Post-chemotherapy (N=15)	*P*-Value*
BM evaluated time (peri-chemotherapy days)	0	24	
Blood cell count			
Median WBC (×10^9/L) (range)	8.80(5.67-11.70)	3.83(0.62-6.9)	0.0002
Median ANC (×10^9/L) (range)	8.03(2.34-10.9)	3.63(0.51-6.0)	0.0009
Median Hb (g/L) (range)	120(93-142)	77(71-121)	0.0006
Median PLT (×10^9/L) (range)	171(79-274)	42(12-129)	0.0001
Underlying disease			
AML	9		
ALL	6		
Donor match			
HLA-partially matched related donor	15		
Conditioning			
BU/CY+ATG	15		
Source of stem cell			
PB	15		
Transplanted total nucleated cell dose (×10^8/ kg, median, range)	8.80(5.97-11.46)		
Transplanted $CD34^+$ cell dose (×10^6/ kg, median, range)	2.49(1.84-6.99)		
Sex mismatch			
No	12		
Female to male	2		
Male to female	1		
ABO mismatch			
No	5		
Minor	7		
Major	3		

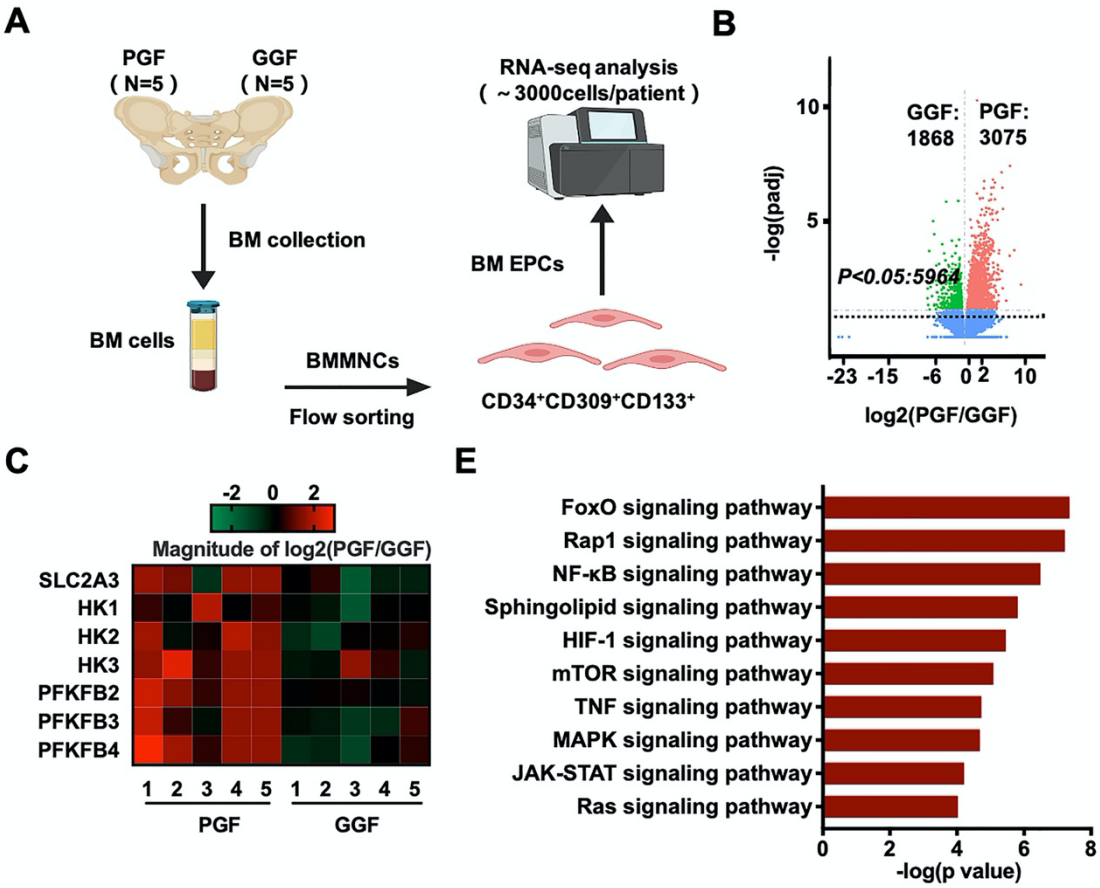
***** The blood cell counts were compared using the Wilcoxon matched-pairs signed rank test. The criterion for statistical significance was $P<0.05$.

Abbreviations: BM, bone marrow; WBC, white blood cell; ANC, absolute neutrophil cell; Hb, hemoglobin; PLT, platelet; AML, acute myelogenous leukemia; ALL, acute lymphocytic leukemia; BU/CY, busulfan, cyclophosphamide and cytarabine.

Supplementary Table 3. Antibody information

Antibody name	Provider	Catalog number
CD45	Becton Dickinson	560777
CD34	Biolegend	343522
CD133	Miltenyi	130-113-184
CD309	Becton Dickinson	560494
PFKFB3	Aabcam	Ab181861
p21 Waf1/Cip1	Cell Signaling Technology	2947
p27 Kip1	Cell Signaling Technology	3686
FAS	Proteintech	13098-1-AP
FOXO3A	Cell Signaling Technology	12829
β-actin	Cell Signaling Technology	8457
a-tubulin	Sigma	T6199
Phospho-NF-κB p65	Cell Signaling Technology	3033
NF-κB p65	Cell Signaling Technology	8242
E-selectin	Proteintech	20894-1-AP
CXCL12/SDF-1	Proteintech	17402-1-AP
cKIT	Biolegend	105818
SCA1	Biolegend	108138
CD150	Biolegend	115926
CD48	Biolegend	103406
Lineage	Biolegend	133313
CD45	Biolegend	103116
CD11B	Biolegend	101263
GR1	Biolegend	108428
B220	Biolegend	103222
CD3	Biolegend	100237
TER119	Biolegend	116218
CD31	Biolegend	102449
VE-Cadherin	Becton Dickinson	562243

Figure S1



D

GO BP overrepresented in PGF BM ECs

GO BP	Fold	P-value
positive regulation of neutrophil activation	13.84	1.15E-03
toll-like receptor TLR1:TLR2 signaling pathway	13.84	1.15E-03
positive regulation of neutrophil degranulation	13.84	1.15E-03
fructose 2,6-bisphosphate metabolic process	11.07	1.97E-03
TRAIL-activated apoptotic signaling pathway	11.07	1.97E-03
positive regulation of Fc-gamma receptor signaling pathway involved in phagocytosis	11.07	1.97E-03
regulation of neutrophil degranulation	10.38	1.74E-04
regulation of neutrophil activation	10.06	1.61E-05
positive regulation of Fc receptor mediated stimulatory signaling pathway	9.88	7.33E-04
negative regulation of interleukin-12 production	7.78	2.13E-05

Figure S1. RNA-seq analysis indicated that FOXO pathway, NF-κB pathway and glycolysis pathway are activated in the damaged BM EPCs of PGF patients. (A) Schematic diagram of RNA-seq analysis of the sorted of BM PGF EPCs and their matched GGF EPCs. **(B)**Distributions and quantifications of the

genes in PGF EPCs and GGF EPCs. The x axis shows the log2 of gene expression change between PGF EPCs and GGF EPCs, whereas the y axis shows the -log10 of the adjusted *P*-value. **(C)**Heatmap of selected glycolysis genes in PGF EPCs and GGF EPCs. (**D)**PANTHER GO biological progress analysis showed the top significant overrepresented GO terms in PGF EPCs according to the fold enrichment score(Fold). **(E)**KEGG metabolism pathway enrichment analysis showed the significant enriched pathways in PGF EPCs which were sorted according to the *P*-value. The y axis shows the -log10 of the *P*-value.

Figure S2

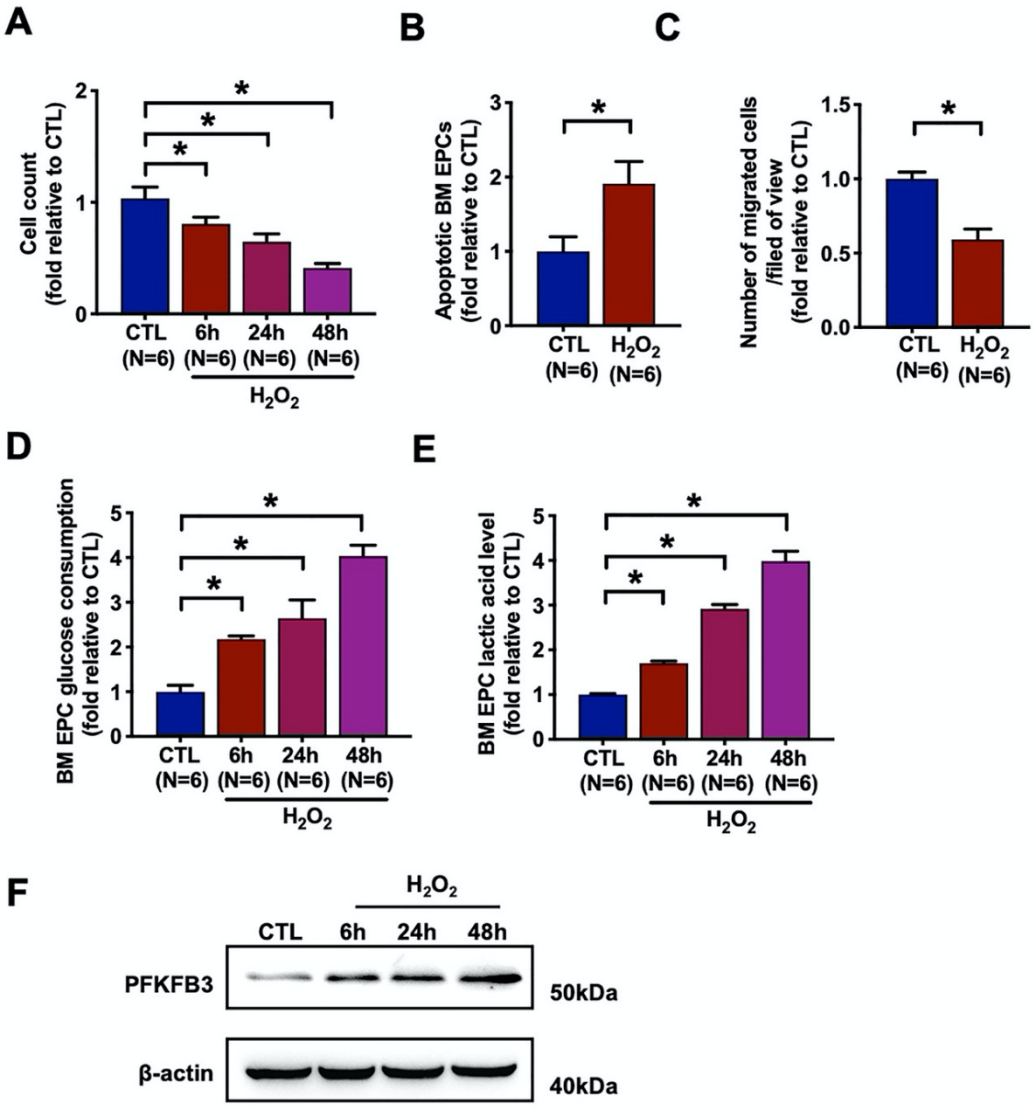


Figure S2. Upregulated PFKFB3 in the damaged BM EPCs induced by hydrogen peroxide *in vitro*. The cultivated BM EPCs from HDs were incubated with hydrogen peroxide(200 μM). Cell number**(A)**, apoptosis**(B)** and migration**(C)** were analyzed in EPCs. **(D-E)**The media of BM EPCs with or without hydrogen peroxide treatment were analyzed for glucose consumption**(D)** and lactate production**(E).** The protein levels of PFKFB3 in BM EPCs with or without hydrogen peroxide treatment were analyzed using western blot analyses**(F).** Data are expressed as fold-change relative to the control group (CTL). The data represent the mean±SEM. *represents $P \leq 0.05$.

Figure S3

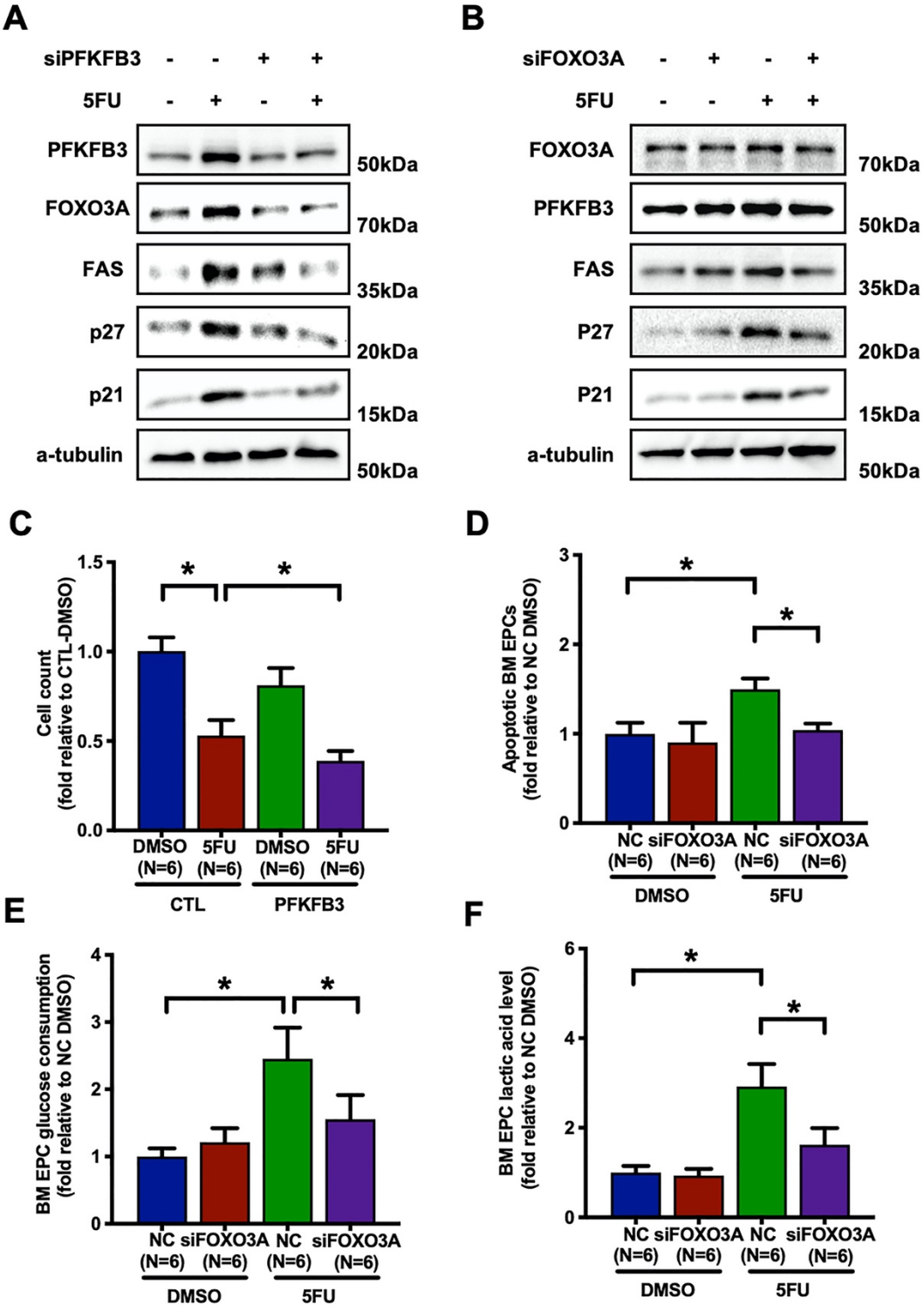


Figure S3. PFKFB3 or FOXO3A silence reduced pro-apoptotic gene expressions after 5FU treatment *in vitro*. (A)Western blot analyses were

performed in the cultivated BM EPCs transfected with non-targeting siRNA controls(NC) or siRNAs targeting PFKFB3 and with or without 5FU treatment. **(B)**Western blot analyses were performed in the cultivated BM EPCs transfected with NC or siRNAs targeting FOXO3A and with or without 5FU treatment. The western blot analyses data were performed in triplicate at least and the representative imagines were shown. **(C)**The cell numbers are shown in the cultivated BM EPCs transfected with a control vector or a vector expressing PFKFB3 which were treated with DMSO or 5FU. **(D)**The apoptosis rates were shown in the cultivated BM EPCs with or without FOXO3A silence and with or without 5FU treatment. **(E-F)**The media of cultivated BM EPCs with or without FOXO3A silence and with or without 5FU treatment were analyzed for glucose consumption**(E)**and lactate production**(F)**. The data represent the mean±SEM. *represents $P \leq 0.05$.

Figure S4

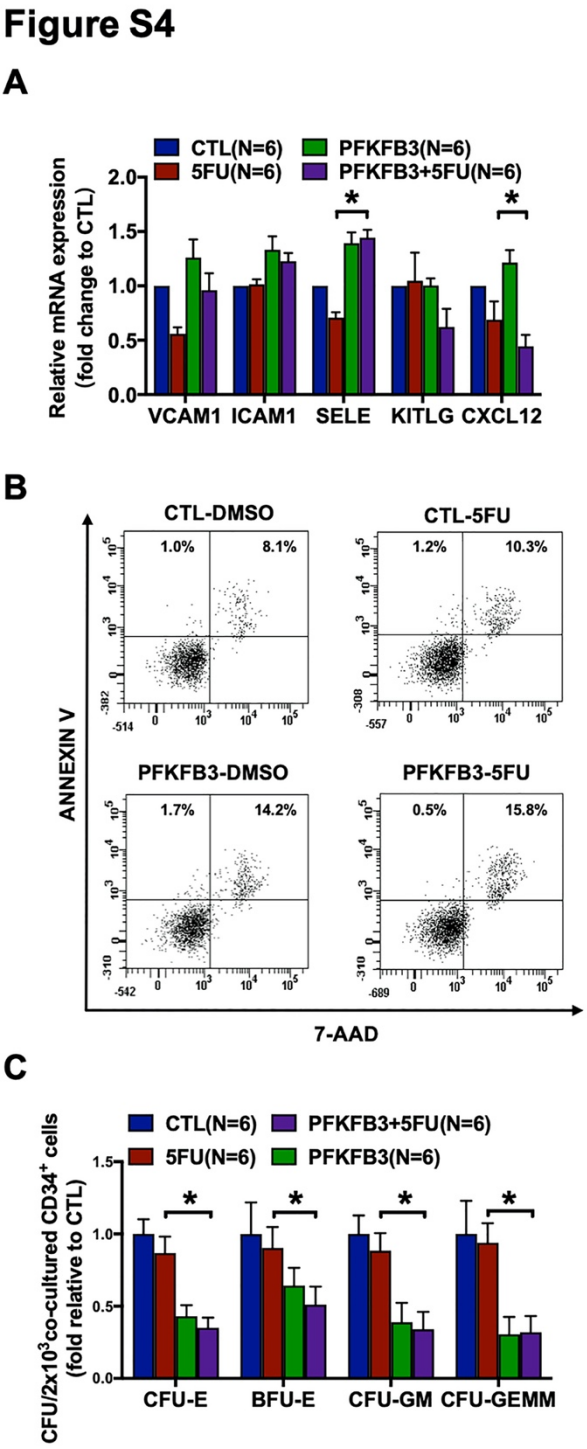


Figure S4. PFKFB3 overexpression impaired the hematopoiesis-supporting ability of BM EPCs *in vitro*. (A)Real-time quantitative polymerase chain reaction(qRT-PCR) analysis of mRNA levels of the indicated gene in the cultivated BM EPCs transfected with a control vector or a vector expressing

PFKFB3 and with or without 5FU treatment were performed. **(B)** Representative flow cytometry analysis of BM $CD34^+$ cells was analyzed after co-culture with BM EPCs transfected with a control vector or a vector expressing PFKFB3 and with or without 5FU treatment. **(C)**The CFU plating efficiency of BM $CD34^+$ cells from HDs was analyzed after co-culture with BM EPCs transfected with a control vector or a vector expressing PFKFB3 and with or without 5FU treatment. *represents $P \leq 0.05$.

Figure S5

A

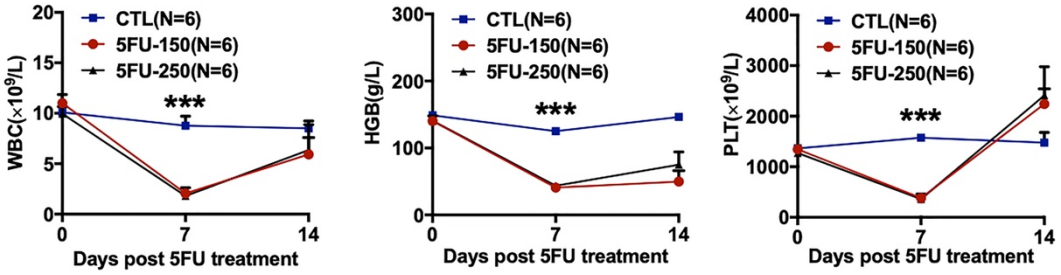


Figure S5. The time course of peripheral blood recovery after 5-FU treatment, including white blood cells(WBCs), hemoglobin(HGB) and platelets(PLT). ***represents $P \leq 0.001$.

Figure S6

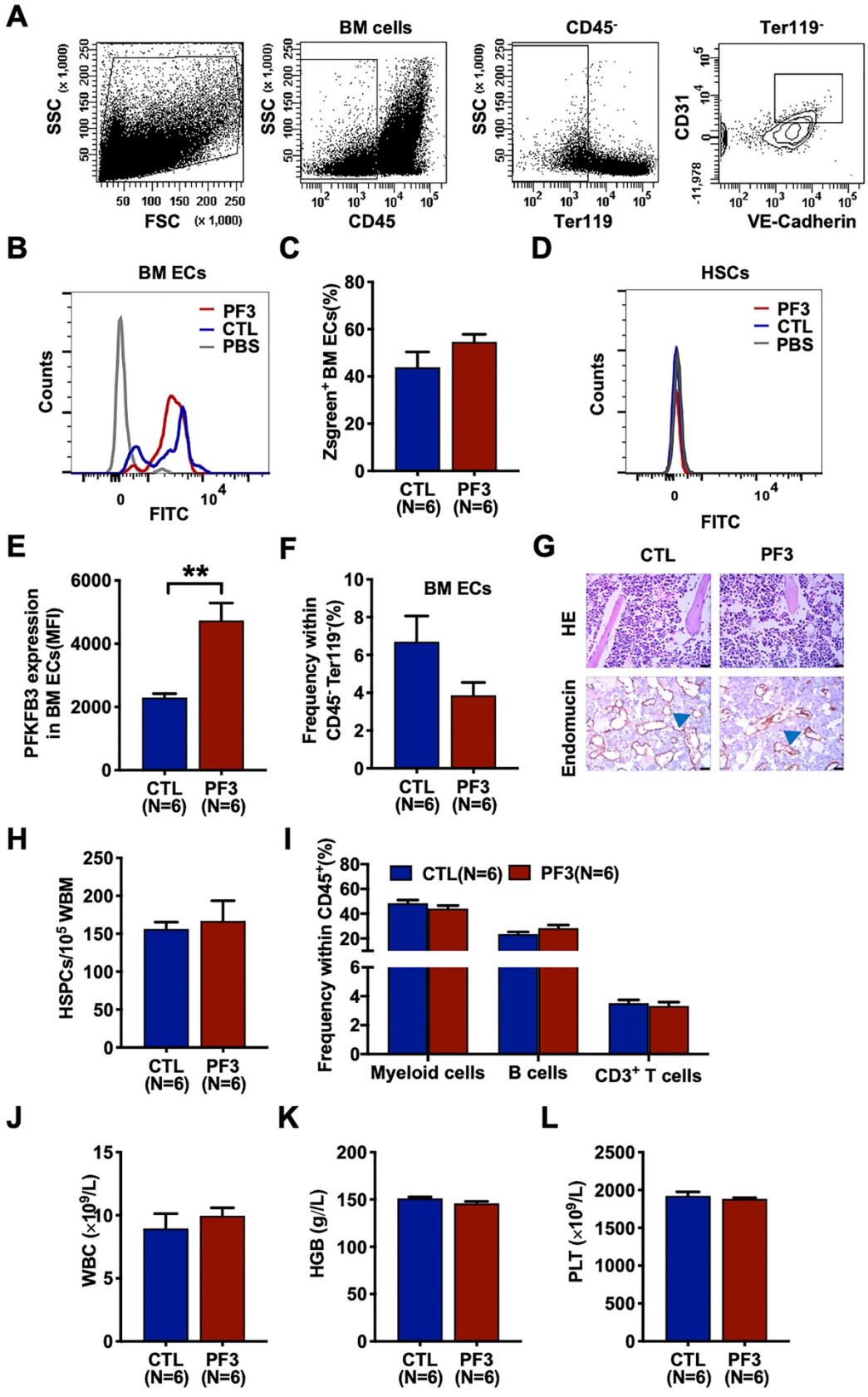

A
BM cells
CD45-
Ter119-
SSC (× 1,000)
FSC (× 1,000)
CD45
Ter119
CD31
VE-Cadherin
B
BM ECs
PF3
CTL
PBS
Counts
FITC
C
Zsgreen+ BM ECs(%)
CTL (N=6)
PF3 (N=6)
D
HSCs
E
PFKFB3 expression in BM ECs(MFI)
**
F
Frequency within CD45-Ter119-(%)
G
CTL
PF3
HE
Endomucin
H
HSPCs/10^5 WBM
I
CTL(N=6)
PF3(N=6)
Frequency within CD45+(%)
Myeloid cells
B cells
CD3+ T cells
J
WBC (×10^9/L)
K
HGB (g/L)
L
PLT (×10^9/L)

Figure S6. The hematopoiesis-supporting ability of BM ECs did not alter in BM EC-specific PFKFB3 overexpression mice in steady state. (A) Gating strategy for quantification of BM ECs by flow cytometry. Representative images **(B)**and quantification **(C)**of Zsgreen$^+$ BM ECs percentage in the total BM ECs(CD45$^-$Ter119$^-$CD31$^+$VE-Cadherin$^+$) from mice with indicated treatment were analyzed by flow cytometry. **(D)**Representative images of Zsgreen$^+$ HSCs in the total HSCs(Lineage$^-$cKIT$^+$SCA1$^+$CD150$^+$CD48$^-$) from mice with indicated treatment were analyzed by flow cytometry. **(E)**The intracellular PFKFB3 level in BM ECs were analyzed by flow cytometry. **(F)**Frequency of CD31$^+$VE-Cadherin$^+$ECs within the BM CD45$^-$Ter119$^-$ cells from mice with indicated treatment were analyzed by flow cytometry. **(G)**H&E and anti-EMCN antibody -stained femur sections showed representative BM cells and BM ECs, respectively. Scale bar(Panels of H&E staining)=10 μm；Scale bar(Panels of anti-EMCN antibody staining)=10 μm. Normal (blue arrowhead) vessels are noted. Frequencies of **(H)**HSPCs (Lineage$^-$cKIT$^+$SCA1$^+$) in whole BM cells(WBM) and**(I)**lineage committed haematopoietic cells within the BM CD45$^+$ cells from mice with indicated treatment were analyzed by flow cytometry. Peripheral blood(PB) cell counts, including white blood cells(WBCs)**(J)**, hemoglobin(HGB)**(K)**and platelets(PLT)**(L)** from mice with indicated treatment. **represents $P \leq 0.01$.

Figure S7

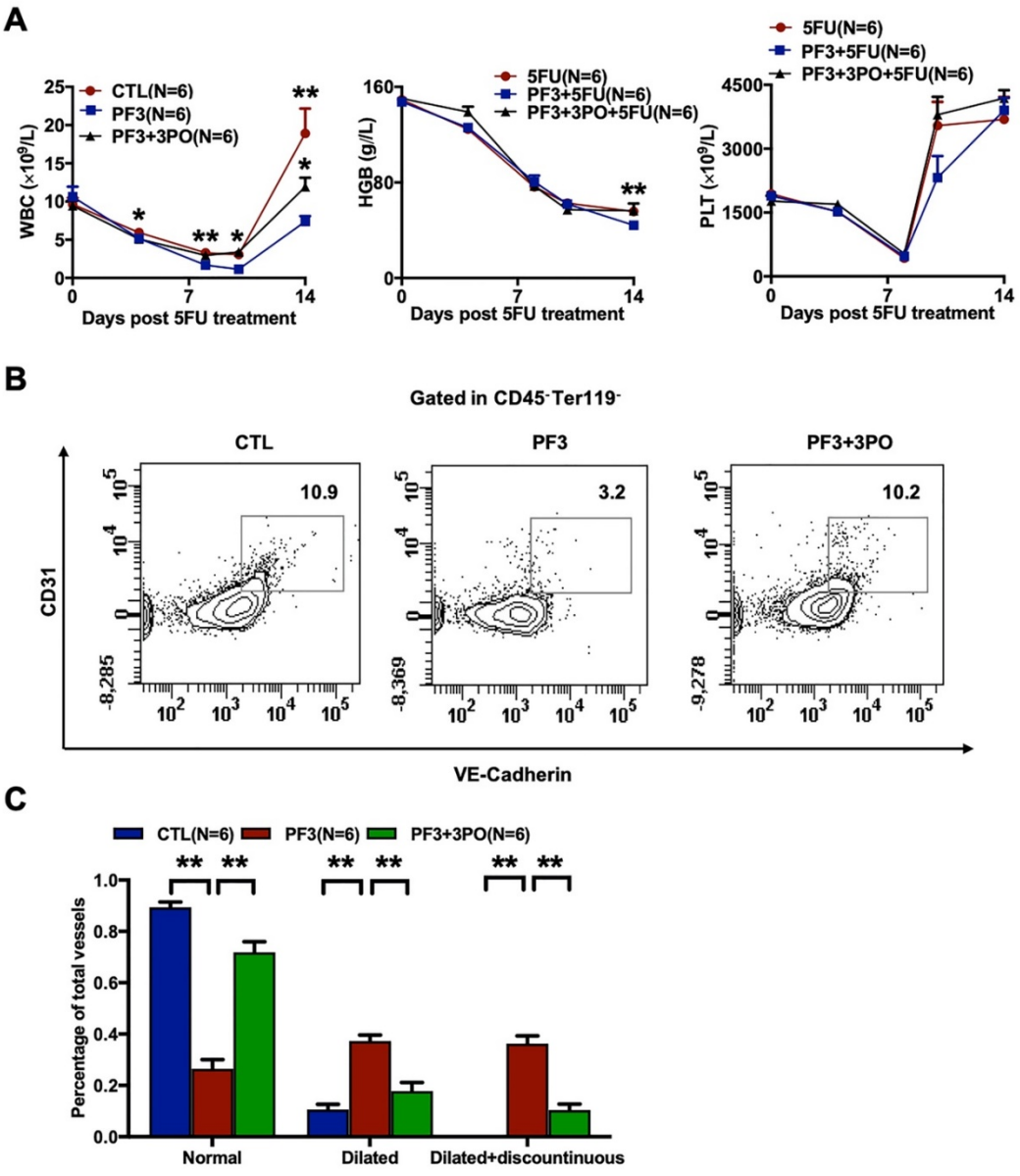


Figure S7. BM EC-specific PFKFB3 overexpression aggravated BM EC damage and delayed hematopoiesis recovery post chemotherapy in mice. **(A)**Recovery kinetics of white blodd cells(WBC), hemoglobin(HGB) and platelets(PLT) after the indicated treatment. **(B)**Representative images of $CD31^{+}VE\text{-}Cadherin^{+}$ ECs within the BM $CD45^{-}Ter119^{-}$ cells from mice with indicated treatment were analyzed by flow cytometry. **(C)** Quantification of damage BM ECs in murine femur stained with anti-EMCN antibody. *represents $P \leq 0.05$. **represents $P \leq 0.01$.

Figure S8

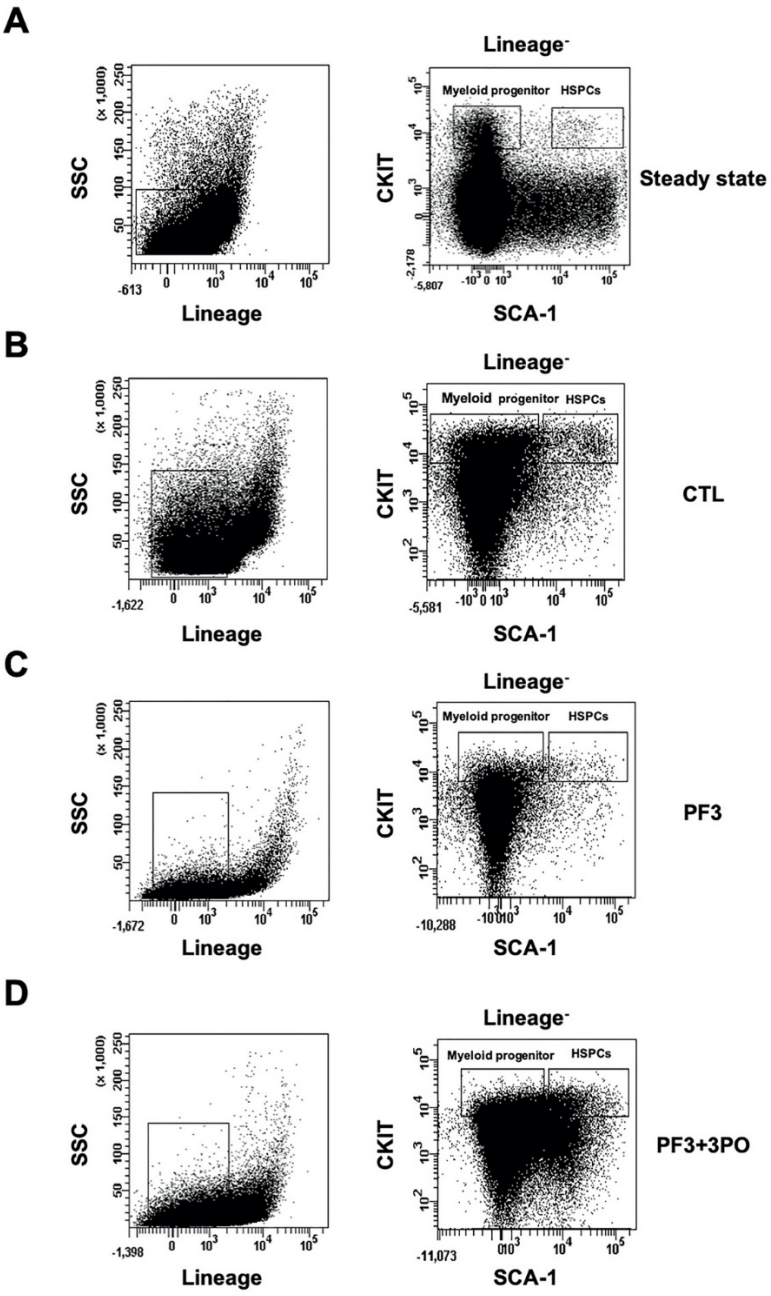


Figure S8. Representative flow cytometry analysis of HSPCs(Lineage$^-$cKIT$^+$SCA1$^+$) and myeloid progenitor(Lineage$^-$cKIT$^+$SCA1$^-$). (A) Mice in steady state. **(B)**Mice with control AAV at day 14 post 5FU treatment(CTL group). **(C)**Mice with PFKFB3-overexpression AAV at day 14 post 5FU treatment(PF3 group). **(D)**Mice with PFKFB3-overexpression AAV and 3PO treatment at day 14 post 5FU treatment(PF3+3PO group).

Figure S9

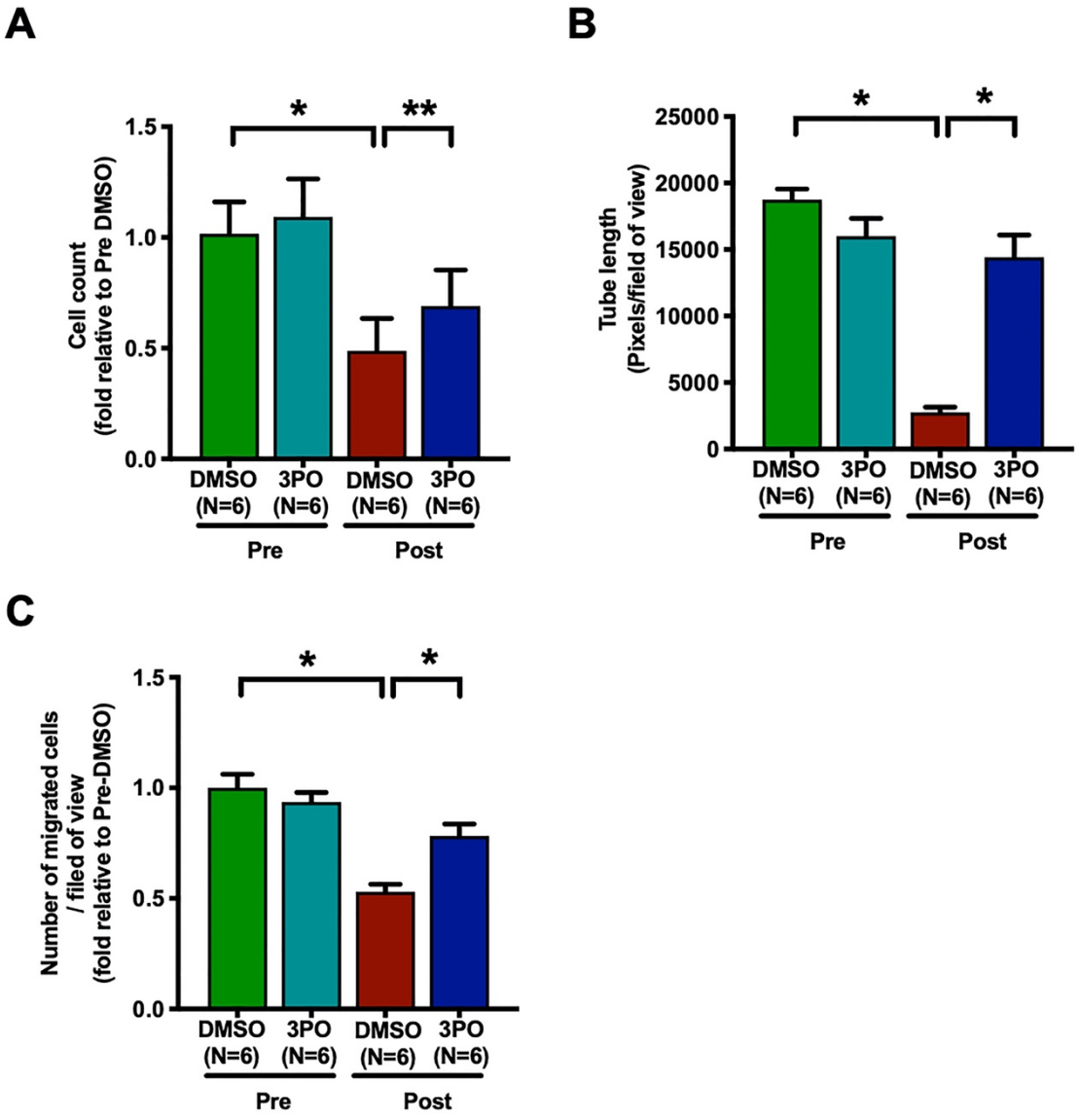


Figure S9. **Glycolysis inhibition improved the number and function of BM EPCs from acute leukemia patients post chemotherapy *in vitro*. (A)**The cell number of the cultivated BM EPCs of patients pre- and post- chemotherapy with or without 3PO treatment were collected for analysis. **(B)**Quantification of the tube length of BM EPCs per field of view was measured in three random low-power fields and averaged. **(C)**Quantification of the migrated BM EPCs per field of view was counted in three random high-power fields and averaged. The data represent the mean ± SEM(right panel). *represents $P \leq 0.05$. **represents $P \leq 0.01$.