# Targeting a thrombopoietin-independent strategy in the discovery of a novel inducer of megakaryocytopoiesis, DMAG, for the treatment of thrombocytopenia

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**Received:** October 1, 2022.
**Accepted:** December 15, 2022.
**Early view:** December 22, 2022.

**https://doi.org/10.3324/haematol.2022.282209**

# Targeting TPO-independent strategy in the discovery of a novel megakaryocytopoiesis inducers DMAG for treatment of thrombocytopenia

**Running title: Targeting TPO-independent thrombopoiesis inducer discovery**

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## Supplementary Methods

### Construction of the drug screening model using gcForest

The model was constructed as shown in Figure 1A. First, the data set was built by 94 compounds, including 43 active compounds and 51 inactive compounds identified by our lab. Two active compounds and 10 inactive compounds were randomly selected as the validation set. The other compounds were used as the training set. Then, their molecular descriptors were calculated by RDKit software (http://www.rdkit.org). The 200 molecular descriptors were obtained, including partial charge (PEOE_VSA-6) descriptors, VSA EState Descriptor (VSA_EState4) and water partition coefficient (MolLogP). To improve the predictive accuracy, the Gini index was used to rank the importance of 200 molecular descriptors (Figure 1B). After removing the redundant feat, 6 data sets with traits of 50%, 60%, 70%, 80%, 90% and 100% were obtained. Finally, the 6 data sets were entered into the gcForest model to validate and predict the activities of the compounds.

### Chemicals

8-Di-O-methylellagic acid 2-O-glucoside (purity ≥ 98%, as determined by HPLC) was purchased from Chengdu DeSiTe Biological Technology Co., Ltd. (Chengdu, China) and reconstituted according to the manufacturers' instructions.

### Cell culture

The human erythroleukemia cell line HEL and megakaryoblastic cell line Meg-01 were purchased from American Type Culture Collection (ATCC) (Bethesda, MD, USA).

HEL and Meg-01 cells were cultured in RPMI-1640 medium (Gibco, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin and maintained at 37°C in a humidified incubator with 5% $CO_2$.

**Analysis of cell morphology**

Cells were incubated with DMAG (10, 20 and 40 μM) for 6 days. These cells were smeared on microscope slides by centrifugation at 200 r.p.m. using a cyto centrifuge (Lu Xiangyi, Shanghai, China) and then stained with Wright Giemsa dye (Sigma, St. Louis, MO, USA). Cell morphology was observed under a microscope (NIKON, Japan).

**Phalloidin staining**

Cells were spun onto microscope slides and fixed with 4% paraformaldehyde for 10 min. After washing twice with PBS, the cells were permeabilized with acetone for 5 min at room temperature. The sample was then washed twice with PBS and incubated with 200 μL of TRITC working solution for 30 min at room temperature in the dark. Finally, the sample was counterstained with 200 μL of 4',6-diamidino-2-phenylindole (DAPI) for 30 s. Cell samples were observed and photographed under a laser scanning confocal microscope (Leica, Germany).

**Measurement of megakaryocyte ploidy**

Cells were washed with PBS and fixed with 70% ice-cold ethanol overnight. Then, the samples were washed twice with PBS and incubated with RNase (Sigma, St. Louis, MO, USA) for 30 min at 37°C. After that, 20 μg/mL propidium iodide (PI) (Sigma, St.

Louis, MO, USA) was added to the sample and incubated for 30 min on ice in the dark. The cells were finally analyzed by a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA).

**Lactate dehydrogenase assay**

Cells were seeded onto a 96-well microplate ($4.0 \times 10^3$ cells/well) and then treated with DMAG (10, 20 and 40 μM) for 1 to 6 days. Cell cytotoxicity was assessed by determining the release of lactic acid dehydrogenase (LDH) from the cells using an LDH cytotoxicity assay kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions.

**RNA sequencing and data analysis**

After the HEL cells were treated with or without DMAG (40 μM) for 3 days, the cells were collected and diluted in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, the samples were sent to Novogene Bioinformatics Technology (Beijing, China) for RNA extraction, cDNA library construction and RNA sequencing (RNA-seq). RNA-seq was performed using the Illumina HiSeq 4000 platform to generate 150 bp paired-end reads. The raw sequencing data were uploaded to the GEO database (GSE142994). Raw data were normalized by Fragments Per Kilobase per Million mapped fragments (FPKM) using the RSEM tool. EdgeR was used to identify differentially expressed mRNAs, with significance determined by a fold change > 2.0 and a $P < 0.05$. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using KOBAS (2.0).

**Western blot analysis**

Cells with different treatments for the indicated periods were lysed with 1× RIPA lysis buffer (CST, MA, USA) containing protease inhibitor cocktail. Protein concentration was measured by the Quick StartTM Bradford 1 × Dye Protein Assay Reagent (Bio-Rad, CA, USA). Thirty micrograms of total protein per sample was loaded and separated by 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS–PAGE), followed by transfer to a polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA, USA). After blocking with 10% skim milk powder in PBS with 0.1% Tween-20 (PBST), the membrane was probed with the following antibodies: MEK, p-MEK, p-ERK1/2, ERK1/2, HIF-1β, c-MPL, p-JAK2, JAK2, p-STAT3, STAT3, p-PI3K, PI3K, p-AKT, AKT, NF-E2, GATA1, TAL1, RUNX1, FOG1 and β-actin (Cell Signaling Technology, MA, USA). The blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, MA, USA). Blots were detected using UltraSignal Hypersensitive ECL Chemiluminescent Substrate (4A Biotech Co., Ltd., Beijing, China) and visualized by the ChemiDoc MP Imaging System (Bio-Rad, California, USA). The intensity of bands was quantified by using ImageJ software (NIH, USA) and normalized to β-actin as an internal control. The fold change of each group was calculated with respect to control samples.

**Quantitative real-time PCR validation**

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Then, it was reverse-transcribed into cDNA using a

HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) following the procedures provided by the manufacturer. Quantitative real-time PCR (RT–qPCR) was carried out using the FastStart Universal SYBR Green Master (ROX) kit (Roche Diagnostics GmbH, Mannheim, Germany) and performed in a Bio-Rad Real-Time PCR detection system (Bio-Rad Laboratories, Hercules CA, USA). Each reaction was performed in triplicate and repeated at least three times. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and were normalized to the GAPDH reference gene. The primer sequences used in RT–qPCR are listed in *Online Supplementary Table S1*.

**Immunofluorescence microscopy**

Cells treated with or without DMAG (40 μM) for 6 days were collected and transferred onto microscope slides. These cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and blocked with BSA. Then, the cells were incubated with NF-E2 antibodies **(**Cell Signaling Technology, MA, USA**)** or β-tubulin (Proteintech, Rosemont, IL, USA) overnight at 4°C**.** After a wishing step, the cells were incubated with FITC-labeled secondary antibody for 1 hour, and the nuclei were stained with DAPI for 10 min at room temperature in the dark. After that, images were captured using a laser scanning confocal microscope (Leica, Germany).

**RNA interference**

NF-E2 siRNA was designed and synthesized by RiboBio Co., Ltd. (Guangzhou, China). The siRNAs were transfected into HEL cells by using a riboFECT CP Transfection Kit

(RiboBio, Guangzhou, China) according to the manufacturer's protocol. The sequence of NF-E2 siRNA is presented in *Online Supplementary Table S1*.

**Establishment of the thrombocytopenia model and treatment of animals**

The Kunming (KM) mice were randomly divided into 4 groups (6 male mice and 6 female mice in each group): (1) normal control group; (2) irradiation (IR) group; (3) TPO positive control group (3000 U/kg); and (4) DMAG-treated group (5 mg/kg). A single dose of 4 Gy total body irradiation was performed to construct a thrombocytopenia mouse model. The mice in the normal control group, IR group, TPO-positive control group and DMAG-treated group were injected intraperitoneally with normal saline, TPO, or DMAG daily post IR for 17 days.

**Measurement of hematologic parameters**

Forty microliters of blood was collected on the indicated days from the mouse eyes' venous plexus and diluted in 160 μL of diluent. The hematologic parameters were measured by an automatic blood cell analyzer (Sysmex XT-1800i/2000IV; Kobe, Japan).

**Bone marrow nuclear cells count**

The bone marrow (BM) cells were obtained from the femurs on day 10 and bone marrow nuclear cells (BMNCs) count was detected by an automatic blood cell analyzer (Sysmex XT-1800i/2000IV; Kobe, Japan).

**Histology analysis**

Four mice were selected randomly from each group and euthanized after treatment with DMAG for 10 days. The femurs, spleen, heart, liver, lung and kidney were separated and fixed in 10% formaldehyde for at least 24 h. The femurs were decalcified with decalcifying solution for over a month. Specimens were then embedded in paraffin, sliced into 5 μm thick sections, and stained with hematoxylin and eosin (H&E) by standard methods. The sections were photographed under an Olympus BX51 microscope (Olympus Optical), and the number of megakaryocytes was counted from 10 microscopy fields per sample.

**Platelet aggregation**

After the mice were anesthetized, the blood was collected from the inferior vena cava into a syringe containing ACD anticoagulant (51 mM trisodium citrate, 22 mM citric acid, and 74 mM D-glucose). Platelet-rich plasma (PRP) was obtained after centrifugation (100 × g) for 10 min at 22°C, and then the platelets were collected by centrifugation (400 × g, 10 min, 22°C). The platelets were resuspended in 800 μL Modified Tyrode's-Hepes buffer (134 mM NaCl, 0.34 mM $Na_2HPO_4$, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3), and the concentration was adjusted to $2 \times 10^8$ platelets/mL. The platelets were stimulated by collagen (10 μg/mL), and platelet aggregation was analyzed using a turbidimetric aggregation-monitoring device (Helena Laboratories, Beaumont, TX, United States).

**Toxicity**

Blood was sampled from mouse eyes and centrifuged to obtain serum. The serum

concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA), blood urea nitrogen (BUN), lactate dehydrogenase (LDH) and creatine kinase (CK) were measured by an automatic biochemistry analyzer (7600, Hitachi, Japan).

**Flow cytometry analysis of BM and spleen cells**

The BM cells were flushed out of femurs with saline solution and filtered by a nylon net. The spleen was cut into small fragments and prepared for signal-cell suspension through suspension into saline solution and filtering by nylon net. Red blood cell (RBC) lysis buffer (Beijing 4A Biotech, Beijing, China) was added to the cell samples to remove RBCs. After that, the cells were labeled with PE-conjugated anti-CD117 (c-Kit, BioLegend, San Diego, CA, USA) and FITC-conjugated anti-CD41 (BioLegend, San Diego, CA, USA), PE-conjugated anti-CD41 (BD Biosciences, San Jose, CA, USA) and FITC-conjugated anti-61 (BD Biosciences, San Jose, CA, USA), FITC-conjugated anti-CD41 and APC-conjugated anti-CD62P (BioLegend, San Diego, CA, USA), and PE-conjugated anti-Ter119 (BioLegend, San Diego, CA, USA). For megakaryocyte ploidy analysis, BM cells were stained with FITC-conjugated anti-CD41 for 30 min at room temperature and fixed with 75% ethanol for 24 h. The remaining steps were similar to the ploidy analysis of HEL cells.

**Immunohistochemical staining**

Five-micrometer-thick femur sections were incubated with 3% $H_2O_2$ for 10 min at room temperature. Then, the sections were blocked with normal goat serum (Abcam, UK) for

20 min and subsequently incubated with primary antibodies against CD41 (Proteintech, Rosemont, IL, USA) or NF-E2 (Cell Signaling Technology, MA, USA) at 4°C overnight. After that, the sections were incubated with biotin-conjugated secondary antibody (Abcam, UK) for 30 min at 37°C. Finally, the sections were exposed to DBA solution (Beyotime, Nanjing, China) for 2 min, followed by staining with hematoxylin. The sections were observed under an Olympus BX51 microscope (Olympus Optical, Japan).

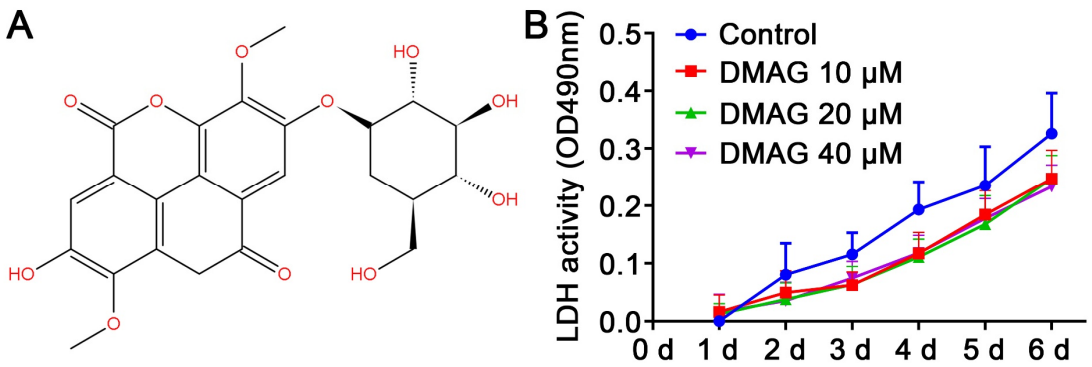
**Enzyme-linked immunosorbent assay**

Enzyme-linked immunosorbent assay (ELISA) was applied to determine the serum TPO level. Blood samples were collected by extirpating eyeballs and the serum was used to measure the serum TPO level using a mouse TPO (Thrombopoietin) ELISA Kit (Elabscience, Wuhan, China) according to the manufacturer's instructions.

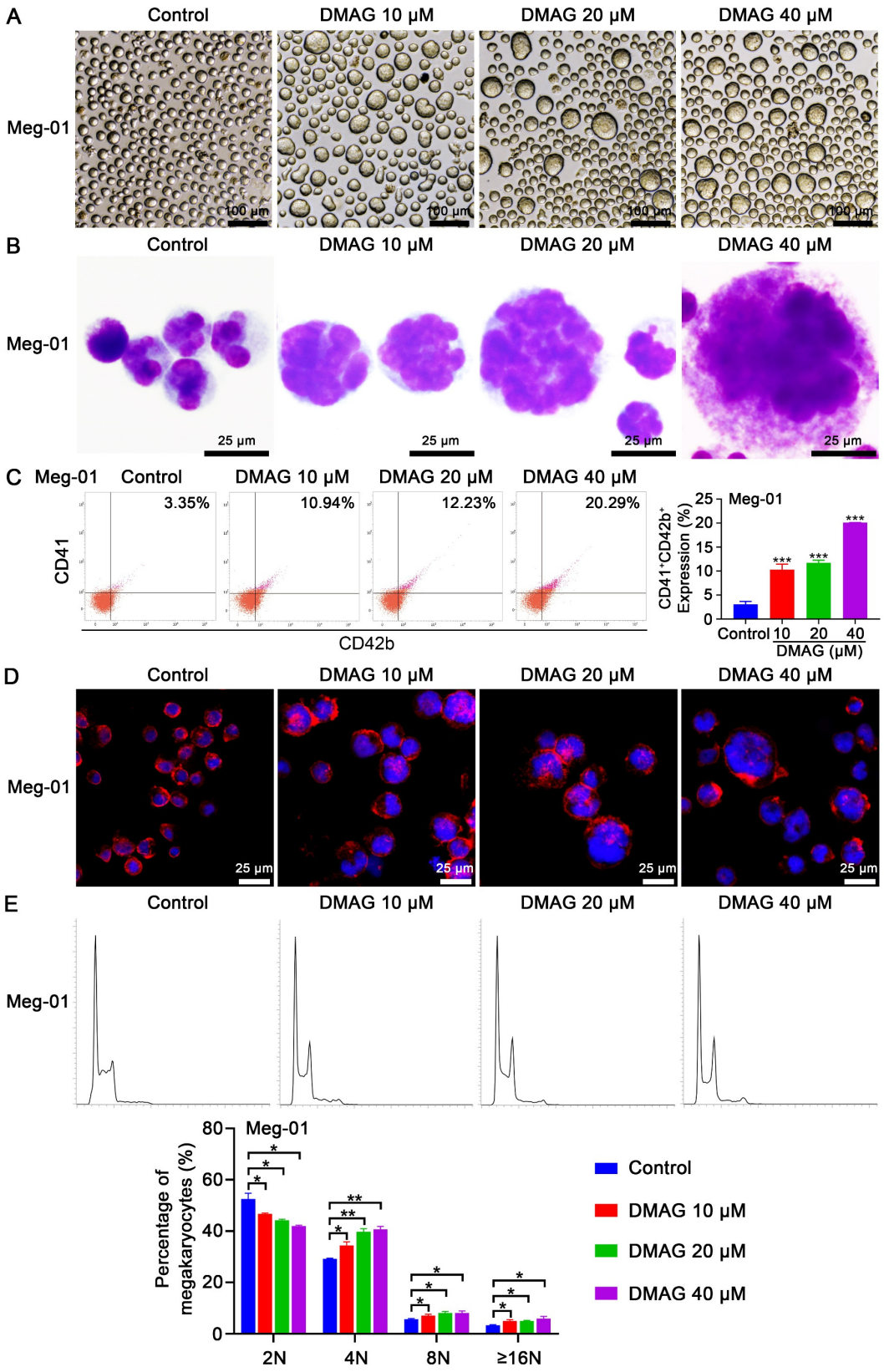
**Zebrafish and treatment**

The larvae of Tg (cd41:eGFP) transgenic zebrafish at 3 days post-fertilization (dpf) were seeded in 12-well plates (20 larvae in each plate) and treated with DMAG (10, 25 and 50 μM). Zebrafish at 5 dpf were fixed with 4% paraformaldehyde overnight at 4°C. Zebrafish larvae were then embedded in agarose in a petri dish for imaging under a laser scanning confocal microscope (Leica, Germany).

## Supplementary Figures

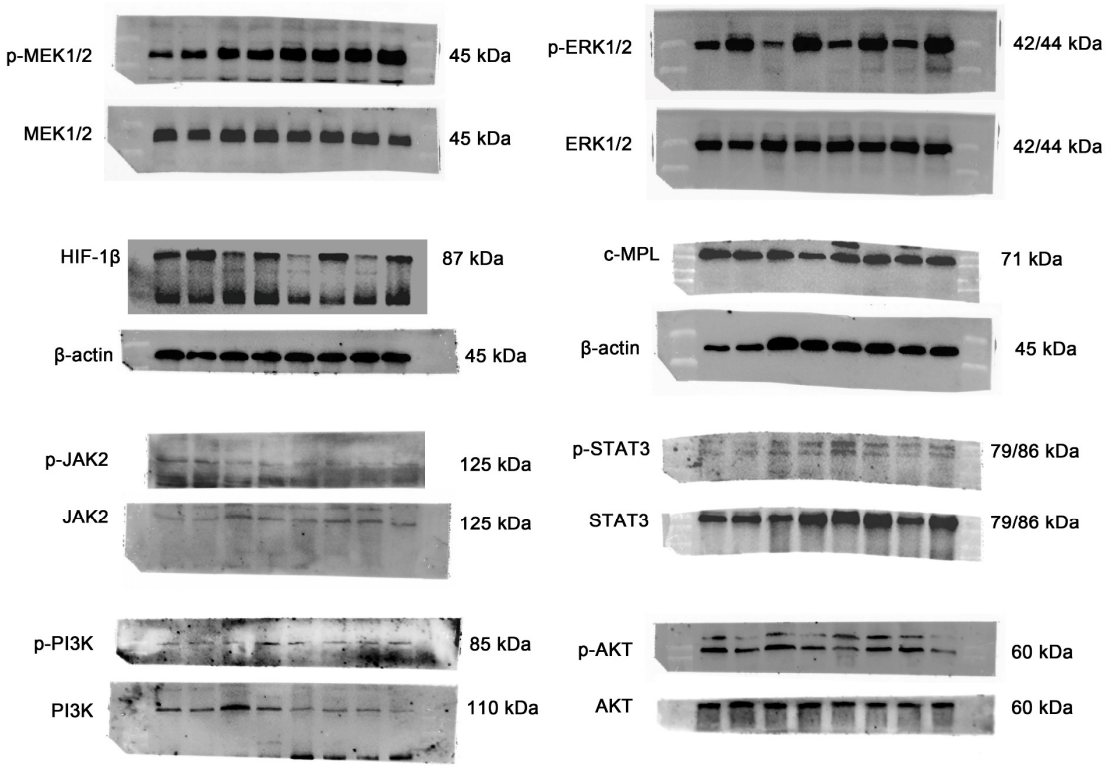



**Supplementary Figure S1. DMAG shows no cytotoxicity *in vitro*.** (A) Chemical structure of DMAG. (B) LDH assay detects the cytotoxicity of DMAG to HEL cells. The data are shown as the mean ± SD from three independent experiments. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; LDH: Lactic acid dehydrogenase.

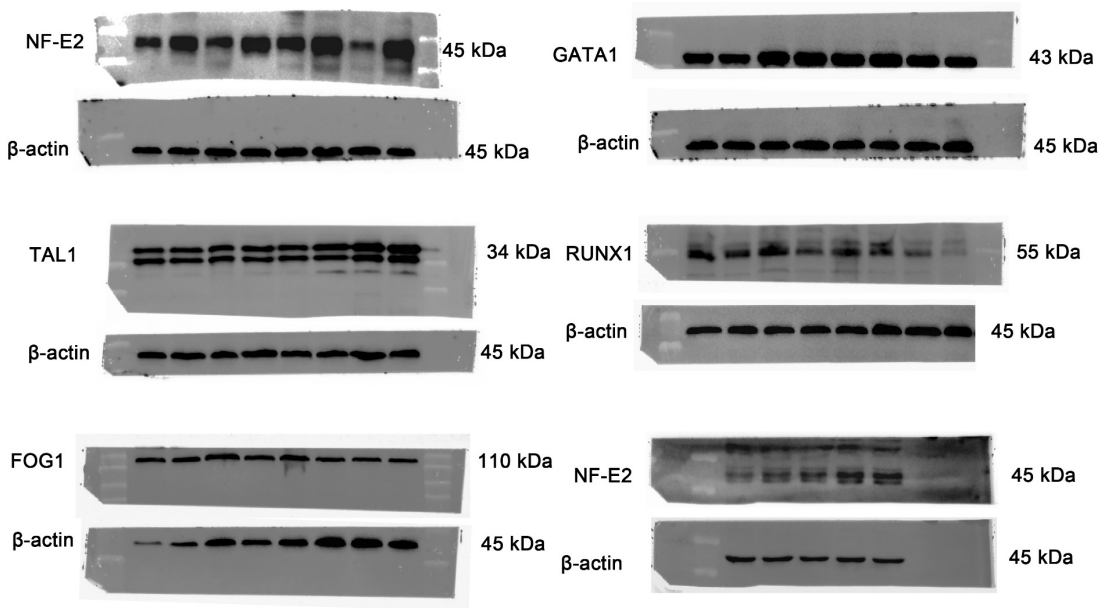



**Supplementary Figure S2. DMAG promotes megakaryocyte differentiation and enhances the DNA ploidy of Meg-01 cells.** (A) Representative images of

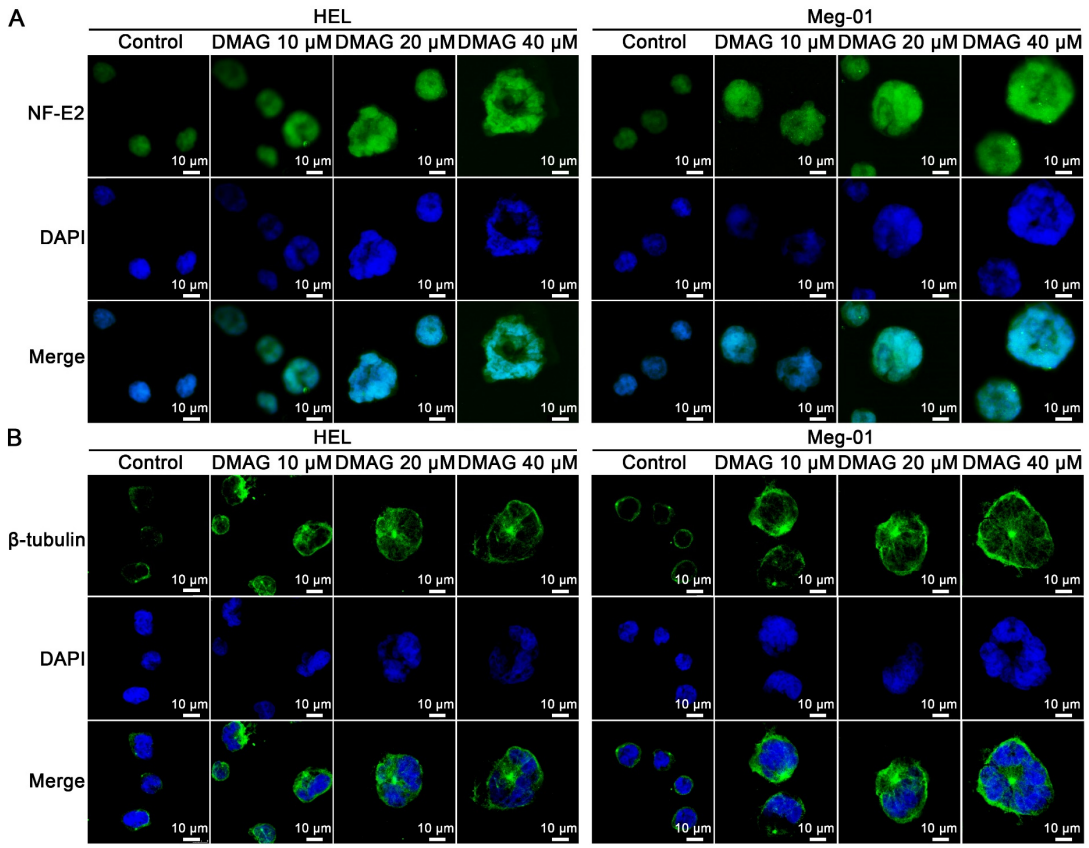
morphological observations of Meg-01 cells. Cells were treated with different concentrations of DMAG (10, 20 and 40 μM) for 6 days. Bars represent 100 μm. (B) Giemsa-Wright staining of Meg-01 cells treated with different concentrations of DMAG (10, 20 and 40 μM) for 6 days. Bars represent 25 μm. (C) Flow cytometry analysis of the expression of CD41 and CD42b after cells were treated with different concentrations of DMAG (10, 20 and 40 μM) for 6 days. The histogram shows the percentage of $CD41^{+}CD42b^{+}$ cells for each group. (D) Phalloidin staining of Meg-01 cells treated with different concentrations of DMAG (10, 20 and 40 μM) for 6 days. DAPI staining (blue) indicates nuclei, and TRITC phalloidin staining (red) of F-actin indicates the boundary of a single cell. Bars represent 25 μm. (E) Flow cytometry analysis of the DNA ploidy of Meg-01 cells treated with different concentrations of DMAG (10, 20 and 40 μM) for 6 days. The histogram shows the percentages of DNA ploidy of Meg-01 cells treated with different concentrations of DMAG (10, 20 and 40 μM) for 6 days. In C and E, data are shown as the mean ± SD from three independent experiments. **P* <.05, ***P* <.01, ****P* <.001, vs the corresponding control. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; DAPI: 4',6-diamidino-2-phenylindole.



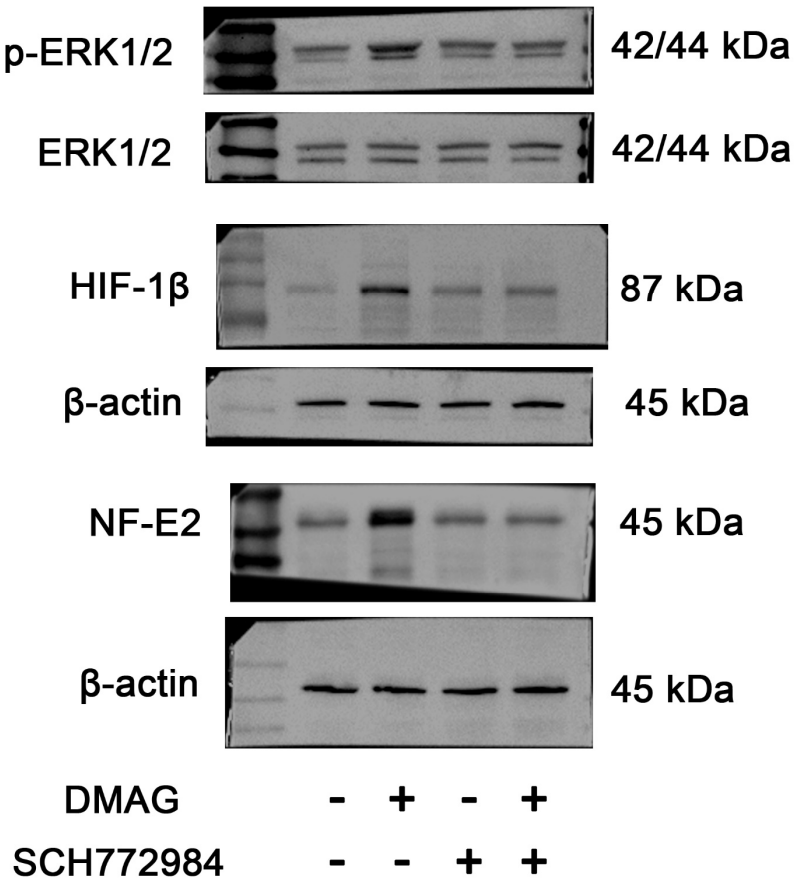

**Supplementary Figure S3. Origin data of western blot analysis in Figure 3.**



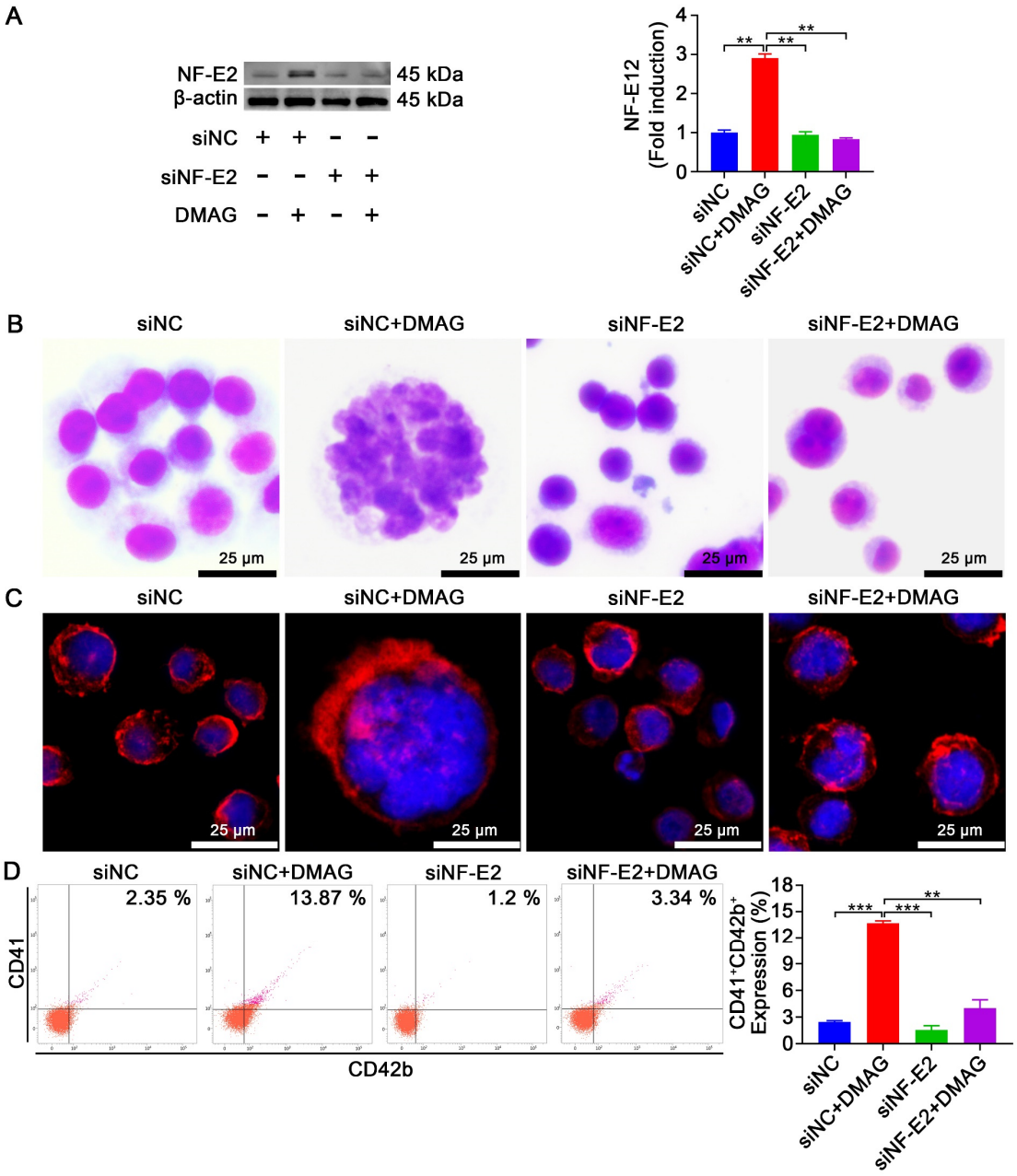

**Supplementary Figure S4. Origin data of western blot analysis in Figure 4.**




**Supplementary Figure S5. Effects of DMAG on the expression of NF-E2 and β-tubulin.** Immunofluorescence analysis of the expression of NF-E2 (A) and β-tubulin (B) induced by different concentrations of DMAG (10, 20 and 40 μM) for 6 days in HEL and Meg-01 cells. Cells were stained with DAPI for nuclei (blue) and antibodies for NF-E2 or β-tubulin (green). Bars represent 10 μm. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; DAPI: 4',6-diamidino-2-phenylindole.

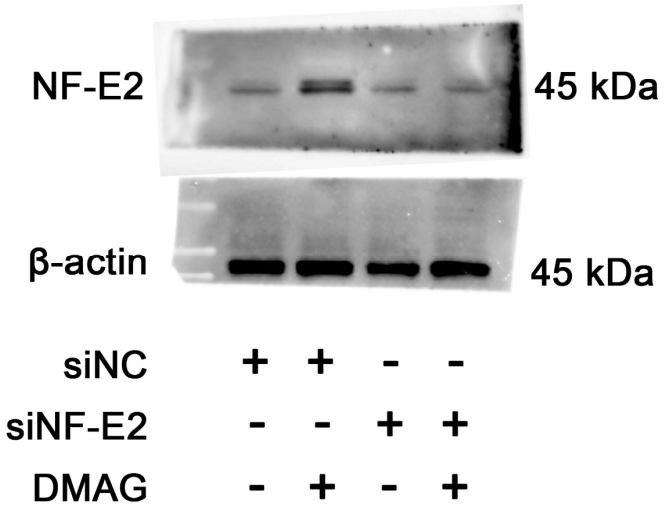



**Supplementary Figure S6. Origin data of western blot analysis in Figure 5.**

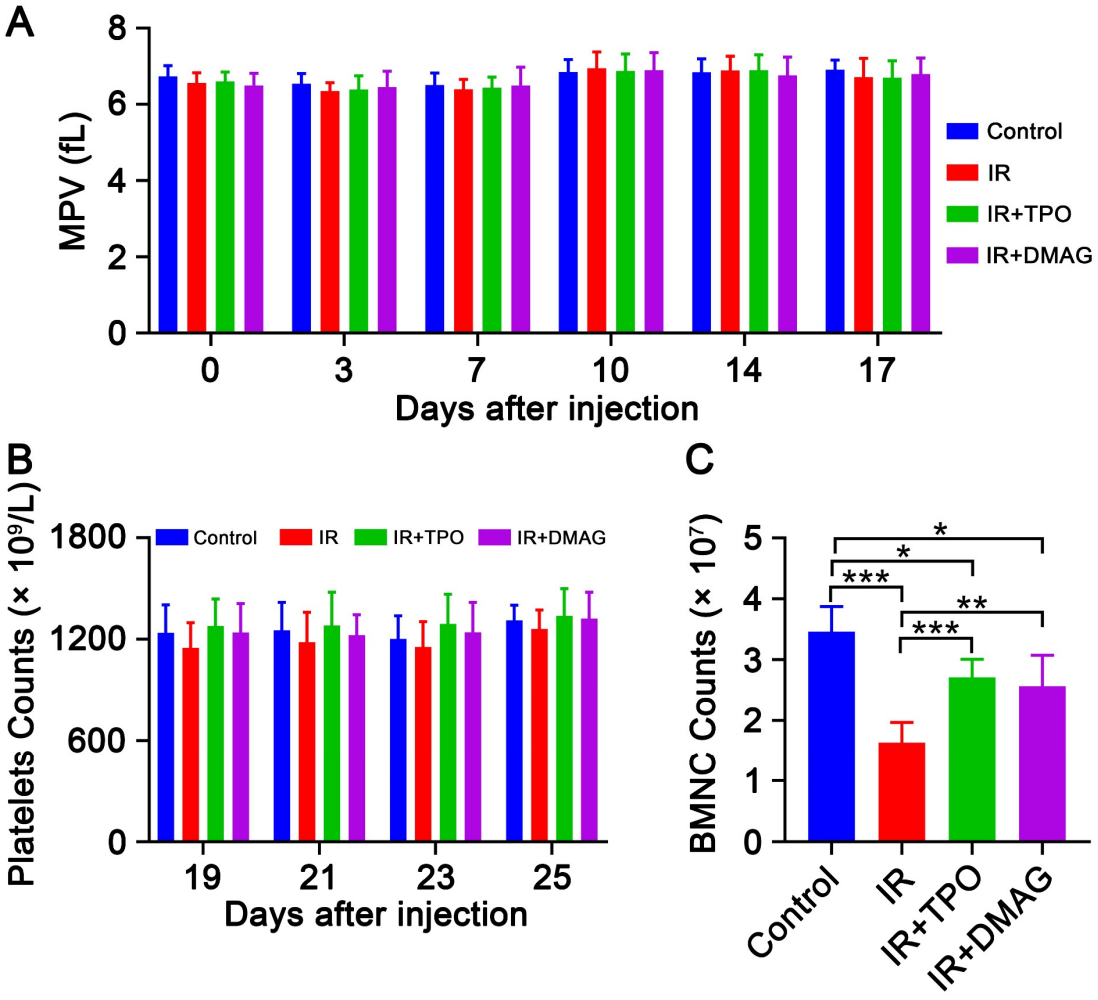



**Supplementary Figure S7. Effects of NF-E2 siRNA on DMAG-induced megakaryocyte differentiation.** (A) Western blot analysis of NF-E2 expression after HEL cells were transfected with NF-E2 siRNA or a negative control followed by DMAG (40 μM) stimulation for 3 days. The histogram shows the expression of NF-E2 after HEL cells were transfected with NF-E2 siRNA. (B) Giemsa-Wright staining detects the effects of NF-E2 siRNA on DMAG (40 μM)-induced cell morphology

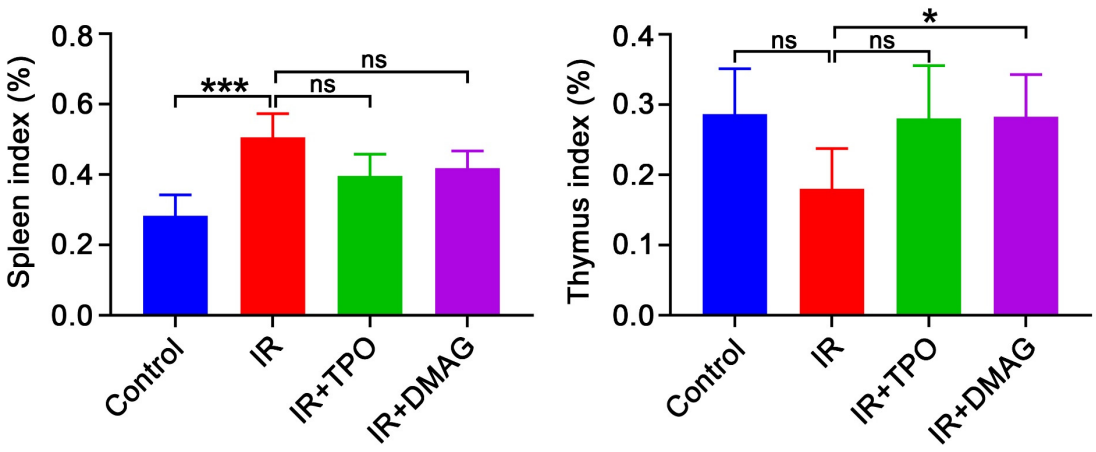
changes. Bars represent 25 μm. (C) Phalloidin staining shows the effects of NF-E2 siRNA on the DMAG (40 μM)-induced increase in the nucleus. Bars represent 25 μm. (D) The effects of NF-E2 siRNA on DMAG (40 μM)-induced megakaryocyte differentiation by detecting the expression of CD41 and CD42b. The histogram shows the proportion of $CD41^{+}CD42b^{+}$ cells for each group. In A and D, data are shown as the mean ± SD from three independent experiments. $^{**}P < .01$, $^{***}P < .001$, vs the corresponding control. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside.



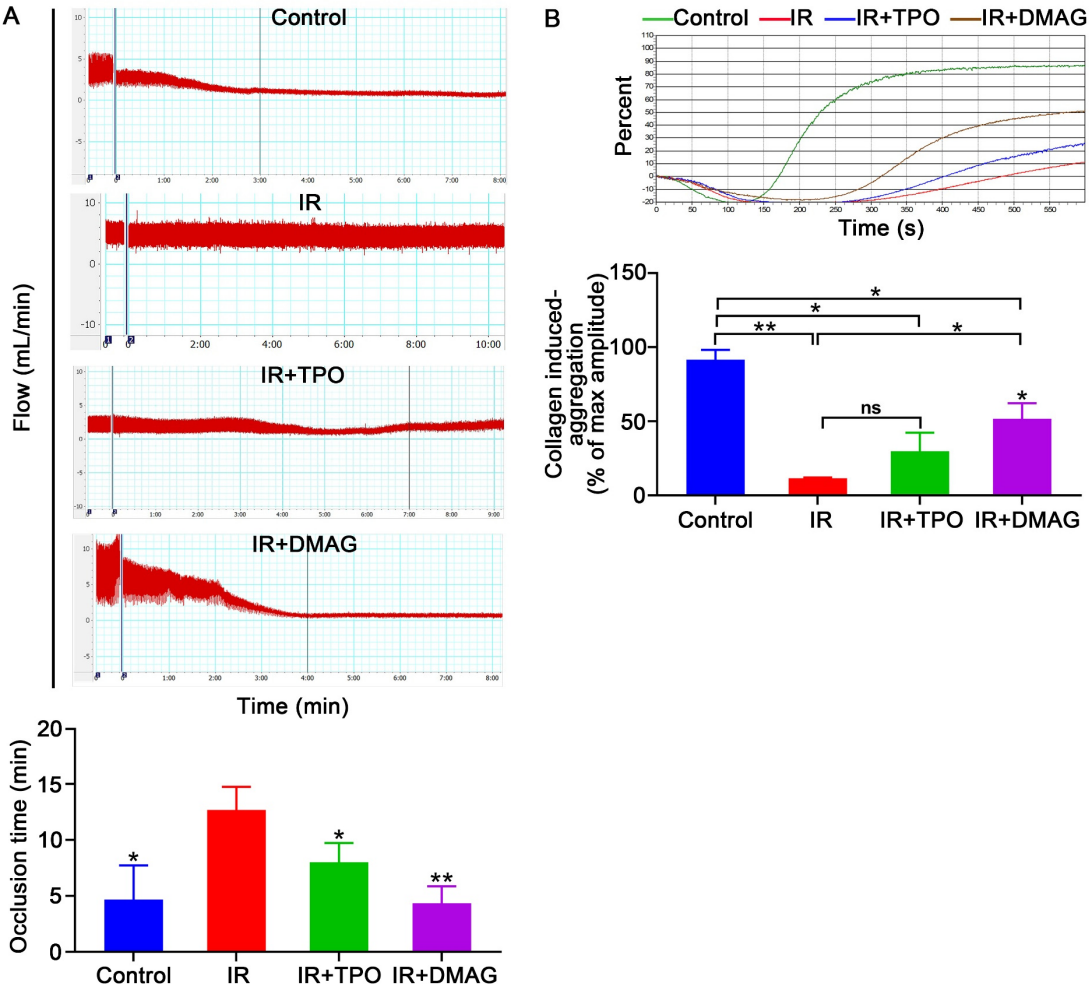

**Supplementary Figure S8. Origin data of western blot analysis in Supplementary Figure S7.**



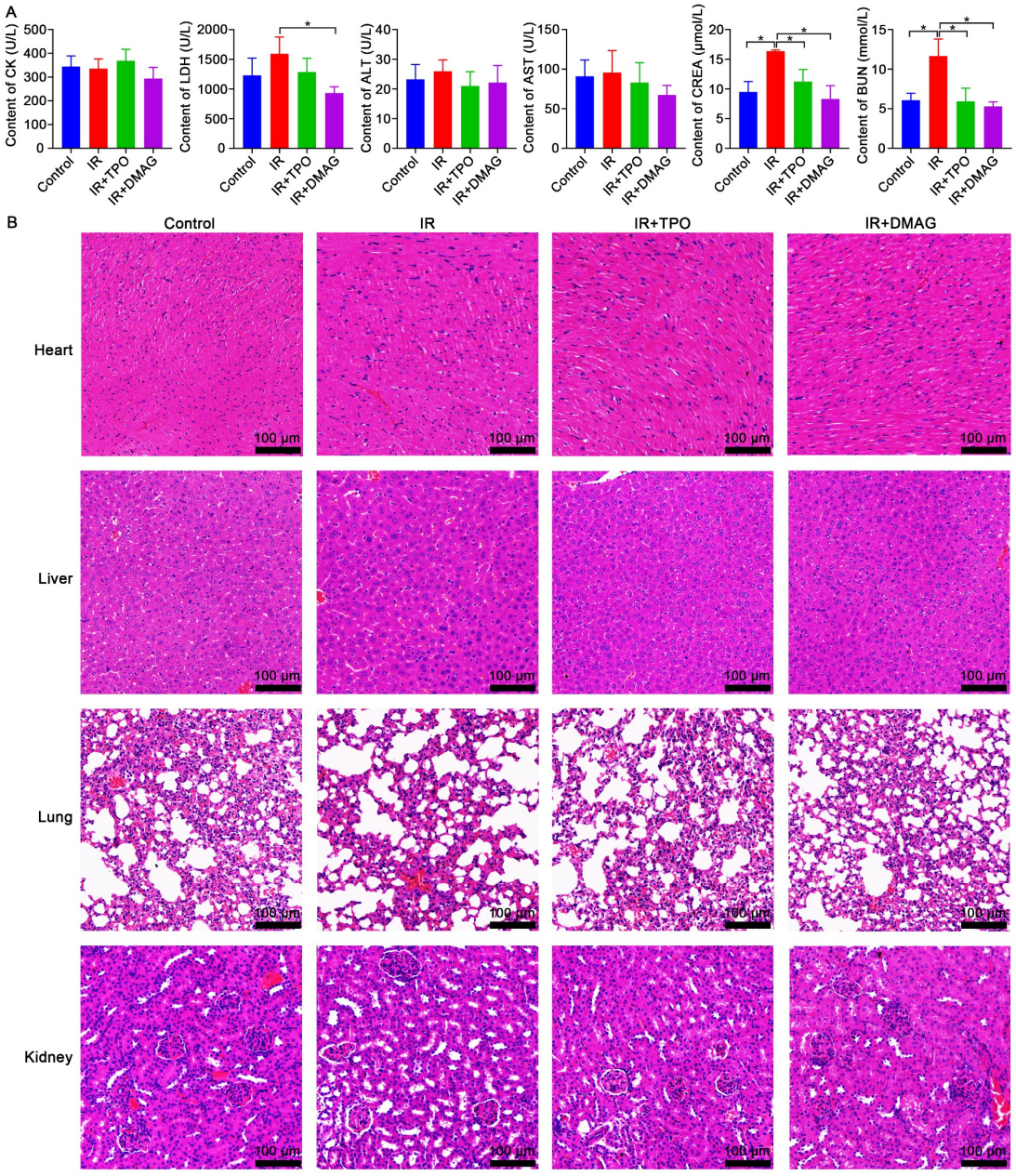

**Supplementary Figure S9. Effects of DMAG on MPV, platelets and BMNC counts of thrombocytopenia mice.** (A) MPV in KM mice at the indicated times after injection with normal saline, TPO (3000 U/kg), or DMAG (5 mg/kg) daily post IR. (B) Peripheral platelet numbers in KM mice at the indicated times after ceasing the TPO and DMAG administration from day 18-25. (C) BMNC counts in KM mice after administrating with TPO (3000 U/kg) or DMAG (5 mg/kg) for 10 days. The data are shown as the mean ± SD from three independent experiments. **P* <.05, ***P* <.01, ****P* <.001, vs the IR group or corresponding control. KM: Kunming; DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; IR: Irradiation; BMNC: Bone marrow nuclear cell.




**Supplementary Figure S10. Effects of DMAG on the visceral index of thrombocytopenia mice.** The data are shown as the mean ± SD from three independent experiments. **P* <.05, ****P* <.001, or indicated as not significant (ns) vs the IR group. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; IR: Irradiation.

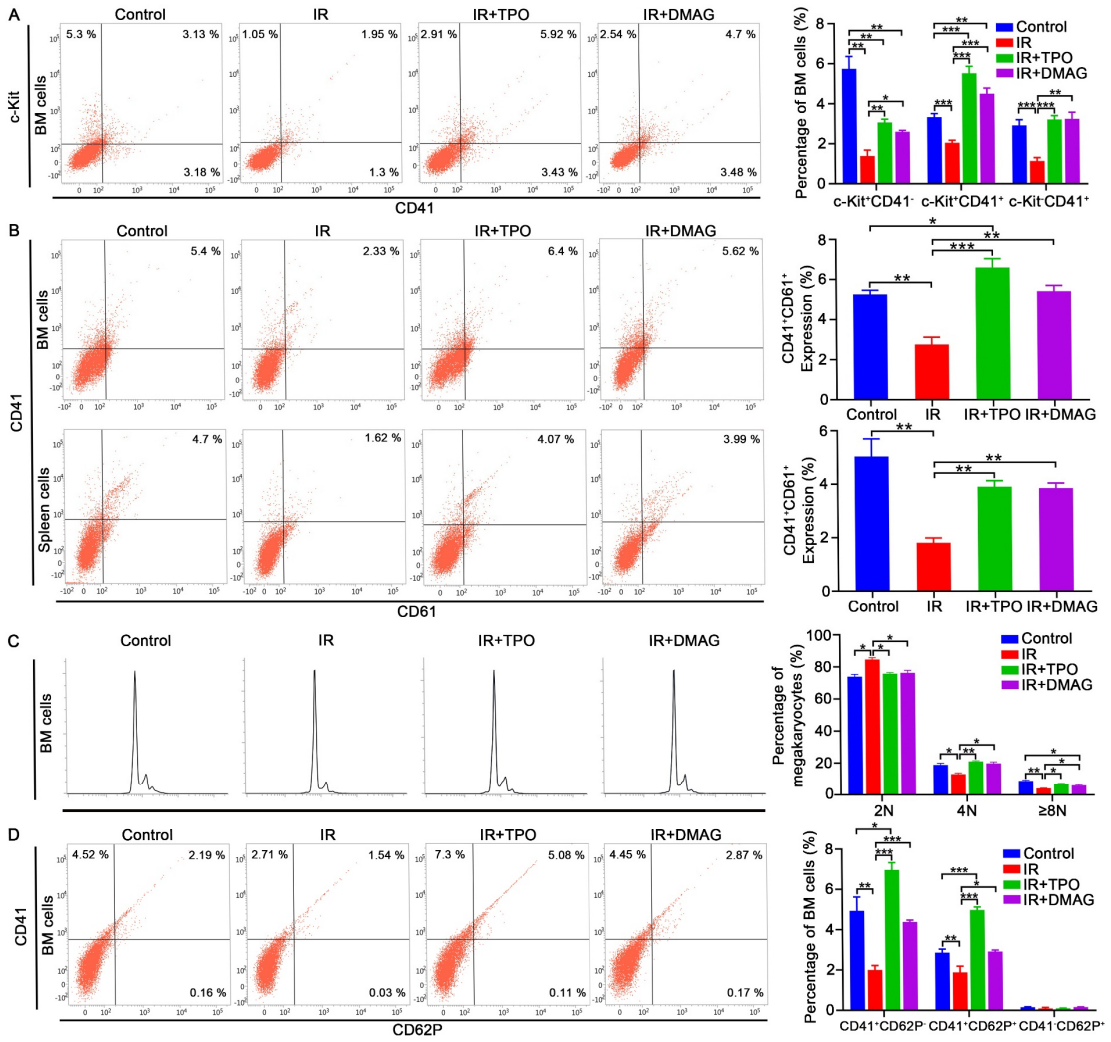



**Supplementary Figure S11. Effects of DMAG on platelet function.** (A) Carotid blood flow tracings in KM mice after administrating with TPO (3000 U/kg) or DMAG (5 mg/kg) for 10 days. The histogram shows the mean carotid artery occlusion times of each group. (B) Aggregometry traces of platelets stimulated with collagen in each group after administrating with TPO (3000 U/kg) or DMAG (5 mg/kg) for 10 days. The histogram represents the aggregation results expressed as the maximal amplitude of aggregation. **P* <.05, ***P* <.01, vs the IR group or corresponding control. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; IR: Irradiation.



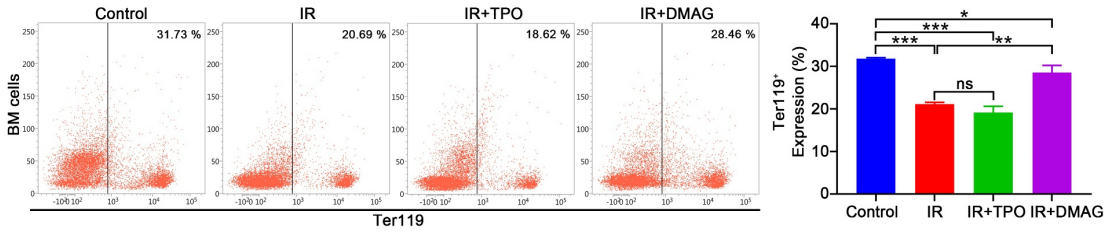

**Supplementary Figure S12. Toxicity evaluation of DMAG *in vivo*.** (A) Blood biochemical analyses of the cardiac function markers CK and LDH, hepatic function markers ALT and AST, and renal function markers CREA and BUN. The data are shown as the mean ± SD from three independent experiments. *$P$ <.05, vs the IR group. (B) H&E staining shows the major organs in each group. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; IR: Irradiation; CK: Creatine kinase; LDH: Lactic acid

dehydrogenase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CREA: Creatinine; BUN: Blood urea nitrogen; IR: Irradiation.

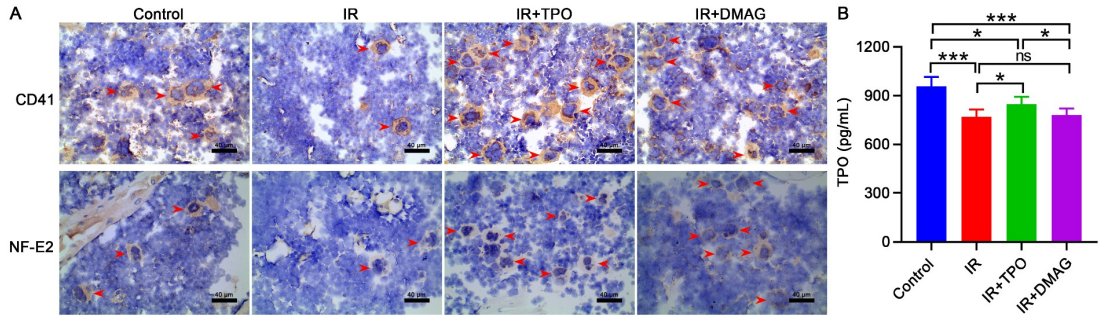



**Supplementary Figure S13. Effects of DMAG on megakaryopoiesis and thrombopoiesis in mice with thrombocytopenia.** (A) Flow cytometry analysis shows the proportions of hematopoietic progenitors (c-Kit$^+$CD41$^-$), megakaryocytic progenitors (c-Kit$^+$CD41$^+$), and megakaryocytes (c-Kit$^-$CD41$^+$) in BM at day 10 post IR. The histogram shows the number of hematopoietic progenitors, megakaryocytic progenitors and megakaryocytes in each group. (B) Flow cytometry analysis shows the expression of CD41$^+$CD61$^+$ in BM and spleen cells at day 10 post IR. The histogram indicates the proportions of CD41$^+$CD61$^+$ cells in each group in BM and spleen cells. (C) Flow cytometry analysis of the DNA ploidy of BM cells at day 10 post IR. The

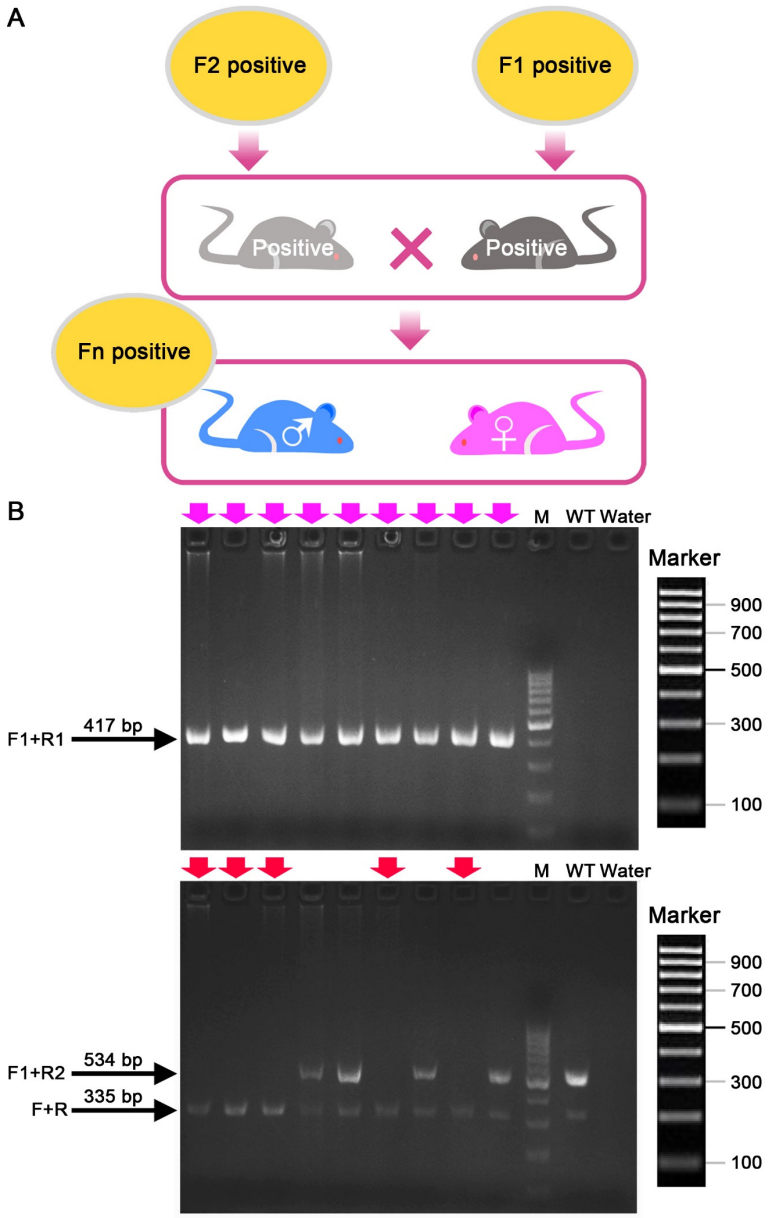
histogram represents the percentages of DNA ploidy of BM cells in each group. (D) Flow cytometry analysis indicates the expression of $CD41^{+}CD62P^{-}$ (megakaryocytes), $CD41^{+}CD62P^{+}$ (platelets) and $CD41^{-}CD62P^{+}$ (activated platelets) at day 10 post IR. The histogram shows the percentages of megakaryocytes, platelets and activated platelets in each group. The data are shown as the mean ± SD from three independent experiments. *$P$ <.05, **$P$ <.01, ***$P$ <.001, vs the IR group or corresponding control. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; BM: Bone marrow; IR: Irradiation.



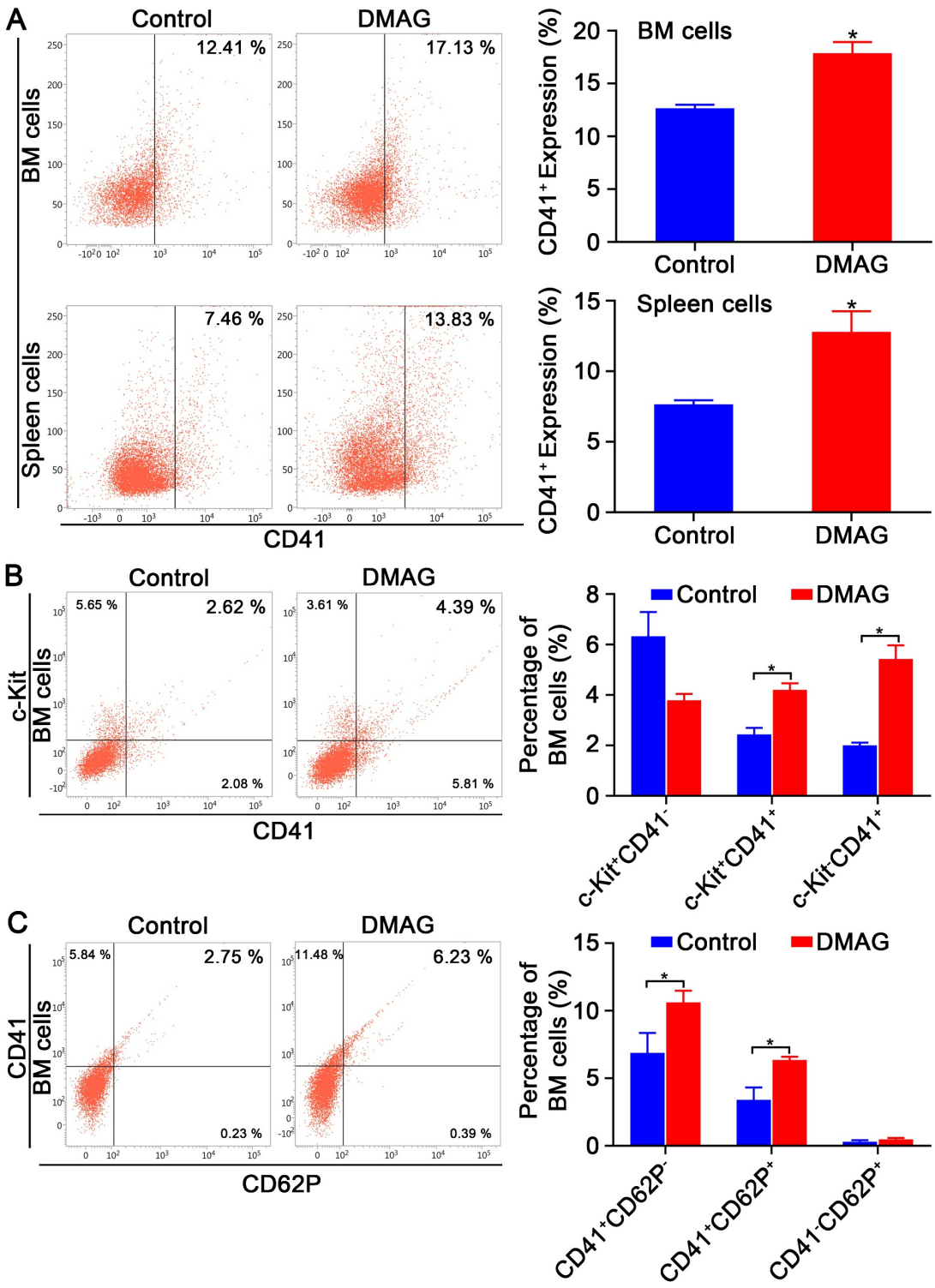

**Supplementary Figure S14. DMAG elevates the number of Ter119$^+$ cells in BM.** Flow cytometry analysis shows the expression of Ter119 in BM after the mice were administered normal saline, TPO (3000 U/kg) or DMAG (5 mg/kg) for 10 consecutive days. The histogram shows the proportion of Ter119$^+$ cells in the BM of each group. The data are shown as the mean ± SD from three independent experiments. **P* <.05, ***P* <.01, ****P* <.001, or indicated as not significant (ns) vs the corresponding control. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; IR: Irradiation; BM: Bone marrow.




**Supplementary Figure S15. Effects of DMAG on the expression of CD41 and NF-E2 in BM and serum TPO level.** (A) Immunohistochemistry staining shows the expression of CD41 and NF-E2 in BM at day 10 post IR in each group. Bars represent 40 μm. The red arrows mark the megakaryocytes. (B) The serum TPO level at day 10 post IR in each group. The data are shown as the mean ± SD from three independent experiments. **P* <.05, ****P* <.001, or indicated as not significant (ns) vs the corresponding control. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; IR: Irradiation; BM: Bone marrow.

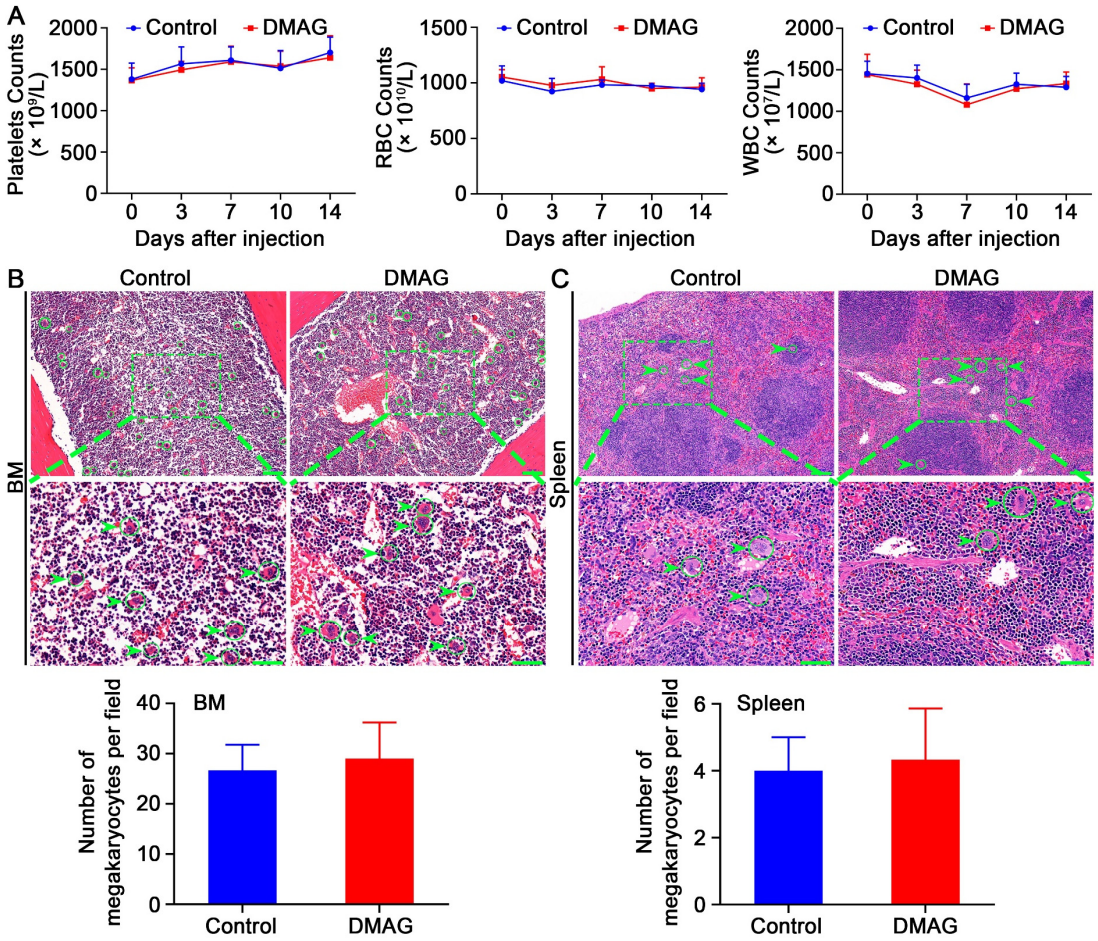



**Supplementary Figure S16. Breeding and screening of c-MPL$^{-/-}$ mice.** (A) Diagram of the breeding scheme of c-MPL$^{-/-}$ mice. (B) PCR screening of c-MPL$^{-/-}$ mice. The pink arrows represent the mice identified as positive by using F1 and R1 primers. The red arrows represent c-MPL$^{-/-}$ mice identified by PCR. M: Marker; WT: Wild type; Water is PCR negative control. F and R primers were used as PCR internal controls. Homozygous: two bands with 417 bp and 335 bp. Heterozygous: three bands with 417 bp and 534 bp and 335 bp. WT: two bands with 534 bp and 335 bp.

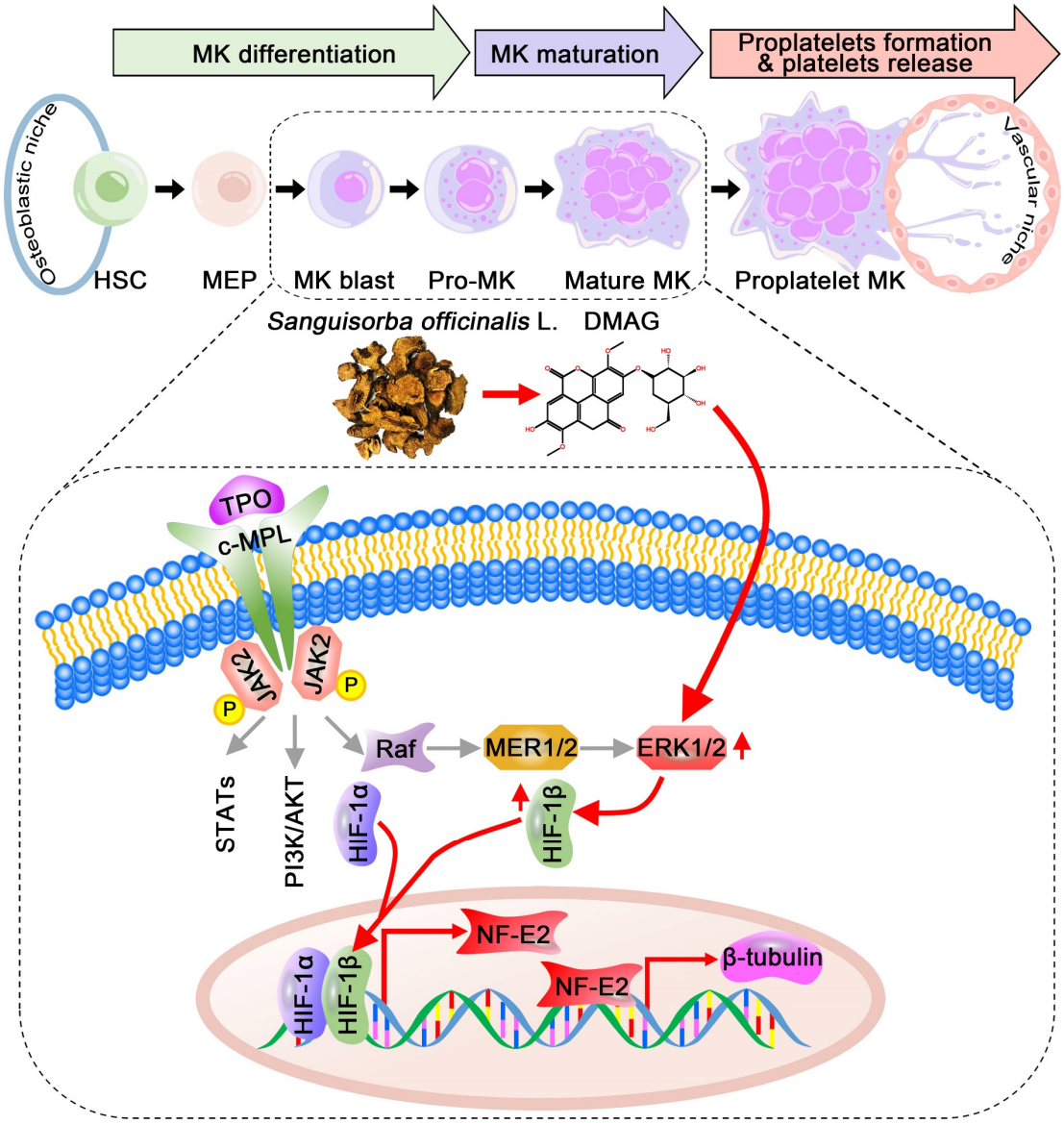



**Supplementary Figure S17. Effects of DMAG on megakaryopoiesis and thrombopoiesis in the c-MPL$^{-/-}$ mouse model.** (A) Flow cytometry analysis indicates the expression of CD41 in BM and spleen cells in c-MPL$^{-/-}$ mice after administration of normal saline or DMAG (5 mg/kg) daily for 10 consecutive days. The histogram

shows the proportions of $CD41^+$ cells (megakaryocytes) in BM and spleen. (B) Flow cytometry analysis indicates the expression of c-Kit and CD41 in BM cells in c-$MPL^{-/-}$ mice after administration of normal saline or DMAG (5 mg/kg) daily for 10 consecutive days. The histogram shows the proportions of c-$Kit^+CD41^-$ cells (hematopoietic progenitors), c-$Kit^+CD41^+$ cells (megakaryocytic progenitors), and c-$Kit^-CD41^+$ cells (megakaryocytes) in BM. (C) Flow cytometry analysis indicates the expression of CD41 and CD62P in BM cells in c-$MPL^{-/-}$ mice after administration of normal saline or DMAG (5 mg/kg) daily for 10 consecutive days. The histogram shows the proportions of $CD41^+CD62P^-$ cells (megakaryocytes), $CD41^+CD62P^+$ cells (platelets) and $CD41^-CD62P^+$ cells (activated platelets) in BM. The data are shown as the mean ± SD from three independent experiments. **P* <.05, vs the corresponding control.




**Supplementary Figure S18. Effects of DMAG on peripheral platelet levels and megakaryocyte numbers in normal C57BL/6 mice.** (A) Peripheral platelet, RBC and WBC numbers in C57BL/6 mice injected with normal saline or DMAG (5 mg/kg) for 14 consecutive days. Histologic analysis of BM (B) and spleen (C) after the mice were administered normal saline or DMAG (5 mg/kg) for 10 days. Bars represent 100 μm (top) and 50 μm (bottom). Ten microscopy fields per sample were counted. The green circles indicate megakaryocytes. The histogram shows the number of megakaryocytes in the BM (B) and spleen (C) in each group. The data are shown as the mean ± SD from three independent experiments. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; RBC: Red blood cell; WBC: White blood cells; BM: Bone marrow.




**Supplementary Figure S19. Schematic representation of the mechanism by which DMAG regulates megakaryocyte differentiation and thrombopoiesis.** DMAG stimulates the phosphorylation of ERK1/2, which activates the expression of HIF-1β. Activated HIF-1β dimerizes with HIF-1α to form fully active HIF-1, causing the activation of NF-E2. NF-E2 subsequently regulates the expression of many genes, such as β-tubulin, leading to megakaryocyte differentiation and thrombopoiesis. In this process, the ERK1/2-HIF-1β-NF-E2 pathway induced by DMAG is independent of the

TPO signaling pathway. MK: Megakaryocyte. DMAG: 3,8-Di-O-methylellagic acid 2-O-glucoside; HSC: Hematopoietic stem cell; MEP: Megakaryocyte-erythroid progenitor cell.

## Supplementary Tables

**Supplementary Table S1. Primers used in this study.**

| RT-qPCR primers | Sequences |
|---|---|
| qNF-E2-F | GGAGAGATGGAACTGACTTGGC |
| qNF-E2-R | GAATCTGGGTGGATTGAGCAGG |
| qGATA1-F | GGCCTCTATCACAAGATGAATG |
| qGATA1-R | ACTGAGTACCTGCCCGTTTACT |
| qGATA2-F | CAAGGCTCGTTCCTGTTCA |
| qGATA2-R | GCCCATTCATCTTGTGGTAGA |
| qFLI1-F | CCAACGAGAGGAGAGTCATCG |
| qFLI1-R | TTCCGTGTTGTAGAGGGTGGT |
| qTAL1-F | CCAAAGTTGTGCGGCGTATC |
| qTAL1-R | CAGGCGGAGGATCTCATTCTT |
| qRUNX1-F | CCTACGCACTGGCGCTGCAACAA |
| qRUNX1-R | GCGGTGGGTTTGTGAAGACAGTGAT |

| siRNAs target sequence | |
|---|---|
| siNF-E2 | TCCTGTGCTACCAAAGGAA |

| CRISPR target sequence | |
|---|---|
| gRNA-A1 | TTTAGTCAACACATGTAGCG-TGG |
| gRNA-A2 | GTCCCCTAGCTTGCTGTTAA-GGG |

| | |
|---|---|
| gRNA-B1 | GTCCCCTAGCTTGCTGTTAA-GGG |
| gRNA-B2 | CCTGTAGCTCCAAACCCTTG-GGG |

PCR screening primers

| | |
|---|---|
| F | CATGCCAATGGTTCACTCTAAGGT |
| R | TCTCTATGTCCCAAAGTGCAGACAC |
| F1 | GATGGACAGAGCGTTCCTACC |
| R1 | TCCACACTTCGTAGATGAGAGGG |
| R2 | GGTTCCTGTCAGATACA |