Supplementary Materials

Prophylactic Oral N-acetyl-L-cysteine Reduced Poor Hematopoietic Reconstitution by Improving Endothelial Cell after Haplo-identical Transplantation

Authors: Yuan Kong[#], Yu Wang[#], Yuan-Yuan Zhang, Min-Min Shi, Xiao-Dong Mo, Yu-Qian Sun, Ying-Jun Chang, Lan-Ping Xu, Xiao-Hui Zhang, Kai-Yan Liu, Xiao-Jun Huang*

Supplementary methods

Transplantation protocols

Donor selection, human leucocyte antigen typing, graft harvesting, conditioning regimen (ATG and G-CSF based), acute graft versus host disease (aG*v*HD) prophylaxis and Cytomegalovirus(CMV) prophylaxis and treatment were performed as previously described[1-8]. Comorbidities in HSCT recipients were assessed according to the hematopoietic cell transplantation-specific comorbidity index[5].

Donors were mobilized with recombinant human G-CSF(rhG-CSF) (Filgrastim, Kirin, Japan) at 5μg/kg/d injected subcutaneously for 5 consecutive days. Transplanted grafts were G-CSF-mobilized BM cells and PB cells from haploidentical sibling donors. The minimal mononuclear cells (MNCs) from BM and PB grafts were 6×10^8 to 8×10^8/kg of recipient weight. The minimal $CD34^+$ cells from BM and PB grafts were 1×10^6 to 2×10^6/kg of

recipient weight. The conditioning regimen consisted of cytarabine (4g/m^2/d IV for 2 days) on day -10, -9, busulfan(0.8mg/kg IV every 6h, for a total of 12 doses) on day -8, -7, and -6, cyclophosphamide(1.8g/m^2/d IV for 2 days) on days -5 and -4, oral semustine (250mg/m^2 for 1 day) on day -3, and rabbit anti-thymocyte globulin(ATG;2.5mg/kg/d IV for 4 days; Sanofi, France) on days -5 to -2.

After allo-HSCT, rhG-CSF(5μg/kg/day) was administered to recipients from day +6 until ANCs were >0.5×10^9/L for 3 consecutive days. Platelet and red blood cell transfusions were performed for patients when platelet counts were <20×10^9/L or hemoglobin levels were <70 g/L.

Chimerism analyses were performed by DNA fingerprinting for short tandem repeats in blood samples and/or by chromosome fluorescent in situ hybridization of BM samples. Complete donor chimerism was defined as no recipient hematopoietic or lymphoid cells detected (sensitivity>0.1% recipient signals)[4].

aGvHD prophylaxis and treatment[4,6,7]

Transplant recipients received cyclosporine A(CsA), mycophenolate mofetil(MMF), and short-term methotrexate (MTX) as GvHD prophylaxis. The dosage of CsA was 2.5mg/kg/d IV from day -9 and was switched to oral dosage when bowel function returned to normal. Whole-blood CsA concentrations were monitored weekly and adjusted to maintain a trough concentration of 150 to 250 ng/mL. CsA dosage was gradually decreased in subjects without GvHD on day +180 but was continued in patients with GvHD. MMF was administered

orally, 0.5g every 12h, from day -9, and discontinued until engraftment after allo-HSCT. MTX 15mg/m^2 IV was administered on day +1, and reduced to 10mg/m^2 on days +3, +5, and +11 post-HSCT.

aGvHD was treated with first-line steroids (methylprednisolone 1-2 mg/kg/d) and by resumption of full-dose CsA administration. Second- or third-line immunosuppressive therapies such as CD25 monoclonal antibody (Basiliximab; Novartis Pharma Stein AG, Basel, Switzerland), MMF, tacrolimus, or MTX were administered in cases of steroid-refractory aGvHD.

Cytomegalovirus(CMV) prophylaxis and treatment[7,8]

CMV infection was monitored based on plasma CMV DNA using a real-time polymerase chain reaction(PCR) assay twice weekly for at least up to 12 weeks after the first infusion. For prophylaxis against CMV disease, ganciclovir (10mg/kg/d) was administered IV twice daily from day -9 to day -2 pre-HSCT. Patients began preemptive therapy with ganciclovir(10mg/kg/d) or foscarnet(90-120mg/kg/d) when CMV-DNA was positive for two consecutive tests and discontinued until the CMV DNA monitoring was negative for two occasions.

Quantity and function analysis of BM ECs

The percentage and function of BM ECs were evaluated at -14D, 0D pre-HSCT, and +1M, +2M post-HSCT only in the patients who were willing to provide BM samples after the written consent. The functions of BM ECs and EPCs, including the intracellular ROS levels, double positive staining and migration

assays, were evaluated pre- and post-HSCT. The numbers of patients performed for the independent experiments were labeled in each figure.

Characterization of primary BM ECs and EPCs

As previously described[9-15], the BM ECs were identified by mouse anti-human CD34, mouse anti-human CD45, and vascular endothelial growth factor receptor 2 (CD309) monoclonal antibodies (Becton Dickinson Biosciences, San Jose, CA). The mouse anti-human CD133 monoclonal antibody (Becton Dickinson) and the aforementioned ECs markers were used to identify EPCs[11,12,14]. The percentages of BM ECs and EPCs were analyzed using a BD LSRFortessa cell analyzer (Becton Dickinson), and indicated by the percentages of $CD34^+CD309^+$ cells and $CD34^+CD309^+CD133^+$ cells in total BM mononuclear cells (BMMNCs), respectively. Aliquots of isotype-identical antibodies served as negative controls.

DiI-Ac-LDL uptake and FITC-UEA-1 binding assay

The double positive staining was performed as previously reported[11,12,14,16]. The 7-day cultivated BMMNCs (1×10^6 per well) in EGM-2-MV-SingleQuots (Lonza, Walkersville, MD, USA) and 10% FBS (Gibco, MA, USA) were incubated with 10 μg/ml DiI-Acetylated Low Density Lipoprotein (DiI-Ac-LDL, Life Technologies, Gaithersburg, MD, USA) at 37°C. After 4 h, the cells were washed and fixed in 4% pre-chilled paraformaldehyde for 10 min. After washes, the fixed cells were incubated for 1h with 10 μg/ml FITC-labeled Ulex Europaeus Agglutinin-I (FITC-UEA-I, Sigma, USA) at room temperature. To evaluate the number of double-positive-stained ECs per well, three random

power fields were counted using a fluorescence microscope (Olympus, Tokyo, Japan).

Migration assay

Cell migration using a transwell chamber (Corning, NY, USA) was performed as previously described[11,12,14]. The cells were trypsinized and seeded in the upper chambers while the medium was added to the lower chamber. The cells were cultured for 24 h, and migrated cells were fixed with paraformaldehyde for 30 min. Then, cells on the bottom surface of the membrane were stained with crystal violet for 20 min. Cell images were obtained under a phase-contrast microscope (Olympus), and the migrated cells were counted manually in 3 random fields/sample.

Histological analysis of human BM tissues

Histological immunofluorescence staining of BM trephine biopsies obtained from the posterior superior iliac spine of the patients was performed as previously described[12,14,17]. The frozen sections were used for immunofluorescence staining. The mouse anti-human CD34 (Becton Dickinson) and rabbit anti-human CD133 (Abcam, MA, USA) antibodies were stained in frozen tissue section slides and incubated at 4°C overnight. The goat anti-rabbit 555 and donkey anti-mouse 488 (Invitrogen, OR, USA) antibodies were added at room temperature for 1 h. DAPI was applied to stain the nuclei, and the prepared slides were analyzed under a Leica TCS SP8 microscope (Leica Microsystems, Wetzlar, Germany). The number of EPCs was determined as the number of $CD34^+CD133^+$ cells per high-power field in BM

trephine biopsies. The enumerations were performed by 2 blinded observers.

References

1. Wang Y, Chang YJ, Xu LP, et al. Who is the best donor for a related HLA haplotype-mismatched transplant? *Blood*. 2014;124(6):843-850.

2. Xu L, Chen H, Chen J, et al. The consensus on indications, conditioning regimen, and donor selection of allogeneic hematopoietic cell transplantation for hematological diseases in China-recommendations from the Chinese Society of Hematology. *J Hematol Oncol*. 2018;11(1):33.

3. Wang Y, Liu QF, Xu LP, et al. Haploidentical vs identical-sibling transplant for AML in remission: a multicenter, prospective study. *Blood*. 2015;125(25):3956-3962.

4. Wang Y, Wu DP, Liu QF, et al. In adults with t(8;21)AML, posttransplant RUNX1/RUNX1T1-based MRD monitoring, rather than c-KIT mutations, allows further risk stratification. *Blood*. 2014;124(12):1880-1886.

5. Sorror ML, Maris MB, Storb R, et al. Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. *Blood*. 2005;106(8):2912-2919.

6. Chang YJ, Xu LP, Wang Y, et al. Controlled, Randomized, Open-Label Trial of Risk-Stratified Corticosteroid Prevention of Acute Graft-Versus-Host Disease After Haploidentical Transplantation. *J Clin Oncol*. 2016;34(16):1855-1863.

7. Huang XJ, Liu DH, Liu KY, et al. Treatment of acute leukemia with unmanipulated HLA-mismatched/haploidentical blood and bone marrow transplantation. *Biol Blood Marrow Transplant*. 2009;15(2):257-265.

8. Wang Y, Fu HX, Liu DH, et al. Influence of two different doses of antithymocyte globulin in patients with standard-risk disease following haploidentical transplantation: a randomized trial. *Bone Marrow Transplant*. 2014;49(3):426-433.

9. Kong Y, Chang YJ, Wang YZ, et al. Association of an impaired bone marrow microenvironment with secondary poor graft function after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2013;19(10):1465-1473.

10. Kong Y, Wang YT, Hu Y, et al. The bone marrow microenvironment is similarly impaired in allogeneic hematopoietic stem cell transplantation patients

with early and late poor graft function. *Bone Marrow Transplant*. 2016;51(2):249-255.

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

12. 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.

13. Werner N, Kosiol S, Schiegl T, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med*. 2005;353(10):999-1007.

14. Kong Y, Shi MM, Zhang YY, et al. N-acetyl-L-cysteine improves bone marrow endothelial progenitor cells in prolonged isolated thrombocytopenia patients post allogeneic hematopoietic stem cell transplantation. *Am J Hematol*. 2018;93(7):931-942.

15. Kong Y, Hu Y, Zhang XH, et al. Association between an impaired bone marrow vascular microenvironment and prolonged isolated thrombocytopenia after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2014;20(8):1190-1197.

16. Bao XM, Wu CF, Lu GP. Atorvastatin inhibits homocysteine-induced oxidative stress and apoptosis in endothelial progenitor cells involving Nox4 and p38MAPK. *Atherosclerosis*. 2010;210(1):114-121.

17. Duan CW, Shi J, Chen J, et al. Leukemia propagating cells rebuild an evolving niche in response to therapy. *Cancer Cell*. 2014;25(6):778-793.

Supplementary Table 1. Univariate and multivariate analysis of risk factors for the occurrence of Poor Hematopoietic Reconstitution(PHR) at +2-month after allo-HSCT

Factors	Univariate analysis	Multivariate analysis [a)]		
	P- value	HR	95% CI	*P*- Value
Bone marrow microenvironment pre-HSCT				
[b)] BM ECs, <0.1% *vs*. ≥0.1%	**0.005***	**6.11**	**1.72-21.67**	**0.005****
Demographics				
Gender, female *vs*. male	**0.84**			
Age at HSCT (years) < 32.5 *vs*. ≥32.5	**0.59**			
Disease characteristics pre-HSCT				
Diagnosis, ALL *vs*. AML	**0.18**			
Status, high-risk *vs*. standard-risk	**0.006***			
Chemotherapy Pre-HSCT				
Pre-HSCT cycles, < 4 cycles *vs*. ≥4 cycles	**0.33**			
HCT-CI pre-HSCT	**0.43**			
≥3 *vs*. 0-2				
Transplant characteristics				
HLA mismatch, 1-3 *vs*. 4-9	**0.50**			
Gender mismatch, FM *vs*. FF *vs*. MF *vs*. MM	**0.11**			
ABO mismatch, no *vs*. minor *vs*. major mismatch	**0.57**			
Transplanted total MNCs, <7.95×10^8/ kg *vs*. ≥7.95×10^8/ kg	**0.83**			
Transplanted $CD34^+$ cells, <2.88×10^6/ kg *vs*. ≥2.88×10^6/ kg	**0.24**			
Transplant-related complications				
aGvHD prior to PHR, yes *vs*. no	**0.12**			
CMV infection prior to PHR, yes *vs*. no	**0.04***			
CMV reactivation treated with ganciclovir	**0.22**			

Abbreviations: PHR, Poor Hematopoietic Reconstitution; allo-HSCT, allogeneic hematopoietic stem cell transplantation; HR, hazard ratio; CI, confidence interval; BM ECs, bone marrow endothelial cells; HCT-CI, hematopoietic cell transplantation-specific comorbidity index; HLA, human leukocyte antigen; FM, female to male; FF, female to female; MF, male to female; MM, male to male; GvHD, graft-versus-host disease; CMV, cytomegalovirus.

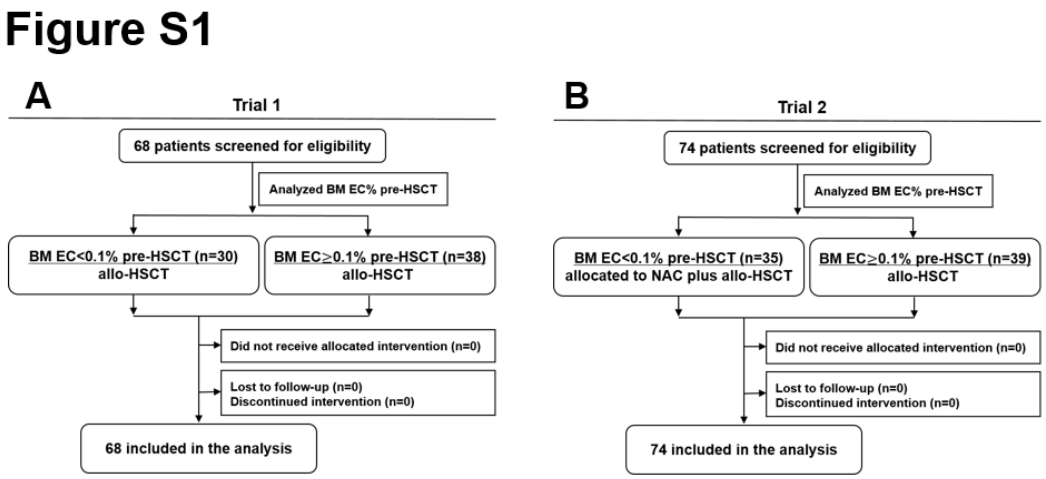
a) To avoid potential confounding factors, multivariate Cox proportional hazard models were assessed for interaction terms with covariates. The variables included in the Cox models exhibited $P<0.10$ after univariate analyses. The final multivariate models were constructed using a forward stepwise selection approach.

b) BM ECs pre-HSCT were separated into 2 risk groups according to the ROC cut-off percentage of BM ECs pre-HSCT (0.1%) with a sensitivity of 80% and specificity of 66%.

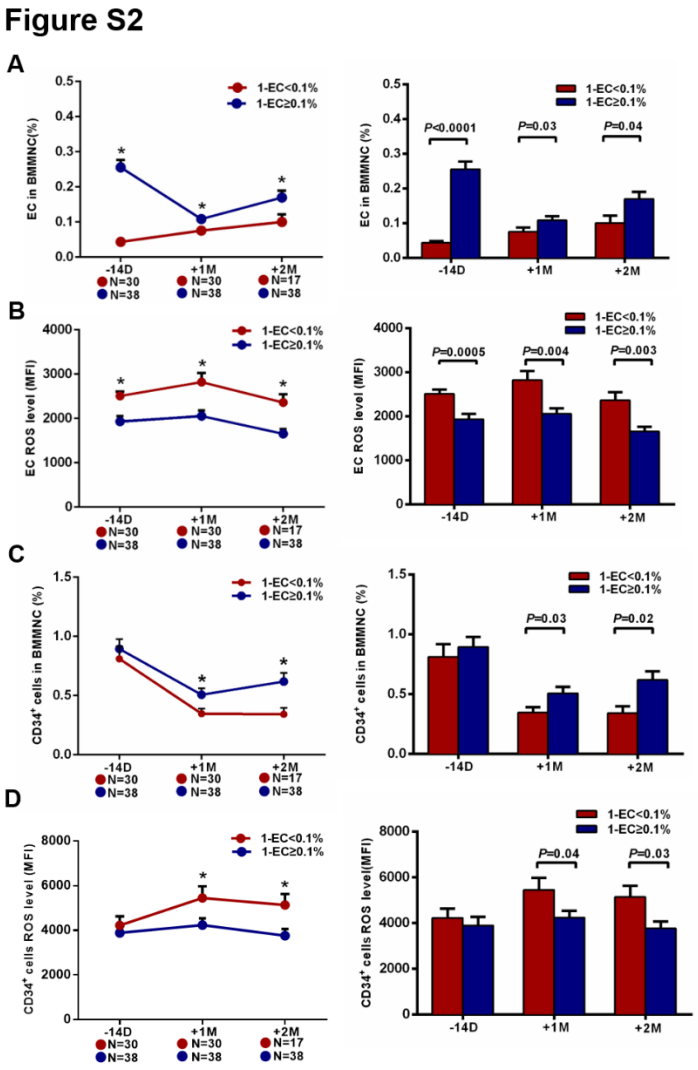
* $P<0.10$ in univariate analysis

** $P<0.05$ in multivariate analysis

Supplementary Figure 1.

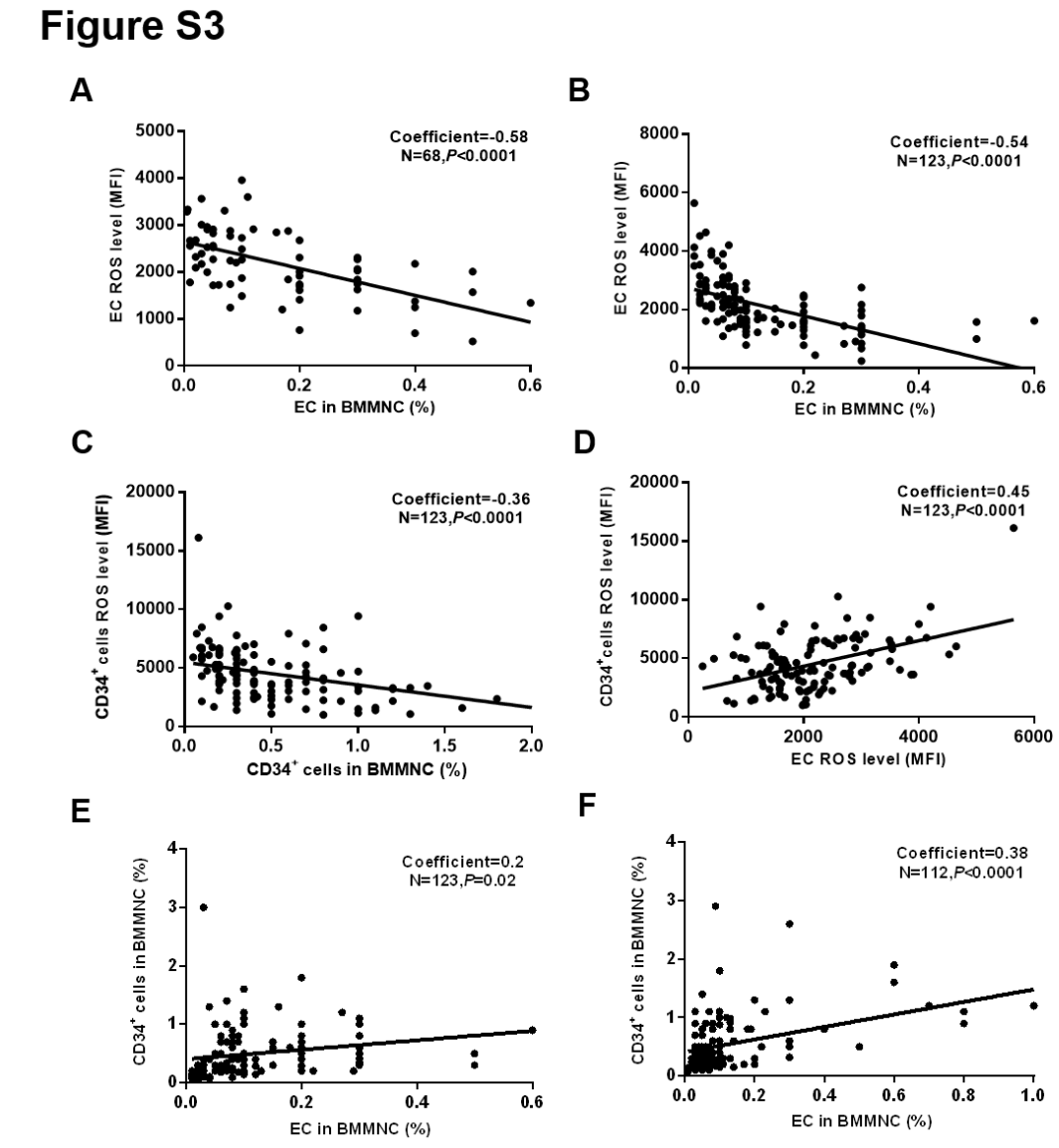


Supplementary Figure2.



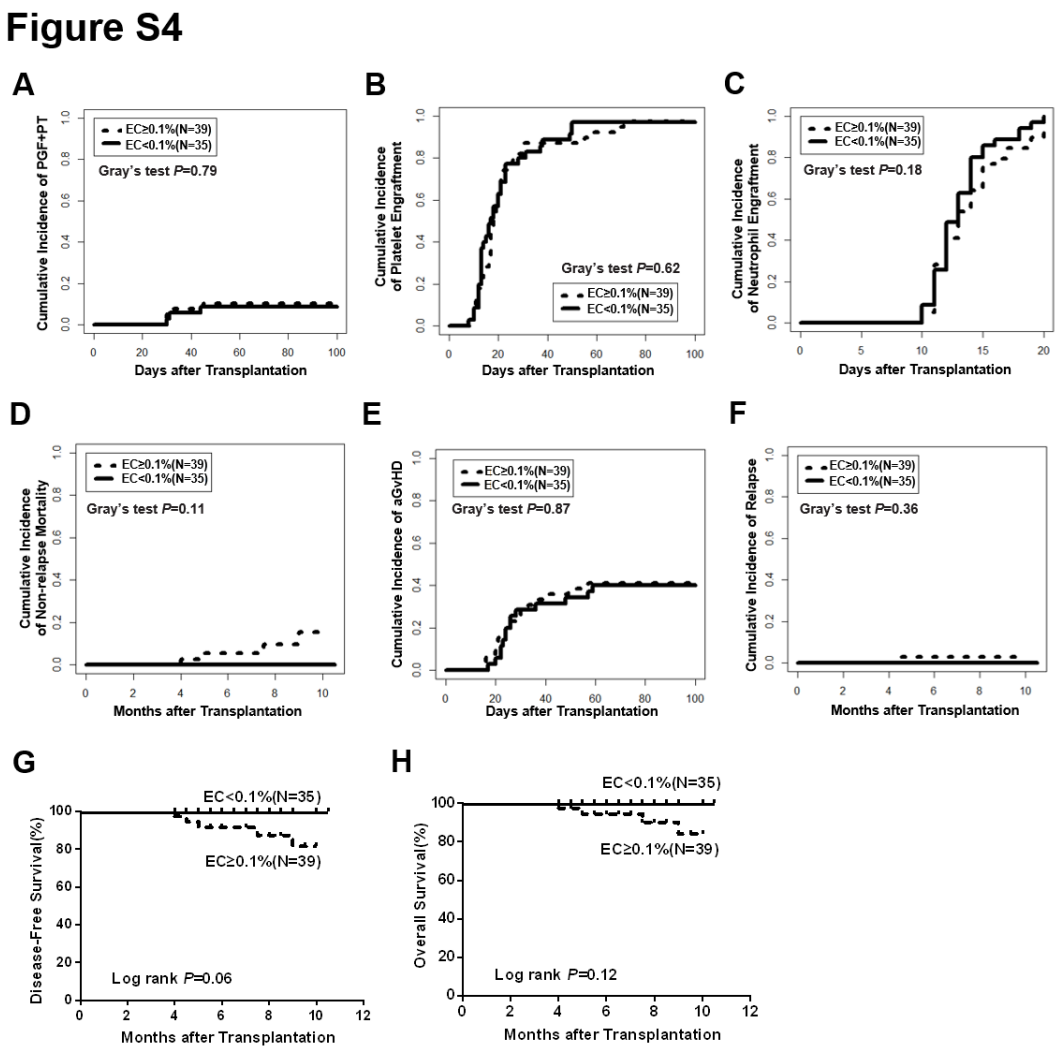
Supplementary Figure 2. Impaired BM ECs pre-HSCT and the defective dynamic reconstitution of BM ECs and CD34$^+$ cells were demonstrated at early time points post-HSCT in EC<0.1% group. The dynamic reconstitution (left panel) and statistical analyses (right panel) of BM **(A)**EC percentages, **(B)**EC ROS levels, **(C)**CD34$^+$ cells percentages, and **(D)**CD34$^+$ cell ROS levels in pre-HSCT EC<0.1% group at -14D pre-HSCT, and +1M, +2M post-HSCT were compared with those in EC≥0.1% group in the first clinical trial. The data were expressed as the mean and SEM. Statistical analyses were performed using one-way analysis of variance for comparisons among the groups. Subject variables were compared using the chi-squared test for categorical variables and the Mann-Whitney U test for continuous variables. All *P*-values <0.05 were considered statistically significant. * means *P* <0.05.

Supplementary Figure 3.



Supplementary Figure 3. Correlation analyses of BM ECs, CD34$^+$ cells, and their ROS levels pre-HSCT and post-HSCT. Inverse correlations were found **(A)**between BM ECs percentages and their ROS levels before allo-HSCT, **(B)**between BM ECs percentages and their ROS levels post-HSCT, **(C)**between BM CD34$^+$ cell percentages and their ROS levels post-HSCT. The positive correlations were demonstrated **(D)**between the ROS levels of BM ECs and BM CD34$^+$ cells, **(E)**between the percentages of BM ECs and BM CD34$^+$ cells post-HSCT in the first clinical trial, and **(F)**the percentages of BM ECs and BM CD34$^+$ cells post-HSCT in the second clinical trial. Correlation analyses were performed using Pearson correlation. All *P*-values <0.05 were considered statistically significant.

Supplementary Figure 4.



Supplementary Figure 4. Similar transplant outcomes were demonstrated between EC<0.1% with prophylactic NAC intervention group and EC≥0.1% group. In the second clinical trial, the cumulative incidences of **(A)**PGF and PT at +2M, **(B)**platelet engraftment, **(C)**neutrophil engraftment within 100 days post-HSCT, **(D)**non-relapse mortality, **(E)**acute GvHD, **(F)**relapse, probabilities of **(G)**disease free survival(DFS), and **(H)**overall survival(OS) were compared between EC<0.1% with prophylactic NAC intervention group and EC≥0.1% group. Cumulative incidence curves were used in a competing risk setting and were compared using the Gray test. Probabilities of DFS and OS were estimated with the Kaplan–Meier method and were compared using the log-rank test. All *P*-values <0.05 were considered statistically significant.