ARHGEF12 regulates erythropoiesis and is involved in erythroid regeneration after chemotherapy in acute lymphoblastic leukemia patients

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Supplementary Data

Supplementary Methods

Cell culture and induced differentiation

The stable ARHGEF12 knockdown (shRNA1, shRNA2) and none target control infected k562 or 32D cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum at 37°C and 5% CO_2. When differentiation was induced, exponentially grown K562 cells were treated with 30 μM hemin (Sigma–Aldrich) for 48 hours. Erythroid differentiation of K562 cells was assessed by the benzidine cytochemical test and the benzidine-positive cells represented more mature erythroid cells.

ShRNA constructs and lentiviral transduction

PLKO.1 lentiviral human ARHGEF12-puro constructs were obtained from Sigma-Aldrich. Lentiviruses were produced in 293T cells by collecting the supernatant and filtering through 0.2μm filter. K562 cells were transfected with fresh made virus containing supernatant at $5x10^6$ cells/ml with centrifuge 1500 rpm for 90 mins and incubated for 18 h. Transfections were repeated at day 2, recovered in fresh media after 8 h incubation. For western blot or Rho-GTP pull down assay, the transduced cells were puromycin selected started at day 3 for 3 days under the desired concentration. Decreased expression of ARHGEF12 was verified by immunoblot analysis.

Zebrafish maintenance and strains

Zebrafish were staged, raised, and maintained as described[1]. The wild-type (Tübingen) and transgenic lines (*cd41:eGFP*[2], *gata1:DsRed*[3], *gata1: eGFP*[4], *pu.1: GFP*[5],*fli1: eGFP*[3], *lyz: GFP*[6]) were used. Embryos were treated with 0.0045% phenylthiourea from Sigma-Aldrich at 12 hours hpf to prevent pigmentation.

o-Dianisidine staining

Staining of hemoglobin by o-Dianisidine was performed as previously described[7].

Cytology assay

Blood cell isolation was performed as described[8]. The 0.9× PBS containing 50 U/mL heparin, 5% fetal bovine serum, and 0.006% tricaine was used as isolation buffer. For cytospin, the cell collections were centrifuged at 300 ×g for 5 minutes onto glass slides. Wright-Giemsa stain was performed according to the manufacturer's instructions (Baso). Foro-Dianisidine staining, the slides were stained with o-Dianisidine solution for 5 minutes (RhoA). The micrographs were taken by a microscope Nikon ECLIPSE 80i with 100× oil immersion lens and Nikon ACT-1 software.

Western blot

Cells were lysed with 1× Cell Lysis Buffer(Cell signaling technology) in the presence of 1 mM PMSF ,1× PhosSTOP (Roche). Soluble lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Bio-Rad). Membranes were probed with specific antibodies. Signals were visualized using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). β-Actin or α- tubulin was used as a loading control.

For zebrafish western blot, embryos at indicated developmental stages were deyolked as previously described[9]. Embryos were homogenized in lysis buffer (20 mM Tris HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 10% glycerol, and 0.1% Triton X-100). Signals were detected with rabbit anti-HA polyclonal antibody (1:1000, Clontech) overnight at 4°C, followed by incubation with horseradish peroxidase–conjugated secondary antibody (1:10,000).

Colony assay

Cultured cells or primary bone marrow cells were plated in triplicate at a density of $1*10^5$cells in 1 ml of MethoCult M3334 classic medium, or $5*10^4$ cells in 1 mL of MethoCult M3436 medium The CFU-E(colony-forming unit-erythroid) and BFU-E(burst-forming unit-erythroid) colonies were defined according to the standard criteria.

Supplementary Figures

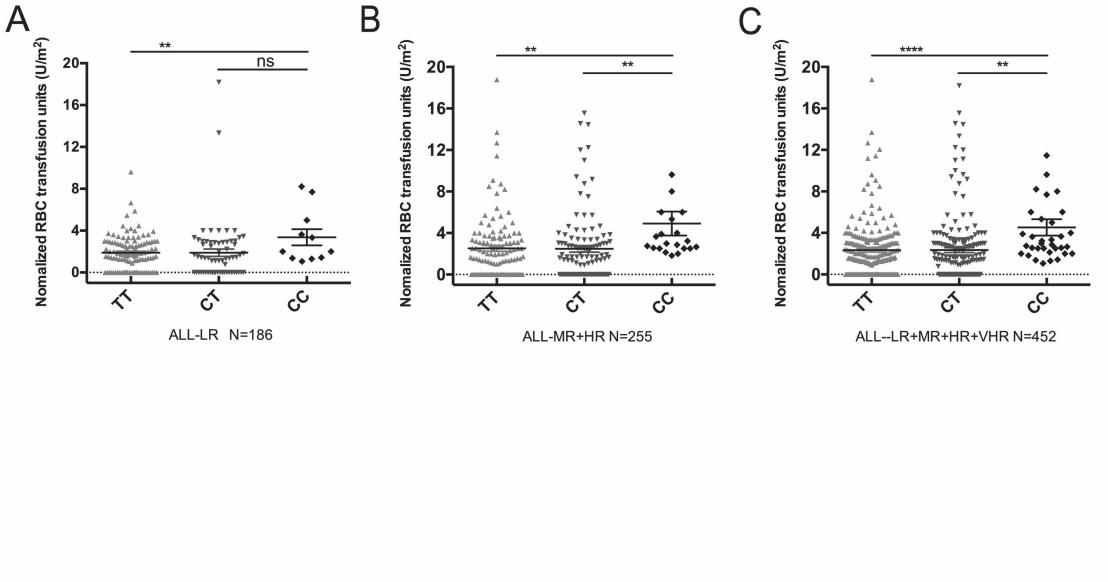

Figure S1. rs10892563 is related to chemotherapy induced anemia.

(A) 182 children with ALL were treated with the SCMC-ALL-2005 low-risk protocol. The average normalized red blood cell (RBC) transfusion units was significantly higher in patients with CC genotype than the CT genotype (P=0.1124) and the TT genotype(P=0.0092).

(B) 102 children with ALL were classified as intermediate-risk group and 153 cases were in high-risk group. They were treated with the similar protocol. The mean normalized red blood cell transfusion units for CC genotype patients was 4.908, significantly higher than 2.475 for CT genotype (P = 0.0065) and 2.526 for TT genotype (P = 0.0044).

(C) 452 children with ALL enrolled in the SCMC-ALL-2005 protocol were genotyped targeting rs10892563. The average normalized RBC transfusion units was significantly higher in patients with CC genotype than the CT genotype (P=0.0011) and the TT genotype(P<0.0001), each point represents one patient's record.

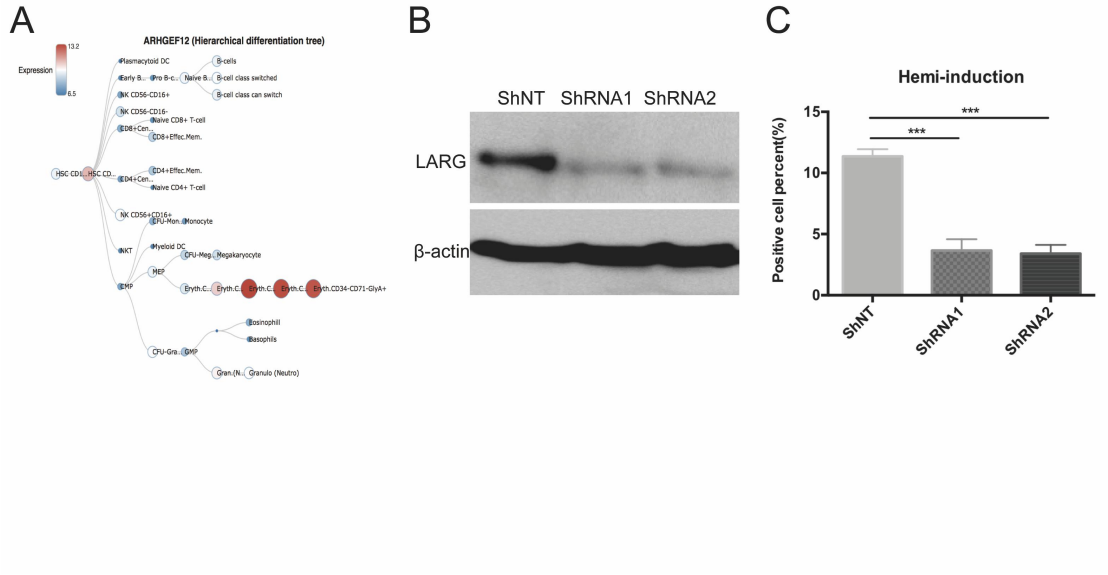

Figure S2. ARHGEF12 is expressed in human erythropoietic cells, and knocking down its expression in K562 cells blocked erythroid differentiation.

(A) ARHGEF12 is exclusively transcribed in the human erythroid lineage according to the Human Differentiation Map Portal database.

(B) We knocked down ARHGEF12 in K562 cells using lentivirus shRNA transduction. Decreased expression of ARHGEF12 was verified by immunoblot analysis.

(C)The benzidine-positive rates in ARHGEF12 deficiency cells (both shRNA1and shRNA2) were significantly lower than in the non-targeted shRNA (NT) cells.

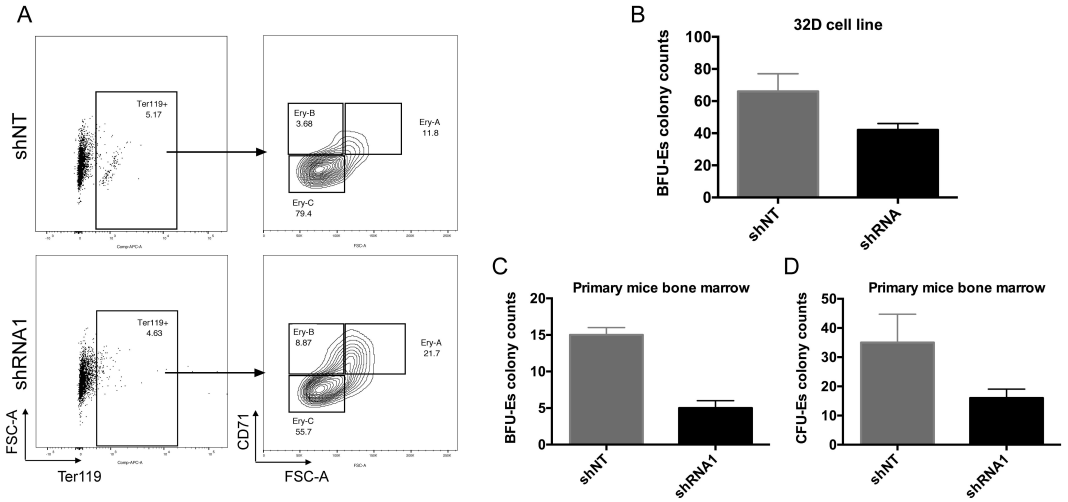

Figure S3. Down-regulation of ARHGEF12 expression blocked erythroid differentiation of the 32D cells and primary murine bone marrow cells.

(A)We used lentiviral shRNA transduction to knock down ARHGEF12 expression in 32D cells. Erythropoietin was added to the medium to a final concentration of 1 U/mL, and after 3 days of induction, erythroid differentiation was detected by flow cytometry using CD71-PE and Ter119-APC antibodies.

(B) 32D cells were transduced with shNT or shRNA lentiviruses and plated in semisolid medium in methyl-cellulose to determine the number of BFU-E colonies. Colonies were scored 12 to 14 days after plating. These results are means of 3 experiments with different samples.

(C, D) Primary mice bone marrow cells transduced with shNT or shRNA lentiviruses were plated in semisolid medium in methyl-cellulose to determine the number of BFU-E and CFU-E colonies .CFU-E colonies were scored 2 days after plating and BFU-E colonies were counted 12-14 days after plating. These results are means of 3 experiments with different samples.

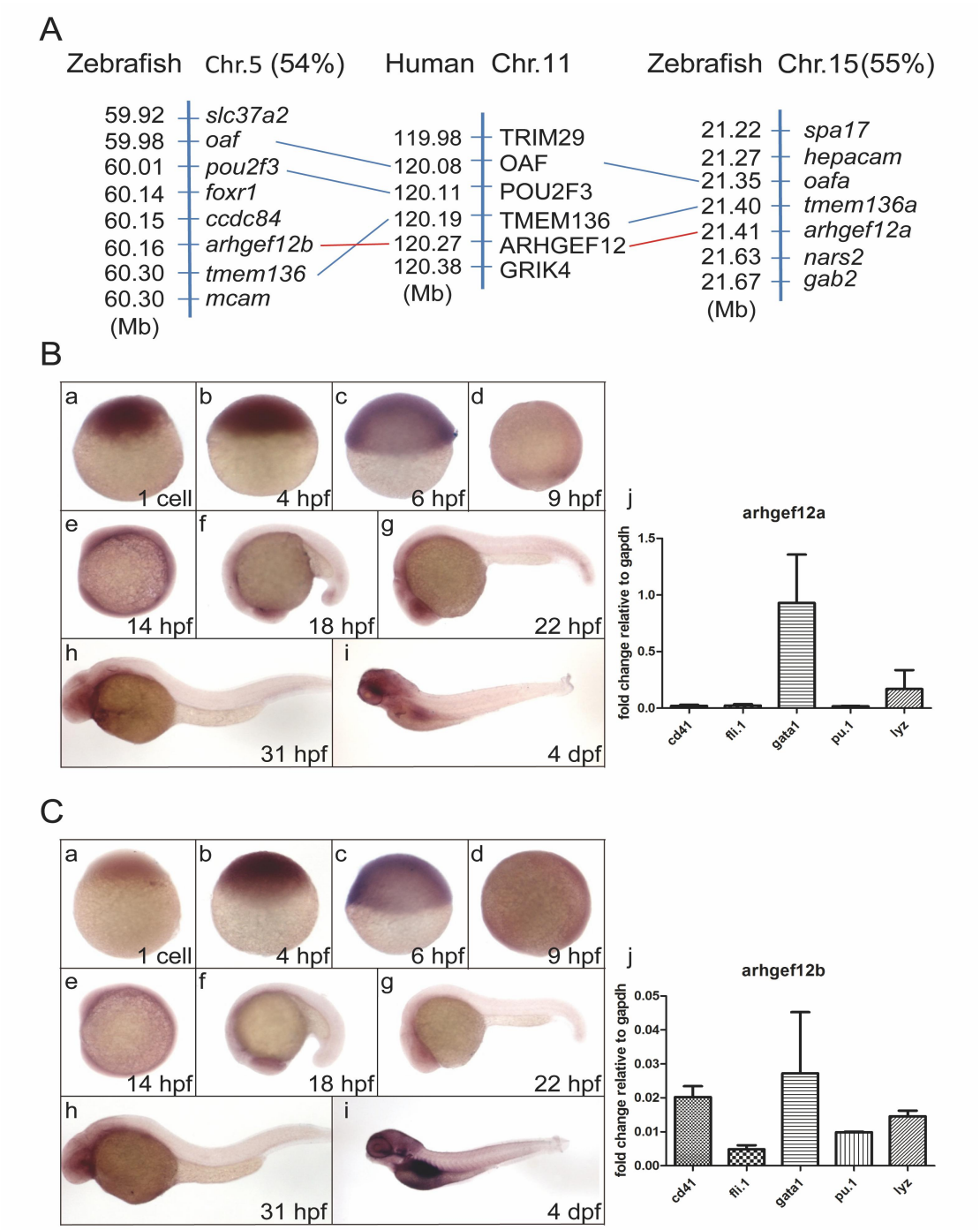

Figure S4. ARHGEF12 orthologous genes in zebrafish are specifically expressed in erythrocytes.

(A) Conserved synteny analysis of ARHGEF12. Comparative maps of the ARHGEF12 gene. Zebrafish-specific ARHGEF12 genes (*arhgef12a* and *arhgef12b*) are also shown.

(B) Expression patterns of *arhgef12a* (a-i) at different indicated developmental stages in wild-type embryos. *arhgef12a* is a maternally derived gene and was widely expressed at 4 hpf (b), 6 hpf (c), 9 hpf (d), and 14 hpf (e) and then reduced from 18 hpf (f-i). The quantitative

RT-PCR results of *arhgef12a* expression showed much higher expression in *gata1*$^{+}$ erythroid progenitors than in other blood cells (*cd41*$^{+}$, *fli.1*$^{+}$, *pu.1*$^{+}$ *or lyz*$^{+}$ cells).

(C) Expression patterns of *arhgef12b* (a-i) at different indicated development stages in wild-type embryos. *arhgef12b* also is a maternally derived gene, widely expressed at 4 hpf (b), 6 hpf (c), 9 hpf (d), and 14 hpf (e) and then reduced from 18 hpf (f-i). The quantitative RT-PCR results of *arhgef12b* expression showed its expression at much higher levels in *gata1*$^{+}$ erythroid progenitors than in other blood cells (*cd41*$^{+}$, *fli.1*$^{+}$, *pu.1*$^{+}$ *or lyz*$^{+}$ cells). The overall expression of *arhgef12a* in blood lineages was higher than that of *arhgef12b.*

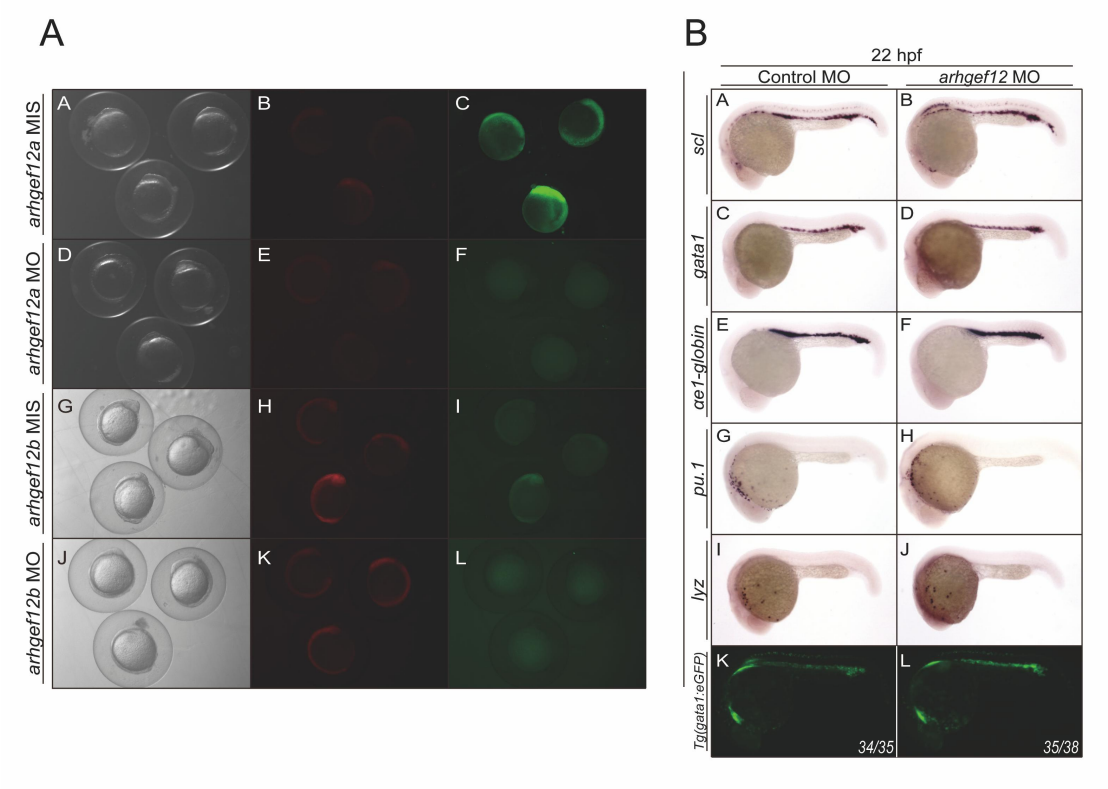

Figure S5. Effects of the *arhgef12a*/*arhgef12b* MO knockdown on the phenotype of primitive hematopoiesis in zebrafish.

(Left panel) Two separate short cDNA fragments of arhgef12a and arhgef12b, which started from ATG and were MO-targeted sequences, were fused in frame with an EGFP reporter. In vitro-transcribed arhgef12a-egfp or arhgef12b-egfp fusion mRNA was co-injected into one-cell-stage embryos with mCherry mRNA (B, E, H, K) as negative control and either mismatch MO (C, I) or specific MO (F, L), respectively. (A), (D), (G), (J) are light microscopy images. Each MO efficiently inhibited the translation of the fusion protein. Mismatch control MO had no such effect.

(Right panel) The whole-mount in situ hybridization (WISH) results of primitive

hematopoietic markers scl (A, B), gata1 (C, D), αe1-globin (E, F), pu.1 (G, H), and lyz (I, J) in both control and arhgef12a and arhgef12b double-MO-injected embryos at 22 hpf. The Tg(gata1: eGFP) transgenic line results for control and arhgef12a and arhgef12b double-MO knockdown at 22 hpf (K, L). All of the primitive markers showed that knockdown of arhgef12a and arhgef12b had no effect on hematopoiesis at the primitive stage.

A

Name	ShRNA1	ShRNA2	ShNT	ShRNA1/ShNT	ShRNA2/ShNT
MAPKAPK2 (Phospho-Thr334)	0.26	0.12	0.96	0.27	0.13
MKK3/MAP2K3 (Phospho-Thr222)	0.96	0.94	1.33	0.73	0.71
p53 (Phospho-Ser15)	2.18	2.10	1.56	1.39	1.34
STAT1 (Phospho-Ser727)	0.74	0.72	1.45	0.51	0.50
Elk-1 (Phospho-Ser383)	2.33	2.49	2.83	0.82	0.88
MEF2A (Phospho-Ser408)	1.39	1.24	0.91	1.53	1.36
Myc (Phospho-Ser373)	1.05	1.08	1.52	0.69	0.71
ATF2 (Phospho-Ser62/ 44)	1.31	1.20	2.00	0.65	0.60
ATF2 (Phospho-Thr71/53)	1.24	1.09	1.52	0.82	0.72

B

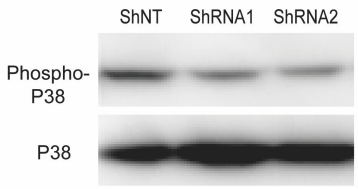

Figure S6. An antibody microarray screen and Western analysis detected reduced phosphorylation activity of the p38 MAPK pathway in the ARHGEF12 knockdown K562 cells.

(A)Phosphorylation antibody microarray screening was performed using ARHGEF12 knockdown (shRNA1, shRNA2) and control (shNT) K562 cells. The most noticeable phosphorylation reduction was seen in members of the p38–MAPK pathway in ARHGEF12 knockdown cells.

(B) Western blot confirmed reduced phosphorylation of p38–MAPK in ARHGEF12-deficient K562 cells (shRNA1 and shRNA2).

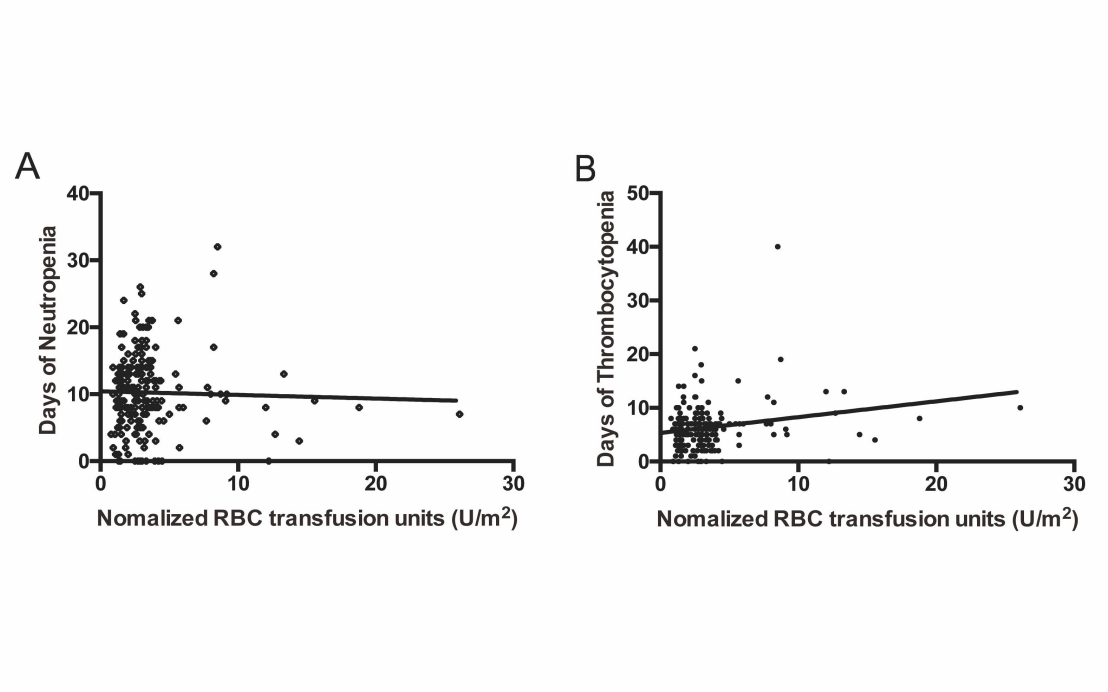

Figure S7. Correlation analysis between RBC transfusion and severity of neutropenia or thrombocytopenia

(A) The days that neutrophil below 1*10^9/L were calculated during the first CAT consolidation therapy (chemotherapy course composed of cyclophosphamide, cytarabine and thiopurine) to MM (LR group) or the second CAT consolidation therapy (MR and HR group) period. RBC transfusion had no significant correlation with neutropenia (r = -0.02891, p=0.6357).

(B) RBC transfusion had positive correlation with thrombocytopenia (platelet below 100*10^9/L), r = 0.2192, p = 0.0003.

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