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

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Abstract

Bone marrow (BM) endothelial progenitor cell (EPC) damage of unknown mechanism delays the repair of endothelial cells (EC) and recovery of hematopoiesis after chemo-radiotherapy. We found increased levels of the glycolytic enzyme PFKFB3 in the damaged BM EPC of patients with poor graft function, a clinical model of EPC damage-associated poor hematopoiesis after allogeneic hematopoietic stem cell transplantation. Moreover, *in vitro* the glycolysis inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) alleviated the damaged BM EPC from patients with poor graft function. Consistently, PFKFB3 overexpression triggered BM EPC damage after 5-fluorouracil treatment and impaired hematopoiesis-supporting ability *in vitro*. Mechanistically, PFKFB3 facilitated pro-apoptotic transcription factor FOXO3A and expression of its downstream genes, including p21, p27, and FAS, after 5-fluorouracil treatment *in vitro*. Moreover, PFKFB3 induced activation of NF-κB and expression of its downstream adhesion molecule E-selectin, while it reduced hematopoietic factor SDF-1 expression, which could be rescued by FOXO3A silencing. High expression of PFKFB3 was found in damaged BM EC of murine models of chemo-radiotherapy-induced myelosuppression. Furthermore, a murine model of BM EC-specific PFKFB3 overexpression demonstrated that PFKFB3 aggravated BM EC damage, and impaired the recovery of hematopoiesis after chemotherapy *in vivo*, effects which could be mitigated by 3PO, indicating a critical role of PFKFB3 in regulating BM EC damage. Clinically, PFKFB3-induced FOXO3A expression and NF-κB activation were confirmed to contribute to the damaged BM EPC of patients with acute leukemia after chemotherapy. 3PO repaired the damaged BM EPC by reducing FOXO3A expression and phospho-NF-κB p65 in patients after chemotherapy. In summary, our results reveal a critical role of PFKFB3 in triggering BM EPC damage and indicate that endothelial-PFKFB3 may be a potential therapeutic target for myelosuppressive injury.

Introduction

Chemotherapy, irradiation and allogeneic hematopoietic stem cell transplantation (HSCT) are all commonly used in patients with hematopoietic cancers. However, many patients who undergo these therapies will suffer poor hematopoietic function, characterized by delayed recovery of hematopoiesis, resulting in prolonged cytopenia and an increased risk of infections, bleeding and hospitalization.[1] The pathogenesis of poor hematopoietic function is unclear, so the optimal therapeutic approaches have not been well established.

Endothelial cells (EC) are responsible for tissue growth and regeneration under homeostasis and stress in multiple organs.[2] In the hematopoietic system, EC are an important constituent of the bone marrow (BM) microenvironment and play an essential role in regulating hematopoietic stem cell (HSC) homeostasis.[3-6] Besides affecting malignant cells, chemo-radiotherapy also damages both HSC and their supportive BM microenvironment, especially EC.[4, 7-12]

As a result, BM EC damage, with a high level of apoptosis and a sustained state of inflammation, limits the recovery of hematopoiesis after chemo-radiotherapy,[4,9,10,13-18] whereas inhibition of EC apoptosis or an infusion of EC accelerates hematopoietic recovery,[10,18,19] indicating that BM EC are a prerequisite for hematopoietic recovery. It is, therefore, critical to identify the mechanism(s) underlying BM EC damage in order to be able to promote hematopoietic recovery.

The endothelial progenitor cell (EPC),derived from BM, is a primitive endothelial precursor with a distinctive potential for differentiation into EC, and is a critical source of EC repair.[20, 21] Murine studies revealed that infusion of EPC can restore BM EC and promote hematopoiesis following radiotherapy.[22] Clinically, in serial studies we showed that BM EPC damage, characterized by higher levels of reactive oxygen species (ROS) and apoptosis, impairs cell migration and angiogenesis, and contributes to BM EC damage and the subsequent occurrence of poor graft function,[13-16,23] defined by pancytopenia after allogeneic HSCT, whereas improvement of BM EPC prior to HSC-transplantation (HSCT) promotes hematopoietic reconstitution of donor HSC after the transplant.[15] However, the mechanism underlying BM EPC damage after chemo-radiotherapy is largely unknown.

There is emerging evidence that EC rely largely on glycolysis in order to produce energy. Glycolysis is relatively low in EC from healthy adults, whereas it is increased in angiogenic EC. Aberrant glycolysis has been reported to be an important pathogenic mechanism in EC-associated diseases, such as pulmonary hypertension and tumor vessels.[24-28] In EC, glycolysis is mainly stimulated by the regulatory enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3),[24] whereas blockade or genetic deletion of endothelial PFKFB3 attenuates the above vascular diseases.[26,27] Glycolysis is, therefore, becoming an attractive target for manipulation in these diseases. However, the metabolic regulatory mechanism in BM EPC, especially the role of defective glycolysis in BM EPC damage by chemo-radiotherapy, remains to be investigated.

We, therefore, used poor graft function, a clinical model of BM EPC damage-associated poor hematopoiesis after allogeneic HSCT, to evaluate the relationship between the expression of the glycolytic enzyme, PFKFB3, and BM EPC damage. Moreover, we studied the mechanistic role of defective glycolysis regulated by PFKFB3 in BM EPC damage, using well-established *in vitro* models of BM EPC damage, murine models of chemotherapy- or irradiation-induced myelosuppression and a murine model of BM EC-specific PFKFB3 overexpression. The PFKFB3-induced BM EPC damage was validated in a cohort of patients with acute leukemia after chemotherapy. Our aim was to uncover the critical role and underlying mechanism of action of PFKFB3 in BM EPC damage and provide a potential therapeutic approach for patients exposed to chemo-radiotherapy in the future.

Methods

Patients and controls

A prospective nested case-control study was performed to compare PFKFB3 levels in BM EPC between patients with poor graft function (n=15) and matched patients with good graft function (n=30) from the same cohort of patients who underwent allogeneic HSCT between February 5, 2018 and October 30, 2020 at Peking University Institute of Hematology (Beijing, China). There were no significant differences between the two groups of subjects with regard to any clinical characteristics (*Online Supplementary Table S1*).

Moreover, PFKFB3 levels were compared in BM EPC collected from acute leukemia patients (n=15) before and after chemotherapy for conditioning prior to haplo identical HSCT. *In-vivo* T-cell-depleted myeloablative chemotherapy-based conditioning regimens administered prior to haploidentical HSCT include busulfan (9.6 mg/kg)/cyclophosphamide (3.6 g/m^2), cytarabine (4 g/m^2), and antithymocyte globulin (10 mg/kg)[29-31] (*Online Supplementary Table S2*).

Forty healthy donors (25 males and 15 females; age range, 25-63 years; median age, 30 years) were enrolled as controls. The study was approved by the Ethics Committee of Peking University People's Hospital, and written informed consent was obtained from all subjects in compliance with the Declaration of Helsinki.

Definitions of poor and good graft function

Poor graft function[13-17,23,32,33] is characterized by hypoplastic or aplastic BM with the presence of two or three cytopenias: (i) an absolute neutrophil count less than 0.5×10^9/L; (ii) a platelet count less than 20×10^9/L; or (iii) a hemoglobin concentration less than 70 g/L on at least 3 consecutive days beyond day +28 after HSCT or requiring transfusion and/or granulocyte colony-stimulating factor administration in the presence of complete donor chimerism. Good graft function[13-17,23,32-35] is defined as an absolute neutrophil count greater than 0.5×10^9/L for 3 consecutive days, a platelet count greater than 20×10^9/L for 7 consecutive days, and a hemoglobin level greater than 70 g/L without transfusion support beyond day +28 after HSCT. Patients with hematologic relapse after allogeneic HSCT were excluded. The transplantation protocols were reported previously.[29-31]

Quantification, culture, characterization and functional analyses of primary bone marrow endothelial progenitor cells

The isolation, quantification, culture, and characterization of BM EPC were performed as previously reported.[13,15,23,35-37] Briefly, BM mononuclear cells were cultured in fibronectin-precoated 24-well culture plates with EGM-2-MV-SingleQuots and 10% fetal bovine serum. Pre-cultured and 7-day-cultured BM EPC were identified by mouse anti-human CD34, CD45, vascular endothelial growth factor receptor 2 (CD309) and CD133 monoclonal antibodies and analyzed using a BD LSRFortessa cell analyzer. Aliquots of isotype-identical antibodies served as negative controls.

Methods to determine the functionality of BM EPC[13,15,23,35-37] include measurements of apoptosis, intracellular levels of ROS or PFKFB3, double-positive staining with both DiI-AcLDL and FITC-UEA-1, tube-formation and migration assays. The hematopoiesis-supporting ability of EPC was evaluated by colony-forming unit assays after co-culture of EPC with BM $CD34^+$ cells from healthy donors. The levels of glucose and lactic acid in culture medium were measured by a glucose assay kit and a lactic acid assay kit, respectively.

In situ immunofluorescence staining of EPC[15,35-37] in BM trephine biopsies was performed with mouse anti-human CD34, rabbit anti-human PFKFB3 antibodies and 4',6-diamidino-2-phenylindole (DAPI), and analyzed under a Leica TCS SP8 microscope.

In vitro models of bone marrow endothelial progenitor cell damage

To construct the models of BM EPC damage, primary BM EPC derived from healthy donors were treated with 5-fluorouracil (5FU; 20 μM)[38] or hydrogen peroxide (200 μM)[39] *in vitro*. To determine the effect of hyperglycolysis or nuclear factor kappa B (NF-κB) inhibition on EPC function, EPC were treated with the glycolysis inhibitor 3PO (10 μM)[27] or the NF-κB inhibitor SC-514 (10 μM).[40]

Establishment of murine models of myelosuppression and of bone marrow endothelial cell-specific PFKFB3 overexpression

Murine models of myelosuppression[4,9,11,41] were constructed using C57BL/6J female mice (6-9 weeks old) treated with 5FU (150 or 250 mg/kg) or irradiation (500 or 650 cGy total body irradiation via X-ray). To generate the murine model of BM EC-specific PFKFB3 overexpression, an adeno-associated virus (AAV)-mediated gene delivery system (recombinant AAV-V_{EC}, an optimized AAV variant for EC transduction), under the control of an EC-specific Tie promoter, was injected intraosseously.[42-45]

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) on day 0 and then with 3PO (25 mg/kg) on days 3, 5, and 7. The kinetics of peripheral blood were analyzed. HSC (lineage$^-$cKIT$^+$SCA1$^+$CD150$^+$CD48$^-$), hematopoietic stem and progenitor cells (HSPC, lineage$^-$cKIT$^+$SCA1$^+$), myeloid progenitors (lineage$^-$cKIT$^+$SCA1$^-$),[46] myeloid cells, T cells, B cells and EC (CD45$^-$Ter119$^-$CD31$^+$VE-Cadherin$^+$), intracellular levels of FOXO3A, NF-κB p65 and PFKFB3 were analyzed in murine BM by flow cytometry. Cells from all mice were stained with hematoxylin and eosin and with an immunohistochemical stain for the EC marker endomucin.

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

Statistical analysis

Prism 6.0 software (GraphPad, San Diego, CA, USA) was used to compute the statistical data. The results are expressed as the mean ± standard error of mean, and the Mann-Whitney U test was used to analyze continuous variables. The Wilcoxon matched-pairs signed rank test was applied to analyze matched or paired data collected from *in vitro* studies. One-way analysis of variance was performed for multiple comparisons. A *P*-value ≤0.05 was considered statistically significant.

Detailed methods can be found in the *Online Supplementary Methods*.

Results

PFKFB3 is enhanced in the damaged bone marrow endothelial progenitor cells of patients with poor graft function

We performed a prospective nested case-control study to compare the expression of PFKFB3, a glycolytic enzyme, in BM EPC from patients with poor graft function (PGF-EPC) and from matched patients with good graft function (GGF-EPC). PFKFB3 expression was significantly higher in PGF-EPC than in GGF-EPC (5782±1094 *vs*. 2584±272.9; *P*=0.0004) (Figure 1A-C). Likewise, glucose uptake was significantly increased in PGF-EPC (2.91±0.35-fold *vs*. 1.30±0.19-fold; *P*=0.008) (Figure 1D). Consistently, *in situ* immunofluorescent staining of BM biopsies revealed that PFKFB3 was increased in PGF-EPC (Figure 1E). Thus, our findings indicate that PFKFB3-induced glycolysis is hyperactivated in PGF-EPC.

To determine whether hyperglycolysis contributes to BM EPC damage in patients with poor graft function, PGF-EPC were treated with 3PO, an inhibitor of glycolysis. 3PO markedly decreased glucose consumption (0.41±0.01-fold; *P*=0.03) (Figure 2A) and lactate production (0.72±0.03-fold; *P*=0.03) (Figure 2B) in PGF-EPC. Moreover, 3PO significantly reduced apoptosis (0.70±0.12-fold;

P=0.03) (Figure 2C), improved tube formation (6672±341.2 *vs*. 3855±390.4; *P*=0.03) (Figure 2D) and enhanced the migration ability (1.60±0.11-fold; *P*=0.03) (Figure 2E) of PGF-EPC. These results suggest that increased PFKFB3 is involved in the damaged EPC of patients with poor graft function.

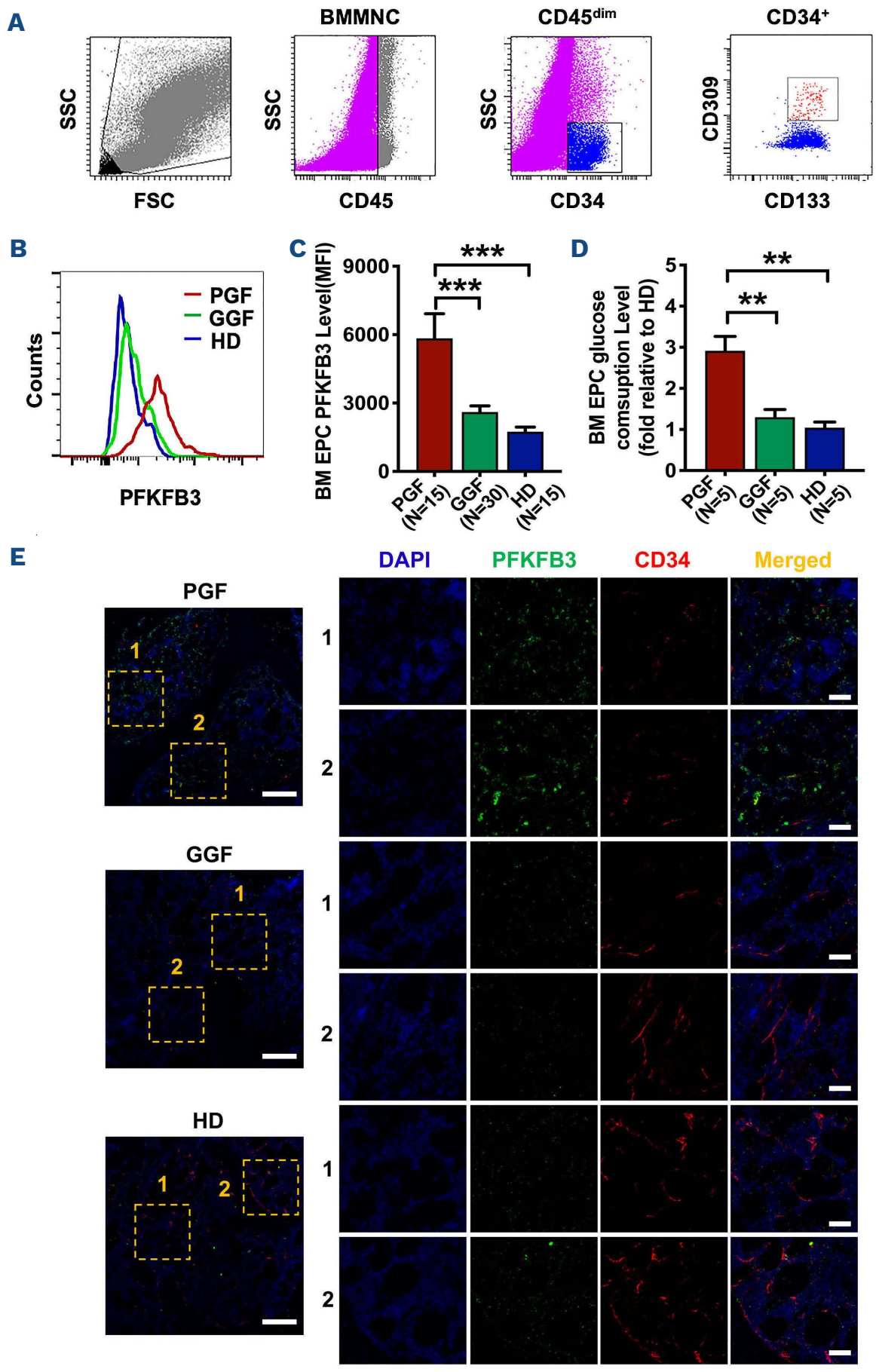

Figure 1. Defective glycolysis in bone marrow endothelial progenitor cells from patients with poor graft function. (A) To detect intracellular PFKFB3 levels in pre-cultured bone marrow (BM) mononuclear cells, BM endothelial progenitor cells (EPC) demonstrating the typical expression of CD34, CD309, and CD133 were first gated by flow cytometry. (B, C) Representative images (B) and quantification (C) of intracellular PFKFB3 levels in the gated pre-cultured BM EPC from patients with poor graft function (PGF), patients with good graft function (GGF) and healthy donors (HD) were analyzed by flow cytometry mean fluorescence intensity (mean ± standard error of mean). (D) Glucose consumption was determined in the media of cultured BM EPC from patients with PGF or GGF and from HD. (E) *In situ* immunofluorescence of BM biopsies (scale bar=50 µm) showed that PFKFB3 was increased in BM EPC from patients with PGF compared to the amounts in patients with GGF or in HD. ***P*≤ 0.01, ****P*≤0.001. BMMNC: bone marrow mononuclear cells; MFI: mean fluorescence intensity.

The FOXO, NF-?B and glycolysis pathways are activated in the damaged bone marrow endothelial progenitor cells of patients with poor graft function

To further understand the metabolic profile and regulatory mechanisms underlying damaged BM EPC, PGF-EPC and GGF-EPC were sorted via fluorescence-activated cell sorting and analyzed by RNA-sequencing (*Online Supplementary Figure S1A*). Markedly different levels of expression between PGF-EPC and GGF-EPC were found for 5,964 genes (adjusted *P*-values <0.05) (*Online Supplementary Figure S1B*). Following further filtering using the 1.5-fold change criterion, 3,075 genes were upregulated in PGF-EPC, whereas 1,868 genes were increased in GGF-EPC (*Online Supplementary Figure S1B*). PGF EPC showed overexpression of genes in the glycolytic pathway, including PFKFB3 (*Online Supplementary Figure S1C*). PANTHER-based analysis of gene on-

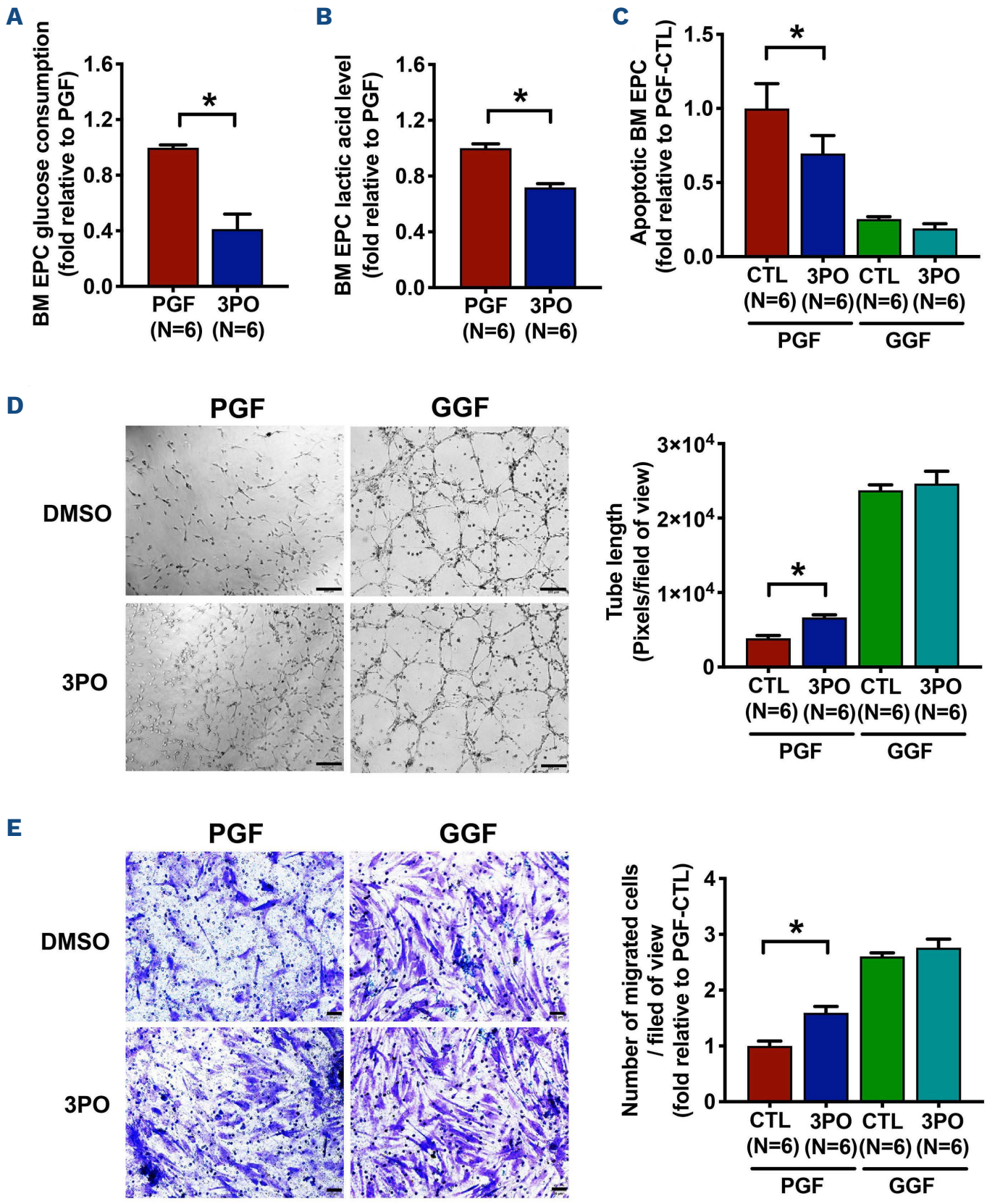

Figure 2. *In vitro* PFKFB3 inhibition increased the number and function of bone marrow endothelial progenitor cells from patients with poor graft function. (A, B) The media of cultured bone marrow (BM) endothelial progenitor cells (EPC) from patients with poor graft function (PGF) which were or were not treated *in vitro* with the glycolysis inhibitor 3PO were analyzed for glucose consumption (A) and lactate production (B). (C) Apoptosis rates of cultured BM EPC from patients with poor or good graft function treated or not with 3PO *in vitro*. (D) Representative images (left, scale bars=200 μm) and quantification (right) of the tube length of BM EPC per field of view, measured in three random low-power fields and averaged. (E) Representative images (left, scale bars= 50 μm) and quantification (right) of migrated BM EPC per field of view, counted in three random high-power fields and averaged. *P≤0.05. PGF: poor graft function; GGF: good graft function; CTL: control; DMSO: dimethylsulfoxide.

tology (GO)-biological processes revealed that inflammation-associated processes, glycolysis- and apoptosis-related pathways were among the top ten significantly overrepresented GO terms in PGF-EPC (*Online Supplementary Figure S1D*). KEGG metabolism pathway enrichment analysis revealed that genes involved in the pro-apoptotic FOXO pathway and the pro-inflammatory NF-κB pathway (a negative mediator of hematopoiesis-supporting ability of BM EC) were enriched in PGF-EPC (*Online Supplementary Figure S1E*). These results indicated BM EPC damage in patients with poor graft function and strongly support the concept that poor graft function is a clinical model of BM EPC damage-associated poor hematopoiesis. Considering the critical roles of the FOXO and NF-κB signaling pathways in the regulation of apoptosis and hematopoiesis-supporting ability of BM EC, studies are needed to determine

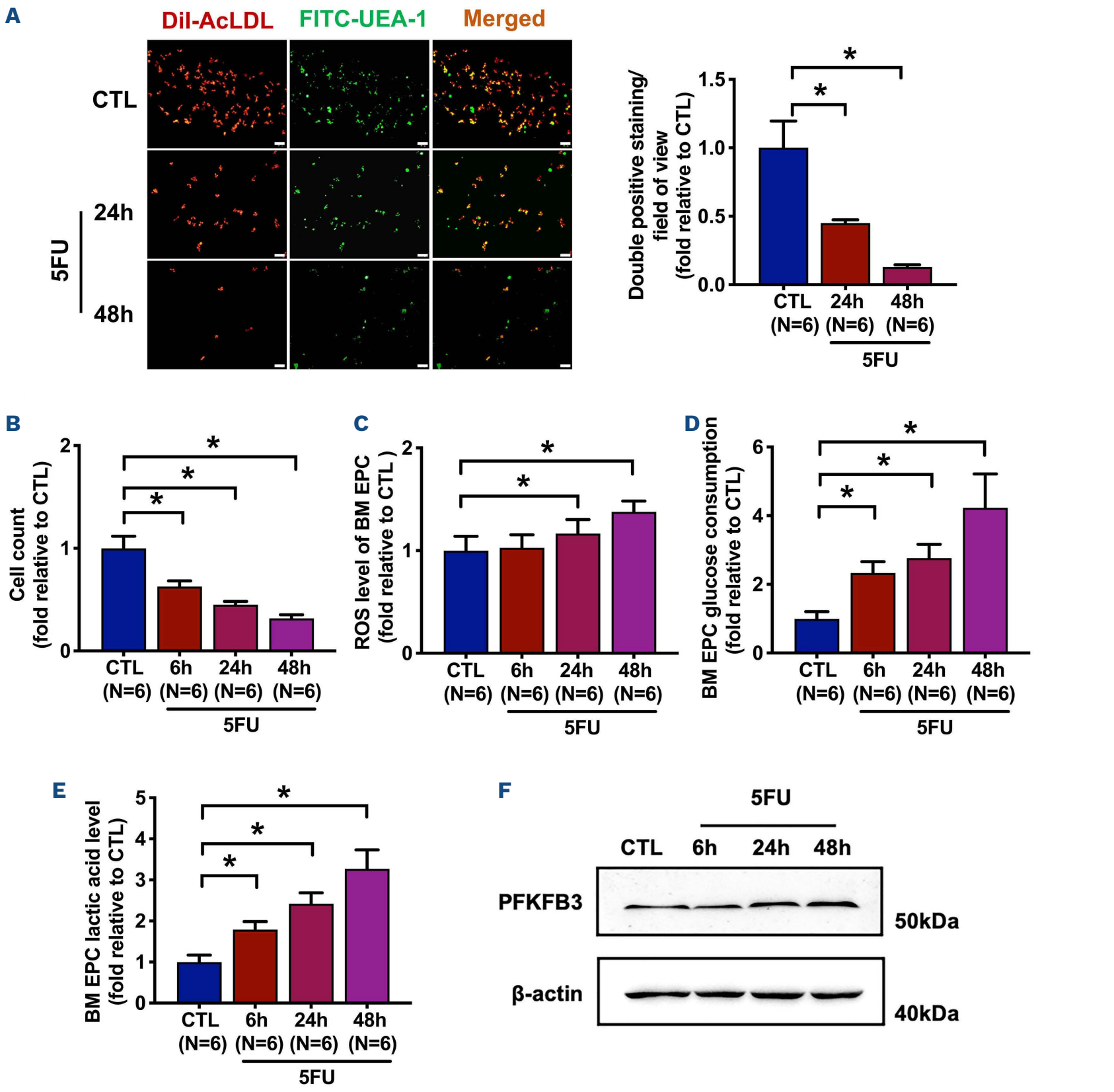

Figure 3. Upregulated PFKFB3 in bone marrow endothelial progenitor cells damaged by 5-fluorouracil *in vitro*. (A-C) Bone marrow (BM) endothelial progenitor cells (EPC) from healthy donors were cultured for 7 days and then incubated with 5-fluorouracil (5FU, 20 μM) for 6 h, 24 h and 48 h *in vitro*. The effects of 5FU treatment on double-positive stained cell number (per field of view) (A), cell number (per well) (B) and level of reactive oxygen species (C) were analyzed. (D, E) The media of BM EPC treated with dimethylsulfoxide (control) or 5FU were analyzed for glucose consumption (D) and lactate production (E). Data are expressed as fold-change relative to the control group. (F) The levels of PFKFB3 protein in BM EPC treated or not with 5FU were determined using western blot analysis. The data represent the mean ± standard error of mean. *$P \leq 0.05$. CTL: control; ROS: reactive oxygen species.

whether activated FOXO and NF-κB signaling pathways contribute to defective glycolysis-induced BM EPC damage.

PFKFB3 promotes FOXO3A and its downstream pro-apoptotic gene expression in bone marrow endothelial progenitor cells after 5-fluorouracil treatment *in vitro*

To further elucidate the mechanism underlying BM EPC damage, *in vitro* models of BM EPC damage triggered by 5FU or hydrogen peroxide were used. Time response effect data indicate that 5FU (20 μM) treatment led to a gradual increase in EPC damage at 6 h, 24 h, and 48 h, with the greatest damaging effect at 48 h, as indicated by reduced double-positive staining of DiI-AcLDL and FITC-UEA-1 (the typical markers of functional EPC) and EPC counts,

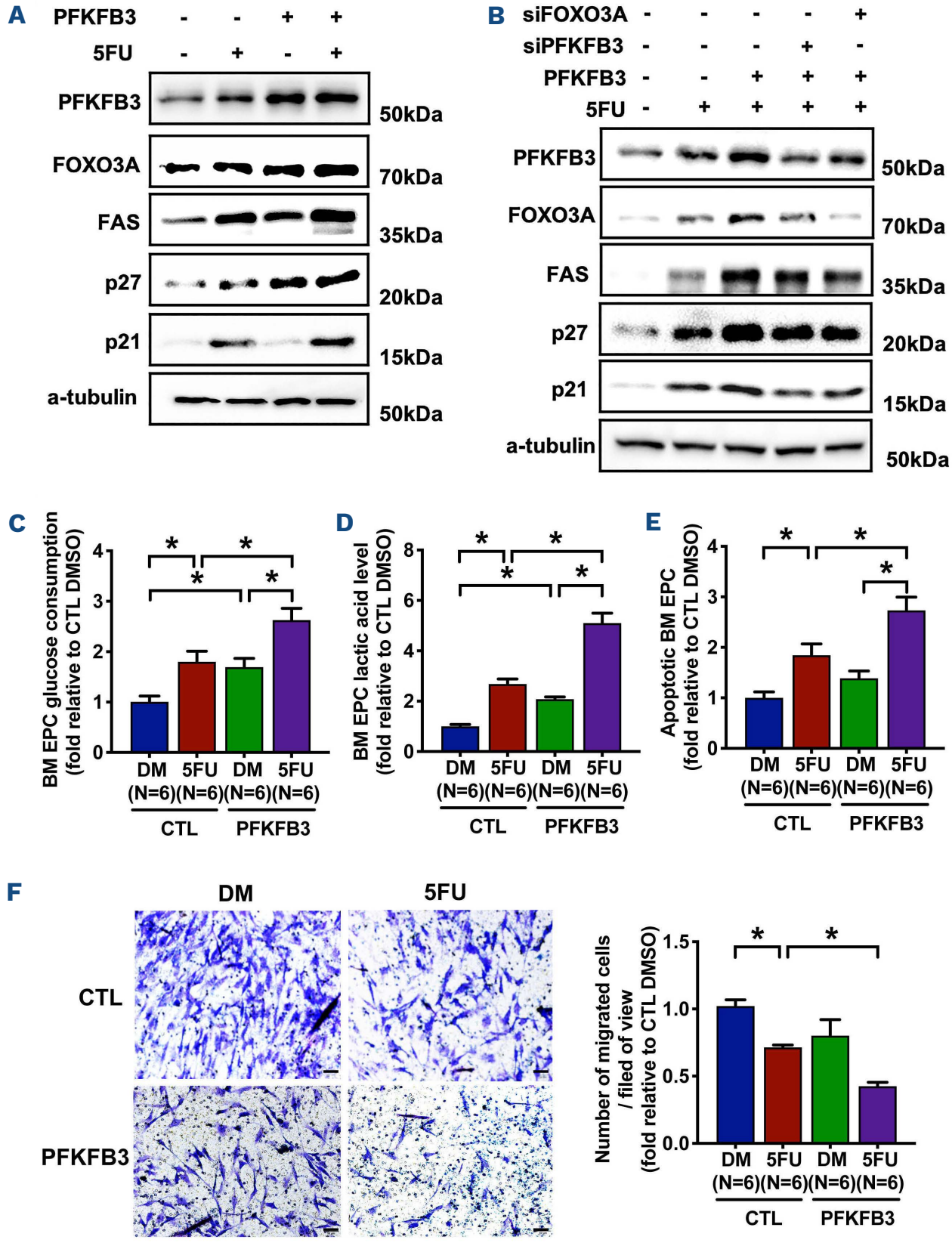

Figure 4. PFKFB3 promoted pro-apoptotic gene expression after 5-fluorouracil treatment via FOXO3A *in vitro*. (A) Western blot analyses were performed on cultured bone marrow (BM) endothelial progenitor cells (EPC) transfected with a control vector or a vector expressing PFKFB3 and treated with dimethylsulfoxide (DMSO, DM) or 5-fluorouracil (5FU). (B) Western blot analyses were performed on the cultured BM EPC transfected with a control vector or a vector expressing PFKFB3, combined with non-targeting siRNA controls or siRNA targeting PFKFB3 or FOXO3A, and with or without 5FU treatment. All the western blot analyses were performed in triplicate at least and representative images are shown. (C, D) The media of cultured BM EPC treated or not with 5FU and with or without PFKFB3 overexpression were analyzed for glucose consumption (C) and lactate production (D). (E, F) Apoptosis (E) and migration (F) (scale bars represent 50 μm) were assessed in the cultured BM EPC transfected with a control vector or a vector expressing PFKFB3 and treated with DM or 5FU. The data represent the mean ± standard error of mean. *$P \leq 0.05$.

whereas ROS levels in the EPC increased (Figure 3A-C).

5FU treatment significantly increased glucose consumption (4.23±0.98-fold at 48 h; *P*=0.03) (Figure 3D) and lactate production (3.27±0.46-fold at 48 h; *P*=0.03) (Figure 3E). Consistently, 5FU treatment increased PFKFB3 expression in EPC (Figure 3F), indicating that enhanced PFKFB3 expression is involved in chemotherapy-induced BM EPC damage.

Hydrogen peroxide markedly triggered BM EPC damage compared with the control (*Online Supplementary Figure S2A-C*). Moreover, hydrogen peroxide significantly increased glucose consumption (*Online Supplementary Figure S2D*) and lactate production (*Online Supplementary Figure S2E*). Consistently, hydrogen peroxide increased the expression of PFKFB3 protein in BM EPC (*Online Supplementary Figure S2F*). These results indicated that enhanced PFKFB3 expression is involved in ROS-induced BM EPC damage.

To investigate the role of the FOXO signaling pathway in defective glycolysis-induced BM EPC damage, BM EPC derived from healthy donors were transfected or not with PFKFB3 plasmids and treated or not with 5FU *in vitro*. PFKFB3 overexpression induced expression of the pro-apoptotic transcription factor FOXO3A and its downstream genes, including FAS, p27 and p21, after 5FU treatment (Figure 4A). Silencing of PFKFB3 decreased expression of these genes after 5FU treatment (*Online Supplementary Figure S3A*), which is consistent with the effects of FOXO3A silencing on expression of these genes after 5FU (*Online Supplementary Figure S3B*). Moreover, knockdown of FOXO3A attenuated the PFKFB3-induced expression of FOXO3A and its downstream genes (Figure 4B). Compared with the effect of 5FU treatment, PFKFB3 overexpression combined with 5FU treatment further increased the levels of glucose consumption (2.63±0.23-fold *vs*. 1.80±0.21-fold; *P*=0.03) (Figure 4C) and lactate production (5.10±0.39-fold *vs*. 2.69±0.20-fold; *P*=0.03) (Figure 4D) in BM EPC. PFKFB3 overexpression combined with 5FU treatment significantly aggravated the effect of 5FU on the number (*Online Supplementary Figure S3C*), apoptosis (2.73±0.26-fold *vs*. 1.85±0.22-fold; *P*=0.03) (Figure 4E) and migration (0.43±0.03-fold *vs*. 0.72±0.02-fold; *P*=0.03) (Figure 4F) of BM EPC, which is consistent with the effects of FOXO3A silencing on these cell events, such as apoptosis level after 5FU (*Online Supplementary Figure S3D*). Considering that FOXO3A silencing alters the expression of PFKFB3 moderately (*Online Supplementary Figure S3B*), these results revealed that FOXO3A may be a downstream factor of PFKFB3 and mediate the effect of PFKFB3 on BM EPC pro-apoptotic gene expression, leading to BM EPC damage.

PFKFB3 inhibits the hematopoiesis-supporting ability of bone marrow endothelial progenitor cells by inducing NF-κB pathway activation *in vitro*

In addition to the effect of PFKFB3-induced glycolysis on FOXO3A and its downstream pro-apoptotic genes, we next sought to identify the regulatory relationship between PFKFB3 and the NF-κB signaling pathway in the damaged EPC *in vitro*. PFKFB3 overexpression increased the levels of phospho-NF-κB-p65 and its downstream adhesion molecule E-selectin and decreased hematopoietic or niche factor CXCL12 (also known as SDF-1) (Figure 5A, *Online Supplementary Figure S4A*) after 5FU treatment, while PFKFB3 knockdown inhibited the levels of phospho-NF-κB-p65 and E-selectin and enhanced SDF-1 expression (Figure 5B, E).

In order to further investigate whether PFKFB3 affects the ability of the damaged BM EPC to support hematopoiesis *in vitro*, we performed co-culture assays using BM CD34$^+$ cells from healthy donors and the EPC from healthy donors with PFKFB3 overexpression after 5FU treatment. Consistent with PFKFB3-induced NF-κB activation and decreased SDF-1 in the damaged BM EPC after 5FU treatment, PFKFB3 overexpression decreased the ability of BM EPC to support CD34$^+$ cells, as determined by the increased levels of ROS (Figure 5C) and apoptosis of the CD34$^+$ cells (Figure 5D, *Online Supplementary Figure S4B*), whereas it decreased colony-forming unit efficiency (*Online Supplementary Figure S4C*). Pharmacological inhibition of NF-κB via SC-514 attenuated the PFKFB3-induced increase in phospho-NF-κB-p65, E-selectin expression, decrease in SDF-1 expression and impaired hematopoiesis-supporting ability of BM EPC (Figure 5E, F). These results indicated that PFKFB3 overexpression could lead to impaired hematopoiesis-supporting ability after chemotherapy by inducing NF-κB pathway activation.

FOXO3A contributes to the PFKFB3-impaired hematopoiesis-supporting ability of bone marrow endothelial progenitor cells

To investigate the regulatory relationship between FOXO3A and the NF-κB pathway in the context of PFKFB3-induced BM EPC damage, we examined the activity of NF-κB and expression of its downstream genes in human BM EPC derived from healthy donors co-transfected with PFKFB3 plasmids and FOXO3A siRNA *in vitro*. FOXO3A silencing mitigated the increase in phospho-NF-κB-p65, E-selectin expression and decrease in SDF-1 expression, which were induced by PFKFB3 overexpression (Figure 5E) and attenuated the PFKFB3-impaired hematopoiesis-supporting ability of BM EPC after 5FU treatment (Figure 5F). These results indicate that FOXO3A contributes to PFKFB3-induced NF-κB pathway activation and impaired hematopoiesis-supporting ability of BM EPC after chemotherapy.

PFKFB3 is highly expressed in the damaged bone marrow endothelial cells of murine models of myelosuppression

To confirm the role of PFKFB3 in the progression of BM EC damage *in vivo*, murine models of myelosuppression in-

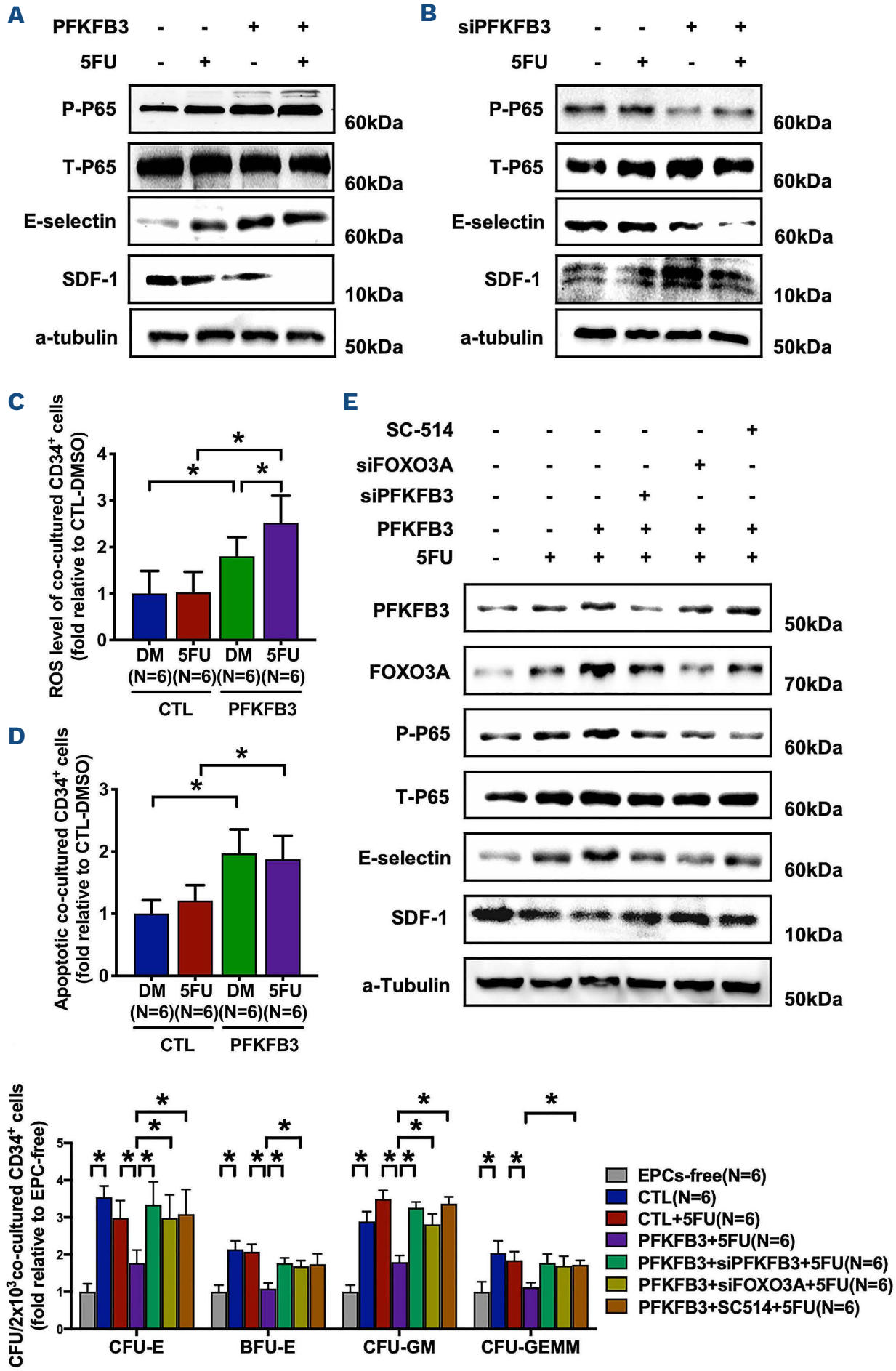

Figure 5. PFKFB3 induced NF-κB pathway activation and impaired the hematopoiesis-supporting ability of bone marrow endothelial progenitor cells *in vitro*. (A) Western blot analyses were performed on cultured bone marrow (BM) endothelial progenitor cells (EPC) transfected with a control vector or a vector expressing PFKFB3 and treated with dimethylsulfoxide (DMSO, DM) or 5-fluorouracil (5FU). (B) Western blot analyses were performed on the cultured BM EPC transfected with a non-targeting siRNA control or siRNA targeting PFKFB3 and treated or not with 5FU. (C, D) Intracellular levels of reactive oxygen species (C), and apoptosis rates (D) of BM CD34⁺ cells from healthy donors were analyzed after co-culture with BM EPC transfected with a control vector or a vector expressing PFKFB3 and with or without 5FU treatment. (E) Western blot analyses were performed on the cultured BM EPC with the indicated treatments. BM EPC in all groups were treated with lipopolysaccharide (100 ng/mL) for 4 h before collection to stimulate the expression of adhesion molecules. All the western blot analyses were performed in triplicate at least and representative images are shown. (F) The colony-forming unit plating efficiency of BM CD34⁺ cells from healthy donors following single culture (BM EPC-free group) and co-culture with BM EPC with the indicated treatments was analyzed. The data represent the mean ± standard error of mean. *$P \leq 0.05$. CFU: colony-forming unit; CFU-E: CFU erythroid; BFU-E: burst-forming unit erythroid, CFU-GM: CFU granulocyte-macrophage, CFU-GEMM: CFU granulocyte, erythrocyte, monocyte, macrophage.

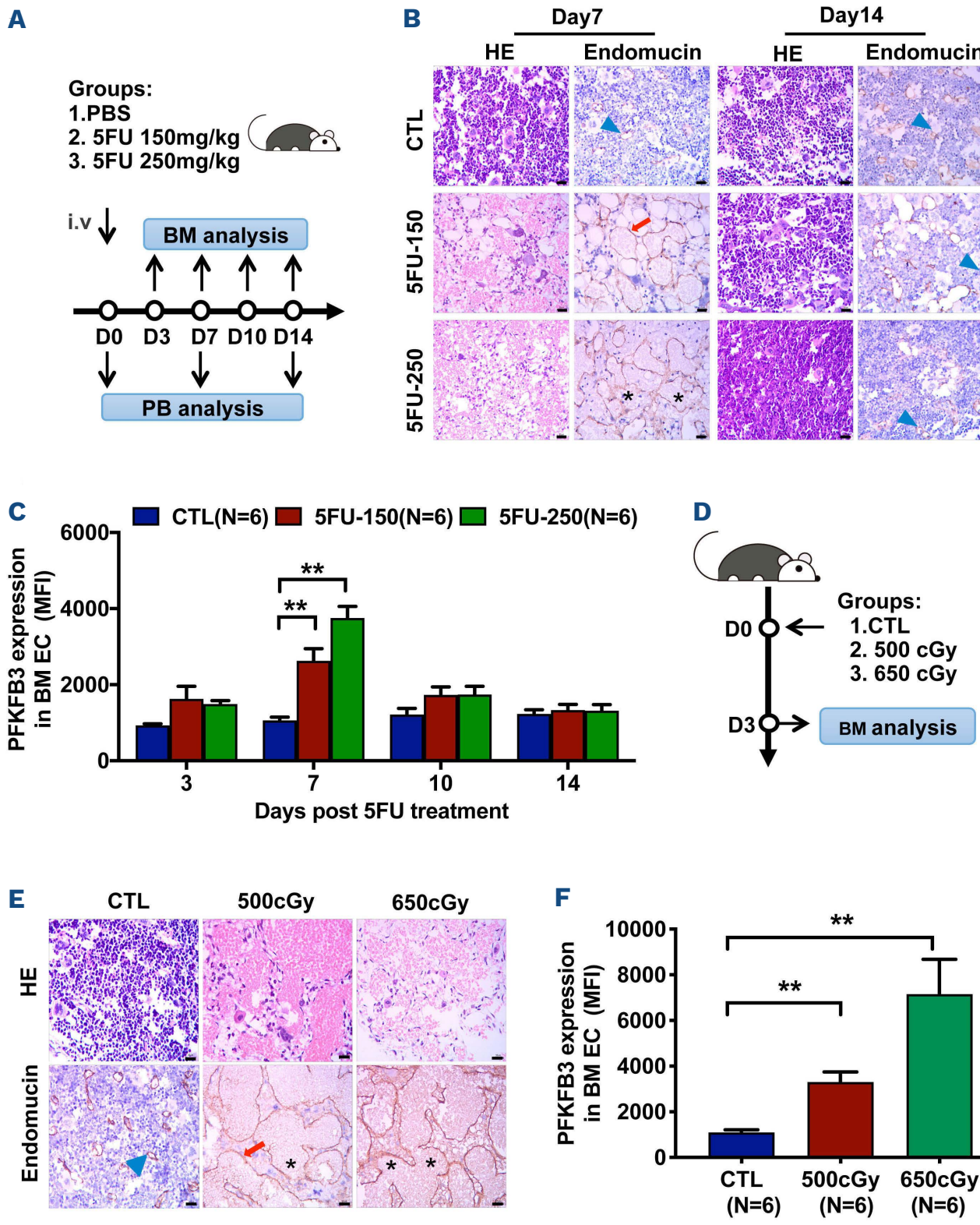

Figure 6. PFKFB3 was increased in damaged bone marrow endothelial cells in murine models of chemotherapy- or irradiation-induced myelosuppression. (A) Schematic diagram of the design of the study on the murine model of chemotherapy-induced myelosuppression. A single dose of 5-fluorouracil (5FU) 150 mg/kg or 250 mg/kg was injected intravenously into C57BL/6J adult female mice. (B) The mice were sacrificed at the indicated times after 5FU treatment. Femoral sections stained with hematoxylin and eosin (HE) and anti-endomucin antibody showed representative bone marrow (BM) damage and BM endothelial cell (EC) damage, respectively, 7 and 14 days after 5FU treatment when compared to that in the steady state control (CTL) group. Scale bar=10 µm. Normal (blue arrowhead), dilated (red arrow) and dilated and discontinuous (black asterisk) vessels were noted. (C) The levels of PFKFB3 in BM EC of the CTL mice and at the indicated times after 5-FU treatment. (D) Schematic diagram of the design of the study on the murine model of irradiation-induced myelosuppression. C57BL/6J female mice (6-9 weeks old) were exposed to a sublethal dose of irradiation of 500 cGy or 650 cGy. The mice were sacrificed at the indicated times after irradiation. (E) Femoral sections stained with HE and anti-endomucin antibody showed representative BM damage and BM EC damage, respectively, 3 days after irradiation, when compared to that in steady state (CTL group). Scale bar=10 µm. Normal (blue arrowhead), dilated (red arrow) and dilated and discontinuous (black asterisk) vessels were noted. (F) The levels of PFKFB3 in BM EC of CTL mice and at day 3 after irradiation. The data represent the mean ± standard error of mean. ***P*≤0.01.

duced by 5FU or irradiation were generated. As shown in Figure 6A-C and *Online Supplementary Figure S5*, the decrease in the levels of white blood cells, hemoglobin and platelets in peripheral blood, and the damage to BM hematopoietic tissue (shown by hematoxylin and eosin staining) and BM EC (visualized by immunohistochemistry) were greatest at day 7 and recovered by day 14 after 5FU treatment in mice. PFKFB3 levels in BM EC were significantly increased at day 7 and reverted to steady-state levels by 14 days after 5FU treatment. Moreover, severe BM EC damage

was observed at day 3 in mice irradiated with 500 cGy or 650 cGy (Figure 6D, E), which is consistent with the significantly increased PFKFB3 expression in BM EC (Figure 6F). These results support the concept that increased PFKFB3 is correlated with the damaged BM EC after chemotherapy or irradiation *in vivo*.

Bone marrow endothelial-specific PFKFB3 overexpression aggravates damage to the bone marrow endothelial cells and delays the recovery of hematopoiesis after chemotherapy in mice

To further assess the role of high expression of PFKFB3 within the BM EC *in vivo*, mice with BM EC-specific PFKFB3 overexpression were generated via an AAV-mediated gene delivery system under the control of an EC-specific Tie promoter, administered by injection into the BM (Figure 7A). The transduction efficiency was similarly high in BM EC from control (AAV-CTL) mice and from AAV-PFKFB3 (AAV-PF3) mice, whereas transduction was not detectable in HSC from these mice (*Online Supplementary Figure S6A-D*). Consistently, PFKFB3 expression was significantly increased in BM EC from AAV-PF3 mice, compared to AAV-CTL mice (*Online Supplementary Figure S6E*). Under steady state, AAV-PF3 mice displayed similar numbers of BM EC and hematopoietic parameters, compared to AAV-CTL mice. No differences were observed in the percentages

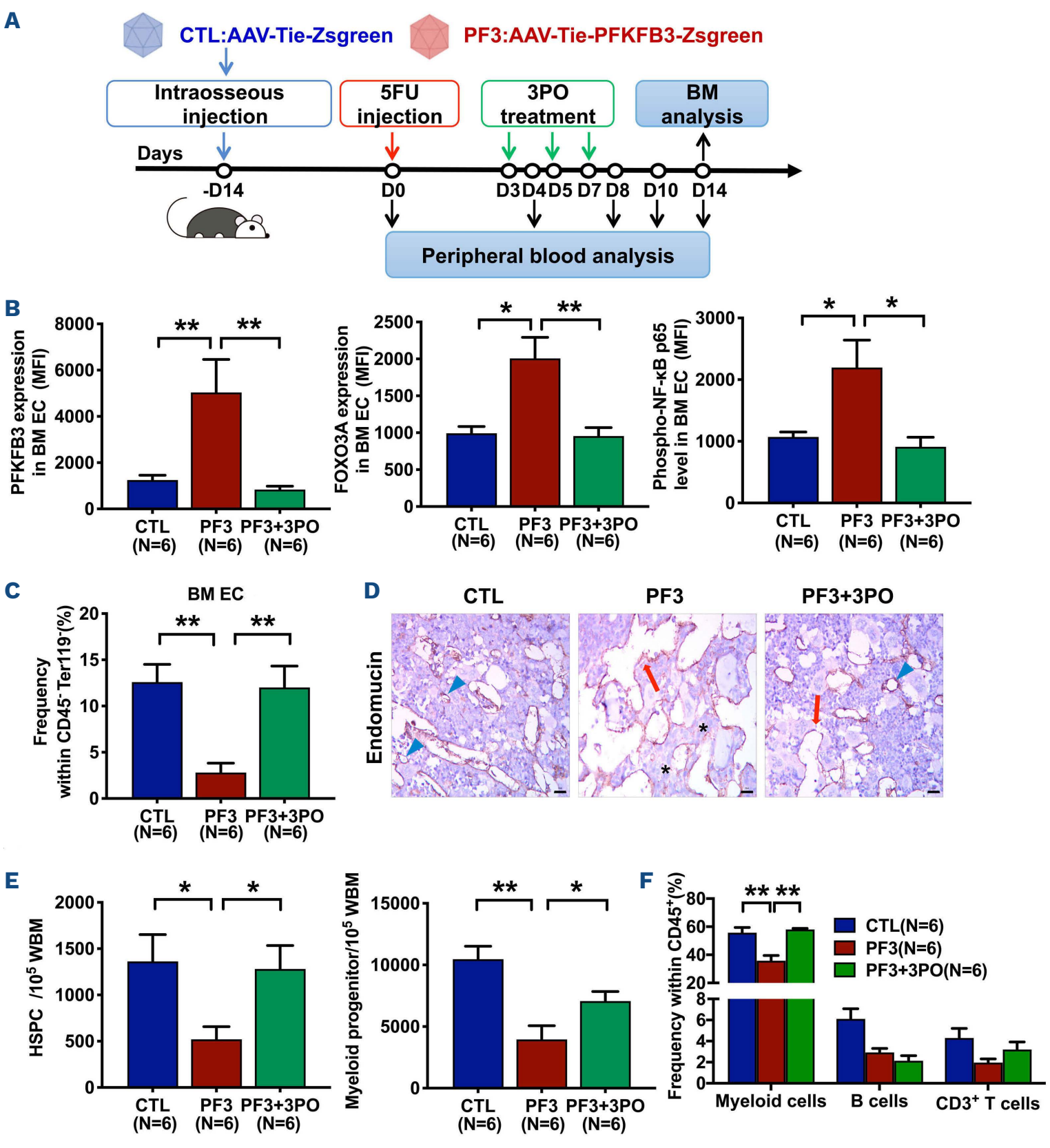

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Figure 7. Bone marrow endothelial cell-specific PFKFB3 overexpression aggravated damage to these cells and delayed the recovery of hematopoiesis after chemotherapy in mice. (A) Schematic diagram of the design of the study on mice with bone marrow (BM) endothelial cell (EC)-specific PFKFB3 overexpression. Adult C57BL/6J female mice (8-10 weeks) were given a single dose of a recombinant AAV-V_{EC} (an optimized adeno-associated virus variant for EC transduction encoding the PFKFB3 and Zsgreen genes under the control of an EC-specific Tie promoter; intraosseous injection 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 the Zsgreen gene under the control of the Tie promoter. Cohorts of mice were treated with 5-fluorouracil (5FU) 250 mg/kg at day 0 and then with 3PO or dimethylsulfoxide control on days 3, 5 and 7. The mice were sacrificed on day 14. (B) The levels of PFKFB3 (left), FOXO3A (middle) and phospho-NF-κB p65 (right) in BM EC were analyzed by flow cytometry. (C) Frequency of $CD31^+VE\text{-}Cadherin^+$ EC within the $CD45^-Ter119^-$ BM cells from mice given the indicated treatments were analyzed by flow cytometry. (D) Representative images of damaged BM EC in a murine femur stained with anti-endomucin antibody. Scale bars represent 10 µm. Normal (blue arrowhead), dilated (red arrow) and dilated and discontinuous (black asterisk) vessels were noted. (E) The frequencies of $Lineage^-cKIT^+SCA1^+$ hematopoietic stem and progenitor cells (left), and $Lineage^-cKIT^+SCA1^-$ myeloid progenitors (right) in whole BM cells were analyzed by flow cytometry. (F) Frequency of lineage-committed hematopoietic cells within the murine $CD45^+$ BM cells was analyzed by flow cytometry. The data represent the mean ± standard error of mean. **P*≤0.05. ***P*≤0.01. HSPC: hematopoietic stem and progenitor cells; WBM: whole bone marrow.

of HSPC and lineage cells, BM vessels and peripheral blood cell counts (*Online Supplementary Figure S6F-L*).

To investigate the role of PFKFB3 in damaged BM EC after chemotherapy *in vivo*, AAV-CTL mice and AAV-PF3 mice were treated with 5FU. At day 14 after 5FU treatment, the levels of PFKFB3, FOXO3A, and phospho-NF-κB-p65 were significantly elevated in AAV-PF3 mice (PFKFB3: 5037±1424 *vs*. 1249±206.9, *P*=0.009; FOXO3A: 2007±285.2 *vs*. 992.8±91.4, *P*=0.02; phospho-NF-κB-p65: 2197±444.3 *vs*. 1071±79.67, *P*=0.03) (Figure 7B) compared to AAV-CTL mice. Analysis of peripheral blood from AAV-CTL mice and AAV-PF3 mice revealed that endothelial-specific PFKFB3 overexpression delayed the recovery of peripheral blood cells following 5FU treatment (*Online Supplementary Figure S7A*). Consistently, we observed a significantly decreased number of BM EC (2.80±1.02 *vs*. 12.58±1.92; *P*=0.004) (Figure 7C, *Online Supplementary Figure S7B*) and impaired BM vessel structure (normal: 0.26±0.04 *vs*. 0.89±0.02, *P*=0.002; dilated: 0.37±0.02 *vs*. 0.11±0.02, *P*=0.002, dilated+discontinuous: 0.36±0.03 *vs*. 0, *P*=0.002) in AAV-PF3 mice (Figure 7D, *Online Supplementary Figure S7C*). In agreement with the BM EC damage in AAV-PF3 mice, at day 14 following 5FU treatment, we observed decreases in the percentages of HSPC (520.0±137.7 *vs*. 1363.0±289.1; *P*=0.04) (Figure 7E, left panel, *Online Supplementary Figure S8*), myeloid progenitors, including granulocyte-macrophage progenitors, common myeloid progenitors and megakaryocyte-erythrocyte progenitors: (3957±1110 *vs*. 10465±1059, *P*=0.004) (Figure 7E, right panel, *Online Supplementary Figure S8*) and hematopoietic lineages, especially myeloid cells (35.87±3.65 *vs*. 55.72±3.75; *P*=0.009) (Figure 7F) in the BM of AAV-PF3 mice. These data support the concept that PFKFB3 overexpression in BM EC triggers the BM EC damage and delays post-chemotherapy recovery of hematopoiesis *in vivo*.

To examine the effect of PFKFB3 inhibition on the repair of BM EC damage *in vivo*, the glycolysis inhibitor 3PO was administered to AAV-PF3 mice after 5FU treatment (Figure 7A). 3PO significantly decreased the expression of PFKFB3, FOXO3A and phospho-NF-κB p65 in BM EC (PFKFB3: 837.3±145.2 *vs*. 5037±1424, *P*=0.002; FOXOX3A: 956.2±112.6 *vs*. 2007±285.2, *P*=0.009; phospho-NF-κB p65: 911.7±155.6 *vs*. 2197±444.3, *P*=0.02) (Figure 7B). Analysis of peripheral blood from AAV-PF3 mice treated or not with 3PO revealed that PFKFB3 inhibition promoted peripheral blood cell recovery following 5FU treatment (*Online Supplementary Figure S7A*). Consistently, an increased number of BM EC (12±2.32% *vs*. 2.8±1.02%; *P*=0.009) (Figure 7C, *Online Supplementary Figure S7B*) and repaired BM vessel structures (normal: 0.72±0.04 *vs*. 0.26±0.04, *P*=0.002; dilated: 0.18±0.03 *vs*. 0.37±0.02, *P*=0.002; dilated+discontinuous: 0.10±0.02 *vs*. 0.36±0.03, *P*=0.002) (Figure 7D, *Online Supplementary Figure S7C*) were found in AAV-PF3 mice treated with 3PO. These mice displayed increased percentages of HSPC (1282.0±252.4 *vs*. 520.0±137.7; *P*=0.04) (Figure 7E, left panel, *Online Supplementary Figure S8*), myeloid progenitors (7057.0±779.9 *vs*. 3957±1110; *P*=0.04) (Figure 7E, right panel, *Online Supplementary Figure S8*) and hematopoietic lineages, especially myeloid cells (58.03±0.80 *vs*. 35.87±3.65; *P*=0.002) (Figure 7F) at day 14 after 5FU treatment, suggesting a BM EC-mediated hematopoietic protection.

3PO diminishes bone marrow endothelial progenitor cell damage in patients after chemotherapy

To further confirm the clinical relevance of the BM EPC damage induced by the glycolytic enzyme PFKFB3, we performed a prospective cohort study of 15 patients with acute leukemia who were scheduled for haploidentical HSCT to investigate the role of PFKFB3 in the damaged BM EPC after chemotherapy (all patients received an *in-vivo* T-cell-depleted myeloablative chemotherapy-based conditioning regimen). BM samples were collected from these patients before and after chemotherapy to compare PFKFB3 levels in their BM EPC. Considering the values from each patient, the PFKFB3 levels were significantly higher before chemotherapy than after it (5344±798.5 *vs*. 2564±377.2; *P*=0.0009) (Figure 8A). Moreover, glucose uptake and lactate production were significantly increased in BM EPC after chemotherapy (2.64±0.69-fold, *P*=0.03; 2.73±0.84-fold, *P*=0.03, respectively) (Figure 8B), suppor-

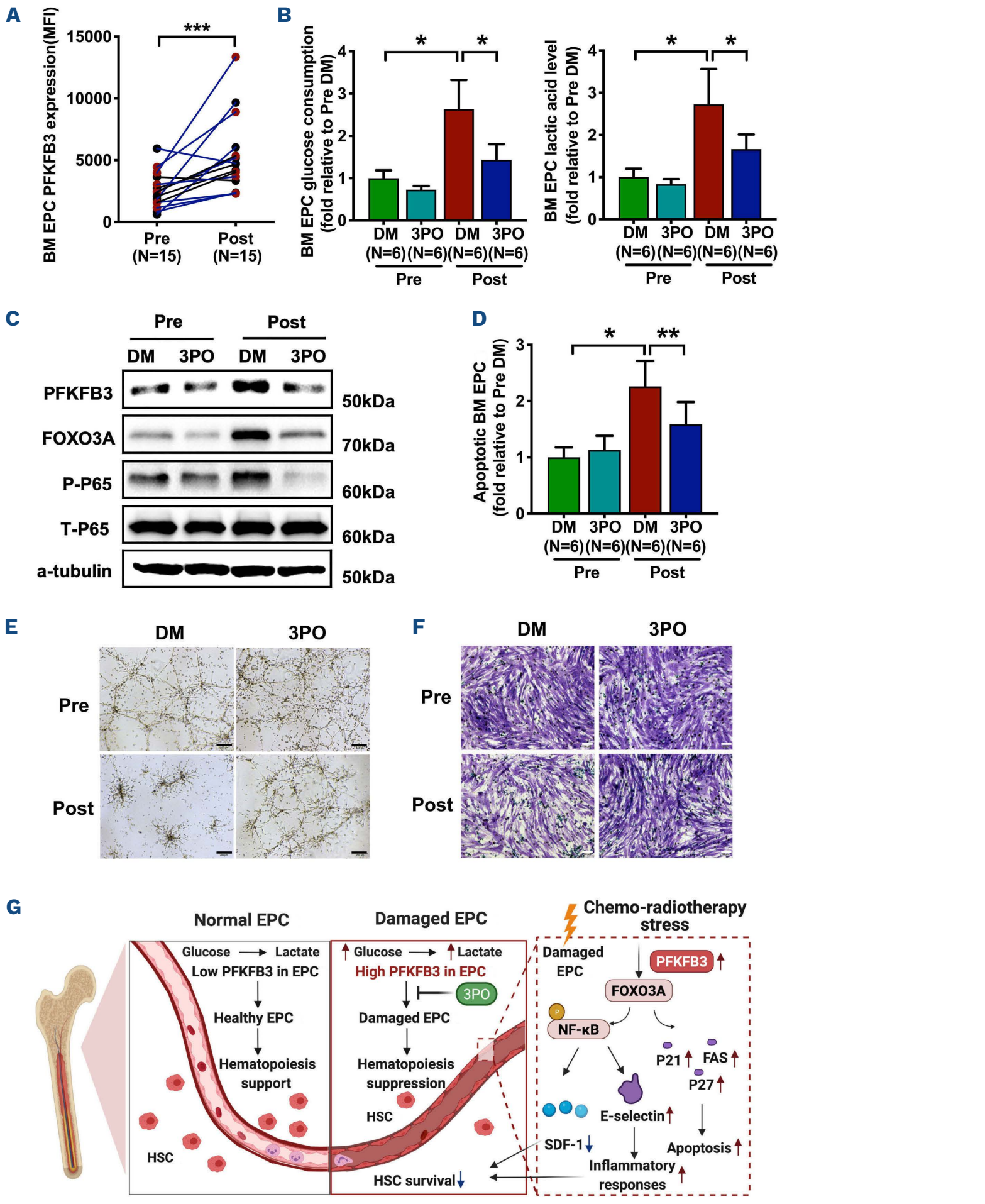

Figure 8. Glycolysis inhibition repaired the bone marrow endothelial cell damage of acute leukemia patients after chemotherapy *in vitro*. (A) The intracellular PFKFB3 levels in gated pre-cultured bone marrow (BM) endothelial progenitor cells (EPC) from acute leukemia patients before and after chemotherapy were analyzed by flow cytometry (mean fluorescence intensity, mean ± standard error of mean). Blue lines indicate patients with poor hematopoietic recovery after chemotherapy (absolute neutrophil count <1×10^9/L or platelet count <50×10^9/L). Red symbols indicate the six patients whose BM EPC were cultured for the following analysis. (B) The media of cultured BM EPC from patients before and after chemotherapy and treated or not with the glycolysis inhibitor 3PO were analyzed for glucose consumption (left) and lactate production (right). (C) Western blot analyses were performed on the cultured BM EPC from patients before and after chemotherapy treated or not with 3PO. All the western blot analyses were performed in triplicate at least and representative images are shown. (D) Apoptosis rates of cultured BM EPC from patients before and after chemotherapy treated or not with 3PO. (E) Representative images (scale bars=200 μm) of the tube length of BM EPC. (F) Representative images (scale bars=50 μm) of migrated BM EPC. (G) Graphical summary of the current study: schematic illustration of the contribution of glycolysis to the damage and repair of BM EPC after chemo-radiotherapy stress. *P≤0.05. **P≤0.01, ***P≤0.001. MFI: mean fluorescence intensity; DM: dimethylsulfoxide; HSC: hematopoietic stem cells.

ting the concept that BM EPC are hyperglycolytic in patients after chemotherapy. The levels of FOXO3A and phospho-NF-κB p65 in BM EPC (Figure 8C) were increased after chemotherapy, accompanied by a decrease in cell number (0.49±0.15-fold; *P*=0.03) (*Online Supplementary Figure S9A*), an increase in apoptosis (2.26±0.45-fold; *P*=0.03) (Figure 8D), as well as impaired tube formation (Figure 8E, *Online Supplementary Figure 9B*) and migration (Figure 8F, *Online Supplementary Figure S9C*), indicating that PFKFB3-induced glycolysis is hyperactivated in the damaged BM EPC of patients after chemotherapy.

The glycolysis inhibitor 3PO markedly decreased glucose consumption (1.43±0.37-fold *vs*. 2.64±0.69-fold; *P*=0.03) (Figure 8B) and lactate production (1.66±0.35-fold *vs*. 2.73±0.84-fold; *P*=0.03) (Figure 8B) in BM EPC in patients following chemotherapy. Moreover, 3PO treatment significantly downregulated the levels of FOXO3A and phospho-NF-κB p65 in BM EPC (Figure 8C), leading to an increased number of cells (0.69±0.16-fold *vs*. 0.49±0.15-fold; *P*=0.008) (*Online Supplementary Figure S9A*), reduced apoptosis (1.59±0.39-fold *vs*. 2.26±0.45-fold; *P*=0.004) (Figure 8D), improved tube formation (Figure 8E, *Online Supplementary Figure S9B*) and better migration (Figure 8F, *Online Supplementary Figure S9C*) of BM EPC after chemotherapy. These results suggest that inhibition of PFKFB3 could attenuate the damaged BM EPC in patients after chemotherapy.

Discussion

BM EC damage is responsible for impaired hematopoietic regeneration following chemo-radiotherapy.[4,9,10,13-18,47] Although targeting BM EC in mice has recently been shown to be a viable strategy to accelerate hematopoietic recovery, the strategy is still in its infancy.[9-11,19,22] The current study demonstrated the critical role and underlying mechanism of action of PFKFB3 in BM EPC damage after chemotherapy or irradiation. The role of PFKFB3-mediated hyperglycolysis in BM EPC damage was confirmed in patients with poor graft function after allogeneic HSCT and in acute leukemia patients after chemotherapy, following *in vitro* studies , *in vivo* studies using murine models of myelosuppression induced by 5FU or irradiation, and a murine model of BM EC-specific PFKFB3 overexpression. Importantly, we first demonstrated that the glycolytic enzyme PFKFB3 mechanistically triggers BM EPC damage after chemotherapy via FOXO3A-induced pro-apoptotic gene expression and NF-κB pathway activation (Figure 8G). Thus, our findings may indicate a potential therapeutic target for patients exposed to chemo-radiotherapy. Glycolysis-induced hyperproliferation was reported and became an attractive target in vascular diseases, such as pulmonary hypertension and tumor vessels.[24,25] BM EPC are considered to be the primitive precursor of EC and a critical contributor to vascular repair.[20,21] However, the metabolic regulatory mechanism in BM EPC, especially in the damaged BM EPC after chemo-radiotherapy, is largely unknown. In the current study, the role of glycolysis in BM EPC, especially in BM EPC damage following chemo-radiotherapy, was investigated using clinical models of BM EPC damage-associated poor hematopoiesis, *in vitro* and *in vivo* studies. We found that PFKFB3 was upregulated in the damaged BM EPC in patients with poor graft function after allogeneic HSCT and acute leukemia patients after chemotherapy. Moreover, PFKFB3 overexpression aggravated BM EPC damage both *in vitro* and in murine models of chemo-radiotherapy damage. By contrast, PFKFB3 inhibition attenuated BM EPC damage both *in vitro* and *in vivo*. These results provide direct evidence that hyperglycolysis induced by PFKFB3 contributes to BM EPC damage after chemotherapy or irradiation.

With regard to the mechanism underlying PFKFB3-induced BM EPC damage, we found that PFKFB3 facilitated pro-apoptotic gene expression via FOXO3A after 5FU treatment *in vitro* and *in vivo*. As previously reported,[48,49] the transcription factors of the FOXO family, especially FOXO3A, are involved in the regulation of many cellular processes, including apoptosis. They play critical roles in the response to environmental stress, such as genotoxic and metabolic stress, rather than being indispensable mediators of normal physiology. Our data revealed for the first time that the glycolytic enzyme PFKFB3 could trigger BM EPC damage by inducing EPC apoptosis via the activation of FOXO3A. Moreover, our work suggests a link between PFKFB3 and the activated NF-κB signaling pathway and the impaired ability of BM EPC to support hematopoiesis after injury, which is consistent with previous reports indicating the pivotal role of NF-κB in BM EC damage and impaired hematopoietic regeneration in mice.[9]

However, we are aware that the details of the mechanism through which PFKFB3 regulates the hematopoiesis-supportive function of BM-EPC have yet to be fully elucidated: for example, is the effect of 3PO and/or PFKFB3-overexpression in BM EPC on hematopoietic progenitors rather than on HSC or HSPC? The relationship between the immune microenvironment and BM EPC damage also needs to be explored further in the future. Moreover, we noted that PFKFB3 overexpression increased the expression of CXCL12 (SDF-1) mRNA without 5FU treatment, whereas PFKFB3 overexpression significantly inhibited the expression of CXCL12 mRNA and protein after 5FU treatment, indicating that chemotherapy may change the protein function and downstream cell signaling of PFKFB3. Additionally, the mechanism by which chemotherapy upregulates PFKFB3 remains to be clarified. It has been reported that ROS induce PFKFB3 in leukemia cells and promote glycolysis,[50] although the mechanism by which

ROS regulate PFKFB3 on the damaged BM EPC after chemotherapy or irradiation needs to be further investigated.

In summary, the current work demonstrates that increased levels of the glycolytic enzyme PFKFB3 contribute to BM EPC damage after chemo-radiotherapy. Although further validation is required, our data suggest that targeting PFKFB3 in BM EPC is a potential, future therapeutic approach for myelosuppression, not only for patients with leukemia but also for those with other cancers.

Disclosures

No conflicts of interest to disclose.

Contributions

XJH and YK designed the study, supervised the preparation of the manuscript, and contributed equally to the study. ZSL and YK performed the research, analyzed the data and wrote the manuscript. All other authors participated in the collection of patients' data. All of the authors read and approved the final manuscript.

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Data-sharing statement

The data in this study are available from the corresponding author upon reasonable request.

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