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Lysophosphatidic Acid Receptors 2 and 3 Regulate Erythropoiesis at Different Hematopoietic Stages

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Abstract

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Declaration of competing interest

The authors declared that they have no competing interest.

Hematopoiesis, the complex developmental process that forms blood components and replenishes the blood system, involves multiple intracellular and extracellular mechanisms. We previously demonstrated that lysophosphatidic acid (LPA), a lipid growth factor, has opposing regulatory effects on erythrocyte differentiation through activation of LPA receptors 2 and 3; yet the mechanisms underlying this process remain unclear. In this study, LPA_2 is observed that highly expressed in common myeloid progenitors (CMP) in murine myeloid cells, whereas the expression of LPA_3 displaces in megakaryocyte-erythroid progenitors (MEP) of later stage of myeloid differentiation. Therefore, we hypothesized that the switching expression of LPA_2 and LPA_3 determine the hematic homeostasis of mammalian megakaryocytic-erythroid lineage. *In vitro* colony-forming unit assays of murine progenitors reveal that LPA_2 agonist GRI reduces the erythroblast differentiation potential of CMP. In contrast, LPA_3 agonist OMPT increases the production of erythrocytes from megakaryocyte-erythrocyte progenitor cells (MEP). In addition, treatment with GRI reduces the erythroid, CMP, and MEP populations in mice, indicating that LPA_2 predominantly inhibits myeloid differentiation at an early stage. In contrast, activation of LPA_3 increases the production of terminally differentiated erythroid cells through activation of erythropoietic transcriptional factor. We also demonstrate that the LPA_3 signaling is essential for restoration of phenylhydrazine (PHZ)-induced acute hemolytic anemia in mice and correlates to erythropoiesis impairment of Hutchinson-Gilford progeria Symptom (HGPS) premature aging expressed K562 model. Our results reveal the distinct roles of LPA_2 and LPA_3 at different stages of hematopoiesis *in vivo*, providing potentiated therapeutic strategies of anemia treatment.

Keywords

Lysophosphatidic acid; Lysophosphatidic acid receptor; 1-Oleoyl-2-O-methyl-rac-glycerophosphothionate; GRI compound 977143; Erythropoiesis; Anemia

1. Introduction

Hematopoiesis is a dynamic process to continuously replenish blood components and sustain the blood system of vertebrates [1]. In adult hematopoiesis, hematopoietic stem cells (HSC) support more than 10 functional types of blood cells, and give rise to multipotent progenitors (MPP) with minor self-renewal ability but complete lineage differentiation potential [2]. The MPP population is heterogeneous and able to differentiate into oligopotent progenitors, the common lymphoid progenitors (CLP) [3] and the common myeloid progenitors (CMP) [4]. The CLP population further gives rise to T cells, B cells, and natural killer (NK) cells, while CMP cells commit to become either granulocyte-monocyte progenitors (GMP) (ultimately generating dendritic cells, granulocytes, and monocytes) or megakaryocyte-erythroid progenitors (MEP) [5]. Lineage specification of MEP into erythrocytes and megakaryocytes, which comprise 99% of cellular components of blood in the human body, is the most crucial process in hematopoiesis [6]. Therefore, disturbance of myeloid differentiation may cause severe physiological dysfunction. Among these multiple hematological disorders, anemia is a highly frequent hematinic deficiency, with a high risk of bleeding and late mortality clinical outcomes, and determines heart failure due to the amount of oxygen to the myocardium reducing [7, 8]. In addition, multiple physical deteriorations are evidenced that lead to the prevalence of the age-dependent hematological diseases, including immune

function decline and anemia are quite frequently diagnosed in old individuals [9–11]. Moreover, erythrocyte senescence is also observed in chemical-induced premature aging rat [12]. Given these health problems, understanding the differentiation process of the myeloid lineage is extremely important.

Myeloid differentiation is shown to be regulated by lysophosphatidic acid (LPA) through activation of multiple LPA receptors, a subgroup of G protein-coupled receptors [13]. LPA, a lipid growth factor that is enriched in serum, has a structure comprised of a polar phosphate head group, a glycerol backbone, and a fatty acid chain [14]. LPA evokes diverse cellular responses through the activation of six distinct G protein-coupled receptors localized in the plasma membrane of various cells. LPA_1, LPA_2, and LPA_3 belong to the endothelial cell differentiation gene family [15]. LPA_4 and LPA_5 belong to the P2Y purinergic receptor family, as does LPA_6, which was recently identified [16]. Upon binding of LPA, conformation changes in the LPA receptors allow them to act as a guanine nucleotide exchanger factor for one or more of the four classes of heterotrimeric G proteins ($G_{12/13}$, $G_{q/11}$, $G_{i/o}$, and G_s) that initiate signaling cascades through downstream molecules, such as Rho, P3-DAG, Adenylyl cyclase, and the Ras/MAPK pathway [17]. Considering the wide range of downstream signaling, LPA has distinct fundamental cellular functions and has been studied for its ubiquitous roles in cell proliferation, migration, adhesion, survival, differentiation, invasion, and cytoskeletal reorganization [18–22]. Moreover, a broad range of physiological and pathological conditions mediated by LPA have been identified, involving platelet aggregation, angiogenesis, reproduction, cancer progression, and neurite retraction [23–27].

A substantial amount of LPA has been detected extracellularly in the serum [28] and other biological fluids [29, 30], during the highly dynamic processes of formation and degradation [26, 31]. In the bone marrow, the major hematopoietic organ in the human body, LPA and its production enzyme autotoxin are present in the perivascular niche near microvessels and also diffuse directly from blood plasma, thereby inducing the differentiation of multipotent HSC into CMP rather than CLP [32]. Moreover, our previous studies have demonstrated that LPA participates in erythropoiesis through the activation of LPA_3 in various vertebrate models, including zebrafish, mice, and humans [33, 34]. Furthermore, we demonstrated that the activation of different LPA receptors may play opposing roles during fate specification along the myeloid lineage [33, 35]. These findings strongly suggest that alteration of the expression patterns of LPA receptors may contribute to the differentiation fate decision of hematopoietic progenitor cells. However, a limited number of studies have investigated the roles of each receptor in the complicated hematopoietic hierarchy. Therefore, in this study, we aim to reveal distinct roles of LPA_2 and LPA_3 at different stages of hematopoiesis to determine the hematic homeostasis of mammalian megakaryocytic-erythroid lineage. LPA_2 was shown to block myeloid lineage with impaired proliferation and increased apoptosis on CMP. In contrast, activation of LPA_3 promoted the erythroid differentiation through regulating erythroid transcription factors. Mostly, LPA_3 agonist 1-Oleoyl-2-O-methyl-rac-glycerophosphothionate (OMPT) successfully restored phenylhydrazine (PHZ)-induced acute hemolytic anemia in mice. The signaling of LPA_3 was also shown to participate in aging-related anemia in Hutchinson-Gilford progeria Symptom (HGPS) premature aging

expressed K562 model. Our findings suggested the potential for therapeutic strategies targeting LPA receptors for the treatment of anemia.

2. Material and Methods

2.1. Mice and pharmacological treatment.

Aged 4-8 weeks BALB/*c* wild type male mice and 4 weeks female mice were obtained from SPF breeding facility of BioLASCO (Taipei, Taiwan) and Jackson Laboratory (Bar Harbor, Maine, USA), were housed in the experimental animal facility with a 12 h light and dark cycle. The entire animal procedure was performed in accordance with governmental regulations (Guideline for the care and use of laboratory animals, Council of Agriculture, Taiwan) and after approval from the Institutional Animal Care and Use Committee (Approval number B201700206, National Taiwan University, Taiwan). 1-Oleoyl-2-O-methyl-rac-glycerophosphothionate (OMPT) (Cayman Chemicals, Ann Arbor, Michigan, USA) and GRI compound 977143 (GRI) (Genome Research Institute, University of Cincinnati Drug Discovery Center, Cincinnati, Ohio, USA) were separately dissolved in ethanol:chloroform (1:1) and DMSO. Daily treatment was operated by intraperitoneal injection to mice at the concentration of 1 mg/kg GRI and 0.5 mg/kg OMPT for 4 consecutive weeks. Both the agonists were prepared in PBS solution containing 3% fatty acid-free bovine serum albumin (Sigma-Aldrich, Missouri, USA).

2.2. Cell culture and pharmacological treatment

K562 human erythroleukemia cells obtained from ATCC were cultured in RPMI 1640 (Ge Healthcare Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 U/mL) (Lonza, Visp, Switzerland) in a humid atmosphere of 5% CO_2 at 37°C. Cell density was maintained between 10^5~10^6 cells/mL, were dissolved in dimethylsulfoxide (DMSO). For pharmacological treatment, 2×10^5 cells were seeded in 6-well plates and treated by incubation with OMPT (Cayman Chemicals), GRI (Genome Research Institute) or Hemin for 5 to 24h.

2.3. Plasmid construct and transfection

GFP-progerin cDNA was subcloned from our progerin-pAS2.V1 vector, which used in our previous study [36], into pcDNA5.TO vector. To establish Tet-on inducible progerin K562 cell model, GFP-progerin-pcDNA5.TO and pcDNA6.TR vector were co-transfected with Lipofectamine LTX (Thermo Fisher Scientific, Waltham, Massachusetts, USA), followed by Blasticidin (5 ug/ml) and Hygromycin (200 ug /ml) selection for positive clone. In order to study effects of progerin on K562 differentiation, tetracycline (1 ug/ml) was added to turn on expression of GFP-progerin. Induction of GFP-progerin was confirmed by western blot and fluorescent microscopy.

2.4. Hematopoietic colony formation assay.

Clonogenic assays were performed on flow-sorted CMP and MEP (1500 cells/ml), using Methocult GF M3434 media (Stem Cell Technologies, Vancouver, Canada) containing SCF, IL-3, IL-6, IL-11, GM-CSF, Flt3-ligand, TPO and EPO (Peprotech, Rocky Hill, New Jersey,

USA) with or without LPA receptors agonists, 5 μM GRI and 50 nM OMPT. The number of colonies was scored after 14 days in culture, respectively.

2.5. Western blot

K562 cell lysates were extracted using RIPA lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) supplemented with 1% protease inhibitor Cocktail (Set V, Merck, Darmstadt, Germany), followed by centrifugation at 4°C and 14000 rpm for 15 minutes. The supernatants were then collected and concentrations were measured using a Bradford protein assay (Bio-Rad, Hercules, California, USA) using BSA as a standard. Twenty μg of total protein were denatured at 100°C for 10 minutes and resolved by 10% SDS polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to polyvinylidene difluoride membranes (Merck, Darmstadt, Germany), which were blocked by 5% BSA in Tris-buffered saline (20 mM Tris, pH 7.4, 150 mM NaCl) containing 0.1% Tween-20 (TBST) for 1 hour before being probed with antibodies overnight at 4°C. The membranes were then washed in TBST and reacted with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, California, USA) and developed using a WesternBright ECL detection kit (Advansta, California, USA). The membranes were then imaged using a UVP AutoChemi Image System (Ultra-Violet Products Ltd, California, USA). Primary antibodies against full length and cleaved form PARP were obtained from Cell Signaling Technology (Danvers, Massachusetts, USA) (46D11 and D64E10), the antibodies against LPA_2 (ab38322) and Lamin A (ab108595) were obtained from Abcam (Cambridge, UK), the antibody against LPA_3 (sc-390270) was obtained from Santa Cruz, and the antibody against GAPDH (GTX100118) was obtained from Genetex (California, USA).

2.6. Peripheral Blood Analysis

Blood was collected from facial vein and mixed with 2.2 mg/ml EDTA to prevent coagulation at the ratio of 1:1. Complete blood counts were determined on Sysmex XT-2000i Automated Hematology Analyzer (Sysmex) to analyze the number of RBCs, Hemoglobin (HGB, g/dL) and Hematocrit (HCT, %).

2.7. Flow cytometric analysis and sorting

Cells were isolated from the bone marrow and spleen of wild-type BALB/*c* mice and then resuspended in phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS). The cell suspension was filtered through the 40-μm cell strainer (BD Falcon, San Diego, CA, USA) for obtaining single-cell suspension. To obtain megakaryocytes, cells were stained with FITC-conjugated anti-CD61 (130-102-626; MACS, Miltenyi Biotec, Germany) antibody. For acquisition and analysis of different stages of erythroid precursors, cells were stained with PE-conjugated anti-Ter119 (130-102-336; MACS) and FITC- conjugated anti-CD71 (553288; BD Biosciences) antibodies. To obtain myeloid progenitor, PerCP-CyTM5.5 Mouse Lineage Antibody Cocktail (51-9006964; BD Biosciences) was used to label cells from the majority of hematopoietic lineages, and lineage marker-negative (Lin^-) cells were collected, followed by staining with FITC-conjugated anti-Sca-1 (130-102-297; MACS), PE-conjugated anti-c-Kit (130-102-542; MACS), Alexa Fluor® 647- conjugated anti-CD34 (560230; BD Biosciences), and APC-CyTM7-conjugated anti-FcγRIII/FcγRII (580541; BD

Biosciences) antibodies. All the cells were incubated with antibodies at 4°C for 20 minutes. For flow cytometric analysis and cell sorting, BD FACSAria III (BD Biosciences) was utilized. LSKs were defined as $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$, common myeloid progenitor (CMP) as $Lin^{-}Sca\text{-}1^{-}c\text{-}KIT^{+}Fc\gamma RIII/Fc\gamma RII^{lo}CD34^{+}$, granule myeloid progenitor (GMP) as $Lin^{-}Sca\text{-}1^{-}c\text{-}KIT^{+}Fc\gamma RIII/Fc\gamma RII^{hi}CD34^{+}$ and megakaryocyte–erythroid progenitor (MEP) as $Lin^{-}Sca\text{-}1^{-}c\text{-}KIT^{+}Fc\gamma RIII/Fc\gamma RII^{-}CD34^{-}$.

K562 cell proliferation was determined using Ki67 staining. Agonists treatment cells were fixed with 4% paraformaldehyde (PFA, Sigma, USA) in PBS and permeabilized with 0.1% TritonX-100 (Sigma). Cells were blocked with 3% FBS in PBS, followed by staining with Alexa Fluor® 488-conjugated anti-Ki67 (558616; BD Biosciences) for 1 hr in RT incubation. For analysis of K562 apoptosis, live cells were stained with both PE-Annexin V and 7-AAD according to manufacturer's recommended protocol (559763; BD Biosciences). The population of PE-Annexin V^{+} and 7-AAD^{-} were defined as apoptosis cells. For analysis of K562 erythropoiesis, cells were stained with PE-conjugated anti-GlyA (555570; BD Biosciences). All experiments were detected by BD FACSCanto II or BD FACSAria III (BD Biosciences) and analyzed by FCS Express software (De Novo, Los Angeles, CA, USA).

2.8. Immunofluorescence analysis

Mice femurs, and spleens were isolated and fixed in 4% PFA (Sigma) for 48h, and subsequently embedded in Optimal Cutting Temperature (O.C.T) (Trajan, Australia), sectioned at 10μm, and Immunofluorescence stained. Before embedding, femurs needed to be further decalcified in 5% nitric acid for 48 hours. Spleen and femurs sections were blocked with 1% BSA and 5% goat serum in PBST (0.1% tween-20 in PBS) for 1 hour and incubated with rabbit anti mouse CD61 (Abcam) overnight. After incubation, cells were stained with Cy3-labeled goat anti–rabbit IgG (Jackson ImmunoResearch, USA) for 1hr, followed by 5 min staining of 4, 6-diamidino- 2-phenylindole (DAPI, Sigma-Aldrich).

Tetracycline (tet)-on inducible control and HGPS K562 cells were harvested, followed by fixation with 4% PFA and permeabilization by 0.5% Triton X. Fixed cells were incubated with anti-LaminA, anti-LPA_3 (ab108595 and ab23692; Abcam) and anti-GFP (sc-9996; Santa Cruz) primary antibodies overnight and then labeled with Alexa Fluor 647 and FITC fluorescent secondary antibody (A-31573 and A16018; Invitrogen, Carlsbad, CA, USA) for 1 hr. All slides were mounted with Fluoromount-GTM (Emsdiasum, Fort Washington, PA, USA) and imaged by Zeiss AxioPlan 2 fluorescence microscope (Zeiss, Jena, Germany).

2.9. RNA extraction, RT-PCR, and real-time qPCT

Sorted progenitor cells and homogenized spleen samples from mice were added with 400 μL TRIzol (Invitrogen) and stored at −80˚C. 1 μg of total RNA was converted to cDNA by reverse-transcription polymerase chain reaction (RT-PCR) with ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). RT-PCR with reagent Phusion® High-Fidelity DNA Polymerase (New England Biolabs, USA) was applied to quantify the expression levels of target mRNA with cycling conditions: 98˚C for 30s, followed by 35 cycles of 98˚C for 10s, 60˚C for 30s, and 72˚C for 15s, then final extension 72˚C for 5 min. The PCR products were then run on 1% agarose gel and image with E-box image system (VILBER, France). Real-

time qPCR with reagent SYBR® Green Mater Mix (Bio-Rad, Hercules, CA, USA) was applied to quantify the expression levels of target mRNA with cycling conditions: 95˚C for 3 min, followed by 45 cycles of 95˚C for 30s, 60˚C for 30s, and 72˚C for 30s. Each gene was normalized to the amount of internal control Rpl-7 or Gapdh for each samples. Specific primer sequences of mice for Lpa1 were: forward: 5'-TGG GTA TCG CGC CCA TAA CA-3', reverse: 5'-TGG CCA TTG CAA TCG AGA GG-3', Lpa2 were: forward: 5'-CCA GCC TGC TTG TCT TCC TA-3', reverse: 5'-GTG TCC AGC ACA CCA CAA AT-3', for Lpa3 were: forward: 5'-CTG TAG TTG TTT CCG CAT GG-3', reverse: 5'-GAA GTT CAA CCT ACC GGT CAA-3', for Lpa4 were: forward: 5'-ACT GCG TTC CTC ACC AAC AT-3', reverse: 5'-CGA TCG GAA GGG ATA GAC AA-3', for Lpa5 were: forward: 5'-TTT GCA TAT GGT GGT CTA CA-3', reverse: 5'-GCT GTG CAT AGT AGG AGA GG-3', for Lpa6 were: forward: 5'-ATG TAC CCG ATC ACT CTC TG-3', reverse: 5'-AGG TCT GTA GGT TGT GTT GG-3', for Rpl7 were: forward: 5'-GCA GAT GTA CCG CAC TGA GAT TC-3', reverse: 5'-ACC TTT GGG CTT ACT CCA TTG ATA-3', for Eklf were: forward: 5'-GAT GAA ATA AGA GTG GAT CCA AGG-3', reverse: 5'- TCT AGG GGT CCA TTT GAG TTG-3', for Gata-1 were: forward: 5'- CAA GAA GCG AAT GAT TGT CAG-3', reverse: 5'- AGT GTT GTA GTG GTC GTT TG-3', for Hba-a1 were: forward: 5'-AAC TTC AAG CTC CTG AGC CA-3', reverse: 5'-GGC AGC TTA ACG GTA CTT GG-3', for Hbb-b1 were: forward: 5'-CTG ACA GAT GCT CTC TTG GG-3', reverse: 5'-CAC AAC CCC AGA AAC AGA CA-3', for Gapdh were: forward: 5'-TGA CGT GCC GCC TGG AGA AA-3', reverse: 5'- AGT GTA GCC CAA GAT GCC CTT CAG-3'. Specific primer sequences of human for p21 were: forward: 5'-TGA GCC GCG ACT GTG ATG-3', reverse: 5'-GTC TCG GTG ACA AAG TCG AAG TT-3', for BAX were: forward: 5'-CCT TTT CTA CTT TGC CAG CAA AC-3', reverse: 5'-GAG GCC GTC CCA ACC AC-3', for GlyA were: forward: 5'-ACA GAC AAA TGA TAC GCA CAA ACG GG-3', reverse: 5'-GGG CTT TTC TTT ATC AGT CGG CGA-3', for γ-globin were: forward: 5'-GCA GCT TGT CAC AGT GCA GTT C, reverse: 5'-TGG CAA GAA GGT GCT GAC TTC-3', for GAPDH were: forward: 5'-AAG GTG AAG GTC GGA GTC-3', reverse: 5'-TGT AGT TGA GGT CAA TGA AGG-3'.

2.10. Haemolytic anaemia mice experiment and Voluntary Exercise

Aged 4 weeks BALB/*c* wild-type male mice (LASCO) were pretreated with either ethanol:chloroform as a control or 0.5 mg/kg OMPT (Cayman Chemicals) for 3 days followed by treatment of 60 mg/kg phenylhydrazine (PHZ, Sigma). Control solution or OMPT injection were continue for another 7 days. Peripheral blood samples were collected at each day from day 0 to day 4, day 7, and day 9 for complete blood count (CBC) analyses. Function recovery were analyzed by voluntary exercise tests.

Mice were housed individually in cages containing rodent exercise wheels equipped with a digital counter activated by wheel rotation [37], and maintained with 12-h light and dark cycle. The number of revolutions was calculated for each day and recorded by using ClockLab (Actimetrics, USA). The mean counts of revolutions were compared between control and OMPT treatment mice with PHZ-induced haemolytic anemia from days 2 to 4.

2.11. Statistical analyses

Each result represents at least three independent experiments. The data are presented as mean ± SD, and t-test or one-way analysis of variance (ANOVA) were used to determine the statistical differences between control and experimental groups, with $p^{*} \leq 0.05$, $p^{**} \leq 0.01$, $p^{***} \leq 0.001$ considered statistically significant. Fluorescent images were analyzed by image J to determine the strength of fluorescent signaling.

3. Results

3.1. Differential expression patterns of LPA_2 and LPA_3 in myeloid progenitors at different stages of hematopoiesis

We previously reported that LPA_2 and LPA_3 play opposite roles during fate specification of the megakaryocytic-erythroid lineage [33, 35]. To clarify the roles of LPA receptors in mammalian myeloid lineage cells and megakaryocytic-erythroid fate decision, we first evaluated the expression of LPA receptors in myeloid progenitors of different stages. LSK ($Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$; including HSC and MPP), CMP, and MEP were sorted from the bone marrow of wild-type male BALB/*c* mice through surface marker recognition (Fig. 1A). The bone marrow cells of wild-type BALB/*c* mice were first gated using Sca-1 and c-Kit antibodies as well as lineage antibody cocktail (Lin) to obtain the LSK fraction (Fig. 1A, up) [38]. The myeloid progenitors ($Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}$ fraction) were subdivided into cell population of $CD16/32^{low}CD34^{+}$ (CMP), $CD16/32^{hi}CD34^{+}$ (GMP), and $CD16/32^{-}CD34^{-}$ (MEP) respectively, based on the expression profiles of Fcγ receptor II/III (CD16/32) and CD34 (Fig. 1A, down) [4]. The PCR and real-time qPCR results showed that CMP expresses a higher level of LPA_2 in comparison with LSK and MEP. On the other hand, LPA_3 was only detected in MEP, but not in the early stages of myeloid progenitors LSK and CMP (Fig. 1B,C and Fig. S1). The profile of LPA_2 and LPA_3 level were also confirm in female BALB/*c* mice to avoid the gender difference. The results from progenitors of female mice were indeed in line with the male mice (Fig. S1A). The expression switch was also confirmed at the protein level in K562 cells (Fig. S2). Together, these results implied an expression pattern switch between LPA_2 and LPA_3 during the differentiation of myeloid progenitors differentiation. It indicated that these two receptors may play distinct roles in megakaryocytic-erythroid lineage regulation.

3.2. Activation of LPA_2 and LPA_3 regulated myeloid differentiation at distinct stages

To delineate the physiological significance of expression divergence between LPA_2 and LPA_3 during myeloid differentiation, we performed hematopoietic colony-formation assay upon stimulation of the LPA receptors. Sorted CMP and MEP from bone marrow were respectively cultured in methylcellulose medium containing cytokines for the differentiation of blood cells with or without the presence of LPA receptor agonists (Fig. 1D), followed by colony numbers scoring after 14 days culture. GRI977143 (GRI), an LPA_2 agonist, significantly decreased the number of CMP colony-forming units, whereas no effect was observed in MEP colony-forming units. In contrast, treatment with OMPT, an LPA_3 agonist, had no effect on the number of CMP colony-forming units, whereas it increased the clonogenicity of MEP (Fig. 1E). Hence, these results are in agreement with the LPA

expression profiles of each progenitor population, suggesting that LPA_2 and LPA_3 act at different stages of progenitor cells in megakaryocytic-erythroid lineage regulation.

3.3. LPA_2 dominantly inhibited myeloid-erythroid-megakaryocytic differentiation

Duo to the importance of the myeloid-erythroid-megakaryocytic axis as these lineages comprise 99% of the main cellular components of blood in the human body to maintain the whole blood system [6], we aim to examine the effects of LPA2 receptor agonist on proportions of hematopoietic progenitors and downstream erythrocytic and megakaryocytic lineage in mice model. BALB/*c* mice were injected with either DMSO solvent, control, or 1 mg/kg GRI every 2 days. Subsequently, complete blood count analysis and flow cytometry were performed to measure changes in blood components each week for 1 month. One week treatment with GRI led to a decrease in the number of CMP and MEP (Fig. 2A). The numbers of red blood cells (RBC) and levels of hemoglobin were also significantly decreased (Fig. 2B). For detail study about the different stages of erythroblasts, pro-erythroblasts, erythroblasts-A (EryA), EryB, and EryC in the bone marrow and spleen were distinguished based on the cell size and the expression patterns of surface markers (Ter119 and CD71) using flow cytometry [39] (Fig. 2C). After 1 week of treatment with GRI, the number of pro-erythroblasts, and $Ter119^{high}$ precursors was significantly decreased. The number of EryA and EryB precursor cells was also decreased (Fig. 2C–E), indicating that the activation of LPA_2 inhibited erythropoiesis. Moreover, flow cytometry and immunofluorescence staining showed that treatment with GRI resulted in marked impairment of megakaryocytic differentiation resulting in very few $CD61^+$ cells (Fig. 2F–J). Together, these results suggested the specific impairment of early myeloid differentiation by LPA_2, which is evident at all stages of the myeloid-erythroid-megakaryocytic lineage.

3.4. LPA_3 acted on MEP to promote erythropoiesis at the expense of megakaryopoiesis

Given that the results of the expression profile analysis and clonogenic assay revealed the central role of LPA_3 at the MEP stage. Thereby, to further determine the requirements for LPA_3 in the differentiation of bipotent MEP progenitors, BALB/*c* mice were intraperitoneally injected with either chloroform:ethanol solvent, control, or 0.5 mg/kg OMPT through a similar strategy. After 1 week treatment, the number of progenitors was not altered by treatment with OMPT (Fig. 3A). However, activation of LPA_3 was found to increased erythropoiesis at the expense of megakaryopoiesis in the spleen. This was evidenced by the significant increase in peripheral blood with RBC and hemoglobin counts increasing (Fig. 3B) and also erythroid populations enhance in spleen (Fig. 3C,D), accompanied by the decrease in megakaryocyte populations (Fig. 3F–H). In contrast, OMPT had not obvious effects on the bone marrow (Fig. 3E,I), which can be explained by different proportions of each progenitor population in the spleen and bone marrow (Fig. S5). In conclusion, it unveils that LPA_3 affects the fate specification of MEP into erythroid differentiation.

3.5. Activation of LPA_2 distrusted the functionality of myeloid cells

Since LPA_2 showed the wide perturbation of myeloid-erythroid-megakaryocytic lineage development with both the *ex-vivo* clonogenicity and *in-vivo* progenitor frequencies decline, but the lineage specification was not altered (Fig. S3). To better characterize the function

role of LPA_2 on CMP, the human hematopoietic cell line K562 was used as a myeloid progenitor model, which retains the capacity to differentiate into granulocytes, megakaryocytes, and erythrocytes [40, 41]. In K562 cells, the proliferation rate was decreased with GRI treatment compared to control or OMPT treatment (Fig. 4A). In addition, activation of LPA_2 increased proliferation marker Ki67 negative cells after treatment of GRI for 12 h (Fig. 4B). Consistent with the results obtained from the animal model, activation of LPA_2 by GRI decreased the number of CMP. Similarly, another group also suggested that activation of LPA_2 arrests cell cycle and inhibits the proliferation of melanocytes [42]. Moreover, we also evidence that LPA_2 might promote apoptosis of myeloid progenitors. The population of apoptosis cells (Annexin V^+7-AAD^-) increased after treatment of GRI for 12h (Fig. 4C). In addition, the apoptosis markers p21, BAX, and cleaved form of poly-ADP ribose polymerase (PARP) were markedly up-regulated after 5 h of treatment with GRI, confirming the induction of apoptosis after LPA_2 activation (Fig. 4D,E). The results are similar with the previous study which suggested that LPA induces apoptosis through LPA_2/MAPK pathway in neuronal PC12 cell [43]. To sum up, these data suggest that LPA_2 leads to aberrant proliferation and apoptosis in myeloid progenitor to impair downstream lineage differentiation.

3.6. LPA_3 promoted the erythroid lineage through lineage-specific transcription factors

Given that LPA_3 was an essential mediator of erythropoiesis, we set out to further investigate whether LPA_3 governs fate-decision of MEP reflected alterations in lineage-affiliated gene expression signatures. In spleen RNA extract, treatment with OMPT increased the levels of both erythroid fate decision factors Eklf and Gata-1 (Fig. 4F). Consistent with the results of the complete blood count analysis, the expression of the hemoglobin-related genes Hba-a1 and Hbb-b1 were markedly upregulated following the activation of LPA_3 in spleen RNA extract (Fig. 4G). Collectively, these results emphasized that LPA_3 acted at a later stage of progenitor, MEP, to mainly alter the erythroid differentiation fates at the expense of megakaryopoiesis.

3.7. LPA_3 signaling is a potential treatment target for anemia and correlates to erythroid impairment in premature aging cell model

Our findings demonstrated that the LPA_3 agonist significantly increased the number of erythrocytes in wild-type mice, suggesting a potential therapeutic strategy for anemia. Accordingly, it's imperative to investigate whether the activation of LPA_3 by OMPT is sufficient to relieve symptoms of anemia using a PHZ-induced hemolytic anemia model. PHZ damages erythrocytes through automatic oxidation and is widely used to investigate potential treatments for erythrocytic deficiency [44]. To establish the anemia model, wild-type BALB/*c* mice were treated with 60 mg/kg PHZ to induce hemolytic anemia at Day 0, and blood samples were collected daily for 9 days until the number of RBC recovered to the basal level. The body weight of general parameter, number of erythrocytes, and level of hemoglobin in the blood were markedly decreased in response to PHZ (Fig. 5A–C). Treatment with OMPT led to a marked elevation in the level of hemoglobin and increased RBC count compared with control at Day 3, on which with the lowest numbers of erythrocytes caused by PHZ-induced anemia (Fig. 5D,E). The blood oxygen content is referred to as oxygen delivery and associated with the regulation of exercise performance

[45]. Therefore, a wheel rotation assay was performed to analyze aerobic exercise performance during hemolytic anemia to further elucidate the role of OMPT in recovery from anemia. Wheel evolutions demonstrated that treatment with OMPT rescued the athletic ability of mice impaired by treatment with PHZ (Fig. 5F,G). The exercise performance of PHZ treated mice was decreased compared with that observed in the control mice between Days 2-4 (the most severe state of anemia). In contrast, treatment with OMPT alleviated PHZ-induced anemia and improved the exercise performance.

To investigate the participation of LPA signaling in aging-related anemia, premature aging HGPS model was used. In our previous study, we have demonstrated that LPA_3 is declining in the aging process and acts as a key contributor to regulate ROS level and cell senescence in HGPS cells and zebrafish model [36]. HGPS is one of the most severe laminopathies and a rare genetic disorder with several premature aging features and the profiling of signaling pathways shows similar pattern with normal aging cells [46]. The syndrome is typically due to a silent mutation (c. 1824C>T; p. Gly608Gly) of *LMNA* that activates alternative pre-mRNA cryptic splicing site and causes 150-nucleotides, 50 amino acids deletion. The missing sequence includes recognition site for ZMPSTE24 endoprotease, leading to the accumulation of un-cleaved Lamin A isoform, named progerin [47]. To establish the tetracycline (tet)-on inducible HGPS K562 cell model, Tet-on control vector (pcDNA5/TO) and 150-nucleotides deletion LMNA (progerin) with EGFP tagging were stably expressed in K562 cells. With tetracycline induction, western blot analysis with Lamin A antibody showed the expression of progein-EGFP protein (Fig. 6A) and the abnormal nucleus morphology in tet-on progerin cells (Fig. 6B). In addition, overexpression of tet-on progerin declined the erythrocyte marker glycophorin A (GlyA) and γ-globin (Fig. 6C,G,H), confirming the similar hematopoietic defect with normal aging. Noteworthy, the protein level of LPA_3 was down-regulated in progerin expressed K562 cell, whereas level of LPA_2 was not altered (Fig. 6D–F), suggesting the decline of LPA_3 level during aging process might exist to determine the aging-related anemia. Moreover, activation of LPA_3 with 50 nM OMPT for 3 days rescued the declining erythrocyte markers GlyA and γ-globin in progerin expressed K562 cell (Fig. 6G,H). Taken together, these finding strongly suggest that LPA_3 may be a key regulator of the erythropoiesis and the activation of LPA_3 by agonists is a potential therapeutic strategy for anemia.

4. Discussion

Several studies have suggested that LPA signaling participates in hematopoiesis. For instance, high concentrations of LPA and the production enzyme autotoxin have been detected in the perivascular niche near microvessels, where they contribute to myelopoiesis [32]. Moreover, our previous results showed that LPA receptors may play opposing roles during the fate specification of the terminal erythroid-megakaryocytic lineage [33–35]. In addition, during early hematopoiesis, LPA is involved in the critical development process and regulates primitive hematopoietic cell motility through a PI3K- and Vav-dependent mechanism [48, 49]. Besides the direct effect, several studies also provide an emerging picture of the importance of LPA in the hematopoietic niche. The niche cells play an important role in HSC differentiation into mature blood cells, and have been shown to tightly control HSC quiescence, self-renewal, homing, and lineage bias decision [50–52].

LPA regulates the migration of mesenchymal stromal cells through RhoA activation, determines osteoclast differentiation, bone resorption activity, and marrow stromal cell senescence by LPA_1, and regulates hematopoietic stromal cell activity by LPA_4 [53–56]. These findings together with our results suggest that LPA signaling is a potent pathway with wide-ranging effects during erythropoiesis.

Furthermore, alteration of the expression patterns of LPA receptors contributes to the differentiation fate decision of hematopoietic progenitor cells. Thus, it is important to further elucidate changes in the expression of LPA receptors and the functional consequences during hematopoietic cell differentiation. In the EDG family, the level of LPA_1 was not detected during myelopoiesis (Fig. S1B). LPA_2 was shown to be mainly expressed in CMP (Fig. 1B and Fig. S1C). The expression level of LPA_3 was significantly increased in MEP (Fig. 1B and Fig. S1D). The members of the P2Y receptor family ($LPA_{4\text{-}6}$) were also detected in myeloid progenitors. The expression levels of LPA_4 and LPA_5 increased throughout ontogeny, and both GMP and MEP exhibited higher expression levels of these two receptors. Furthermore, LPA_6 may commit the stem cells to the GMP axis, since its expression level was higher in most progenitors than in MEP (Fig. S1E,F). Further investigations are warranted to determine the functions of both $LPA_{4\text{-}6}$ during myelopoiesis.

In addition, we showed that LPA_2 signaling mainly regulated the myeloid-erythro-megakaryopoiesis axis by decreasing the size of the CMP population and its downstream lineage. However, the proportion of GMP was not consistently downregulated by treatment with GRI (Fig. S4). The lineage fate decision of CMP also remained unchanged, as shown in the *ex vivo* colony-forming assay (Fig. S3B). Recent studies provide a reasonable explanation by suggesting distinct road maps of blood differentiation. The reprogramming process between the lymphoid and myeloid lineages from a tyrosine kinase receptor $Flt3^+$ LSK population, lymphoid-primed multipoint progenitor (LMPP), which can give rise to CLP and CMP but lacks erythro-megakaryocytic potential [57]. Furthermore, several studies have also indicated that lymphoid cells can be reprogrammed to functional macrophages through the expression of *C/EBPa* and *PU.1* transcription factor [58, 59]. Moreover, progenitors are revealed present heterogeneous and oligopotent of each fraction, meaning that some defined CMP fractions may not be able to differentiate into GMP [6]. Based on these evidences, we formed two hypothesis. Firstly, LPA may act on the fraction of CMP with only erythro-megakaryopoiesis potential. Secondly, decrease in GMP fraction by the activation of LPA_2 can be compensated through reprogramming of the lymphoid lineage, results in unchanged number of GMP.

Interestingly, the myeloid lineage in both major organs (bone marrow and spleen) were significantly inhibited by treatment with GRI in the mouse model (Fig. 2). In contrast, the beneficial effects of OMPT on the number of erythroblasts was only present in the spleen (Fig. 3). We speculate that, although stem cells can migrate between the bone marrow and spleen, regulation in the spleen may differ from that occurring in the bone marrow. For instance, the major component in the bone marrow niche, osteo-lineage cells, do not exist in the spleen niche. Furthermore, several studies have indicated that the frequency of stem cells in the bone marrow is significantly higher than that observed in the spleen, suggesting that the spleen contains a greater number of working progenitor and functional cells to maintain

the physiological condition [60, 61]. Thus, we analyzed the proportions of each progenitor population in the spleen and bone marrow (Fig. S5). The comparison of populations showed that the number of MEP (LPA_3 target) was lower than that of CMP in the bone marrow. This finding indicated that the enhancement effects of OMPT occurred in the spleen due to the different proportions of later progenitors. These results particularly enhance the evidence that LPA_2 and LPA_3 act at different stages through their distinct expression profiles.

Any perturbation during hematopoiesis potentially leads to multiple hematological disorders. For instance, myeloproliferative neoplasms are diseases associated with the overproduction of platelets, erythrocytes, and other blood cells [62]. In addition, anemia is also a very frequent disorder, arising from congenital abnormality, environmental factors, or exposure to external chemicals, and associated with the patients with inflammatory disease, cancer, congestive heart failure, chronic kidney disease, and the individuals after transplantation [63]. Regarding clinical outcomes, reports have associated the presence of anemia with a high risk of bleeding and late mortality. Moreover, since hemoglobin is an essential oxygen transporter from the lungs to the circulation, thereby anemia reduces the amount of oxygen delivery, lowers the functional capacity of the patient, reduces the amount of oxygen to the myocardium, and determines heart failure. Considering these impaired outcomes in noted in the clinic, the development of new treatment strategies is urgently warranted. In clinical practice, recombinant human erythropoietin (EPO) is the first-line therapeutic for the treatment of anemia. However, EPO has been reported to elevate the risk of numerous side effects in patient, including hypertension, venous thromboembolism, stroke, and progression of cancer [64–66]. Our results demonstrated the beneficial effect of treatment with LPA_3 agonist in a murine model with induced anemia. This effect was achieved by replenishing the number of erythrocytes and level of hemoglobin, and significantly recovering the mobility of mice. On the other hand, multiple molecules around the niche and cell-intrinsic mechanism of stem cell undergo several changes that contribute to the hematological disorder, including immune function decline and anemia, during aging. For instance, DNA replication stress, ROS increasing, and abnormal DNA damage response are all evidenced to correlated with the age-related DNA damage accumulation on HSC [67–69]. These adverse consequences may alter the epigenetic status of chromosome DNA then lead the mutant HSC to leave the quiescent state, decrease the regenerative potential. Moreover, it will reduce bone marrow homing by depolarized stem cell through CDC42 activity activation, results in the aberrant lineage hierarchy [68, 70]. The abnormal differentiation contribute to a skewed lineage to the myeloid lineage, at the expense of a correspondingly diminished lymphoid potential [71, 72]. In this study, our data present strong evidence that the decline LPA_3 level during aging process may critically contribute the aging-related anemia. Moreover, activating LPA_3 by its agonist rescued anemia phenotype in progeria cells. Consistent with our previous study that activation of LPA_3 with LPA_3 agonist also showed the beneficial effects on the aging cells by promoting antioxidant enzymes and ROS reduction [36], the LPA_3 agonists may hold value with the function of erythropoiesis promoting and ROS scavenging to be a potential drug against the acute and aging-related anemia in the future.

This study demonstrated that LPA_2 and LPA_3 are expressed at different hematopoietic stages and play distinct regulatory roles in the fate decisions of the myeloid-erythroid-

megakaryocytic lineage. Firstly, we analyzed the expression levels of LPA receptors in different populations of myeloid progenitors. Interestingly, the mRNA expression level of LPA_2 is higher in CMP, whereas that of LPA_3 is lower in CMP; however, the latter is upregulated after commitment of CMP to MEP. *In vitro* colony-forming unit assays of sorted murine progenitor cells revealed that the LPA_2 agonist GRI reduces the erythroblast differentiation potential of CMP. In contrast, the LPA_3 agonist OMPT increases the production of erythrocytes from MEP. This suggests that LPA_2 and LPA_3 act at different stages of progenitors to regulate myeloid lineage commitment. Furthermore, in our mouse model, reduction of the CMP and MEP populations in response to treatment with GRI indicates that activation of LPA_2 prevents CMP from differentiating into MEP due to proliferation decline and apoptosis increasing. In contrast, activation of LPA_3 promotes the differentiation of MEP into erythrocytes at the expense of megakaryopoiesis. This suggests that the LPA_3 signal is a pivotal erythropoietic regulator of the MEP lineage fate decision. These results further clarify the roles of LPA_2 and LPA_3 during hematopoiesis, and account for the opposing regulation of erythro-megakaryopoiesis observed in our previous studies (Fig. 4H). Notably, we also demonstrated that the decline of LPA_3 mediates aging-induced anemia and the LPA_3 agonist provides insight towards the possible novel treatment strategy for anemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

LPA	Lysophosphatidic acid
LPA_{1-6}	Lysophosphatidic acid receptor 1 to 6
EDG	Endothelial cell differentiation gene
P2Y	Purinergic receptor
HSC	Hematopoietic stem cells
MPP	Multi-potent progenitors
LSK	$Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ stem cells
CLP	Common lymphoid progenitors

CMP	Common myeloid progenitors
GMP	Granulocyte-monocyte progenitors
MEP	Megakaryocyte-erythroid progenitors
EPO	Erythropoietin
GRI	LPA_2 agonist, GRI compound 977143
OMPT	LPA_3 agonist, 1-Oleoyl-2-O-methyl-rac-glycerophosphothionate
PHZ	Phenylhydrazine
HGPS	Hutchinson-Gilford progeria Symptom.

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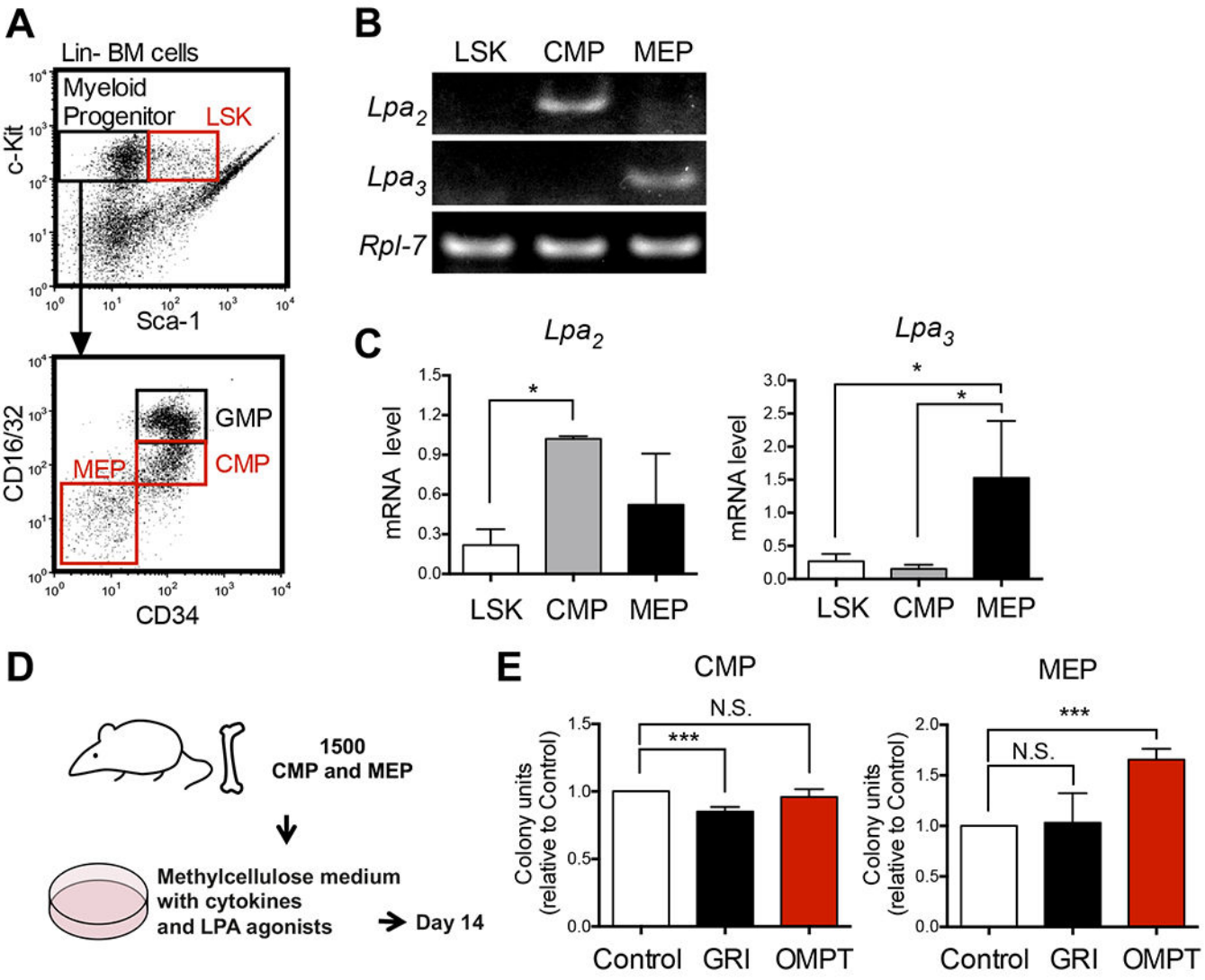

Fig. 1. LPA_2 and LPA_3 determined hematic homeostasis at the different stages of hematopoietic progenitors.

(A) Representative plots show the population of hematopoietic progenitors from the bone marrow of wild-type BALB/*c* mice gated using Sca-1 and c-Kit antibodies and Lineage antibody cocktail (Lin). The $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells were defined as LSK cells. The $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}$ fraction was subsequently divided into $CD16/32^{low}CD34^{+}$ (CMP), $CD16/32^{hi}CD34^{+}$ (GMP), and $CD16/32^{-}CD34^{-}$ (MEP). (B, C) Serial sorted hematopoietic myeloid progenitor cells, LSK, CMP, and MEP, were isolated and described in panel A. The expression of endogenous LPA_2 and LPA_3 in progenitor cells was determined by RT-PCR and normalized with internal control, *Rpl-7*. The mean expression level showed a higher level of LPA_2 in CMP versus LSK and MEP. Of note, a higher level of LPA_3 was observed in MEP versus LSK and CMP. (D) Experimental scheme showing the clonogenic myeloid colony-forming assay in methylcellulose medium. Flow-sorted CMP and MEP (1,500 cells/ml) were seeded in Methocult GF M3434 media containing SCF, IL-3, IL-6, IL-11, GM-CSF, Flt3-ligand, TPO, and EPO, with or without LPA receptor agonists (LPA_2 agonist

GRI and LPA_3 agonist OMPT). The total number of colonies was quantified after 14 days of culture. (E) The clonogenicity of GRI treated CMP was decreased compared with that noted in OMPT treated cells and DMSO treated control cells. An increased number of colonies was observed for OMPT treated MEP versus GRI treated cells and DMSO treated control cells. Histograms represent mean ± SD from three independent biological replicates. *$p \leq 0.05$, ***$p \leq 0.001$. N.S. indicates non-significant.

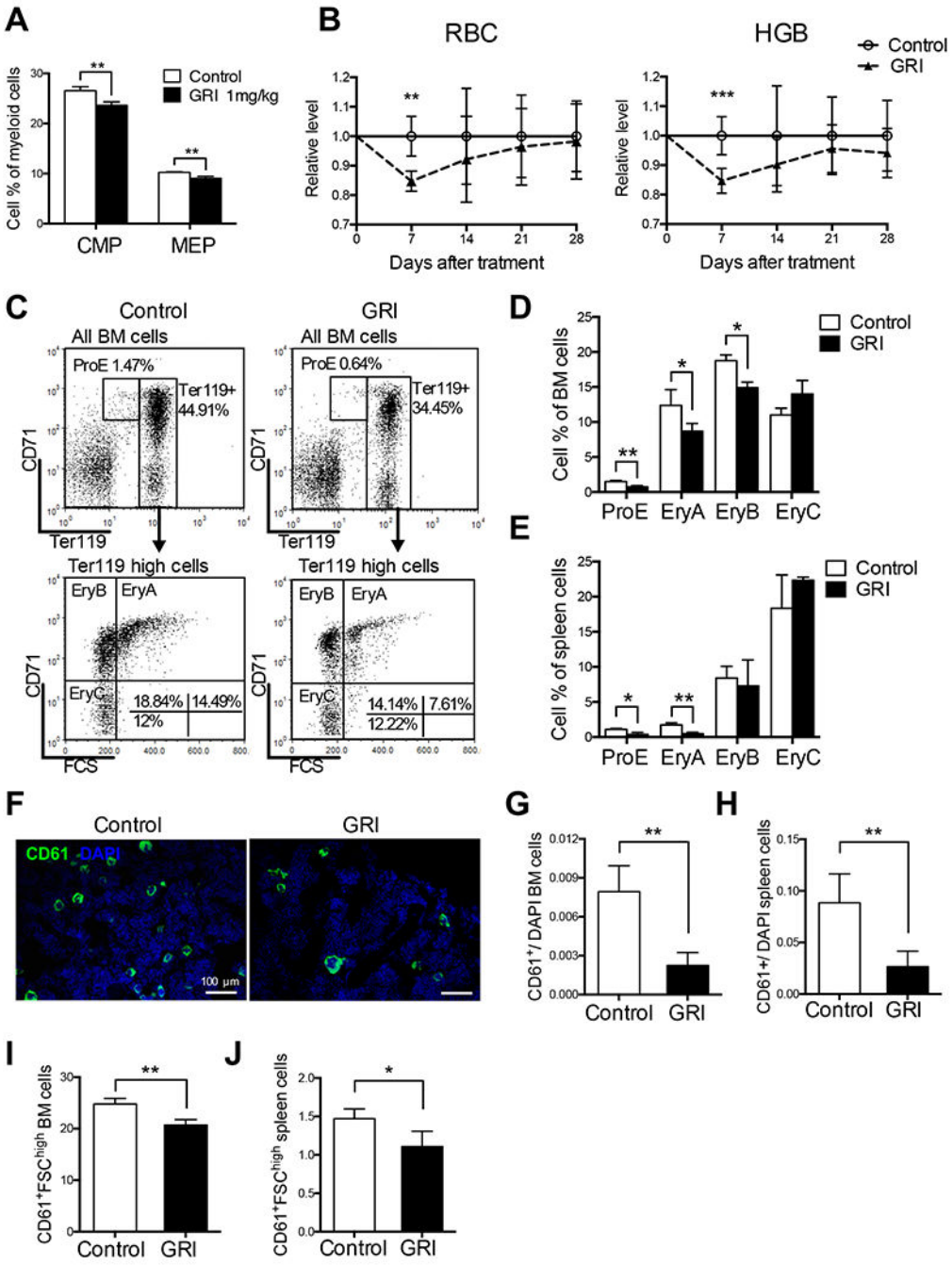

Fig. 2. LPA_2 agonist GRI inhibited erythro-megakaryocytic differentiation.

Wild-type BALB/c mice were injected with either solvent control, DMSO, or 1 mg/kg LPA_2 agonist GRI to activate LPA_2 every other day. (A) Percentage of CMP and MEP among the myeloid progenitors were obtained from the bone marrow. The frequencies of CMP and MEP in GRI treated mice was decreased compared to the control group. (B) Whole blood was drained from a facial vein and analyzed by CBC continually each week for 4 weeks. All the values were first normalized to Day 0. The point on each time point showed the ratio of GRI treated group comparing to control group. After 1 week of treatment with GRI, the

numbers of RBC and levels of hemoglobin were significantly decreased. (C) Representative plots showing change in the population of erythroblasts in the bone marrow, ProE ($CD71^{high}Ter119^{intermediate}$), EryA ($CD71^{high}Ter119^{high}FSC^{high}$), EryB ($CD71^{high}Ter119^{high}FSC^{low}$), and EryC ($CD71^{low}Ter119^{high}FSC^{low}$) after treatment with GRI compared with control through flow cytometric analysis. (D) After 1 week of treatment with GRI, the mean erythroblast precursor frequencies of ProE, EryA, and EryB were reduced in bone marrow (BM), and (E) ProE and EryA were reduced in spleen. (F) After 4 weeks of treatment, bone marrow sections were analyzed through immunofluorescence staining with CD61, a surface marker of megakaryocytes. (G, H) Activation of LPA_2 significantly decreased the number of megakaryocytes, as indicated by the reduced ratio of CD61/DAPI in bone marrow and spleen. (I, J) The mean megakaryocyte frequency revealed by flow cytometry showed reduced $CD61^{+}FSC^{high}$ cells after treatment with GRI in bone marrow and spleen. Each group of mice: $N \geq 3$. Histograms represent mean ± SD. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

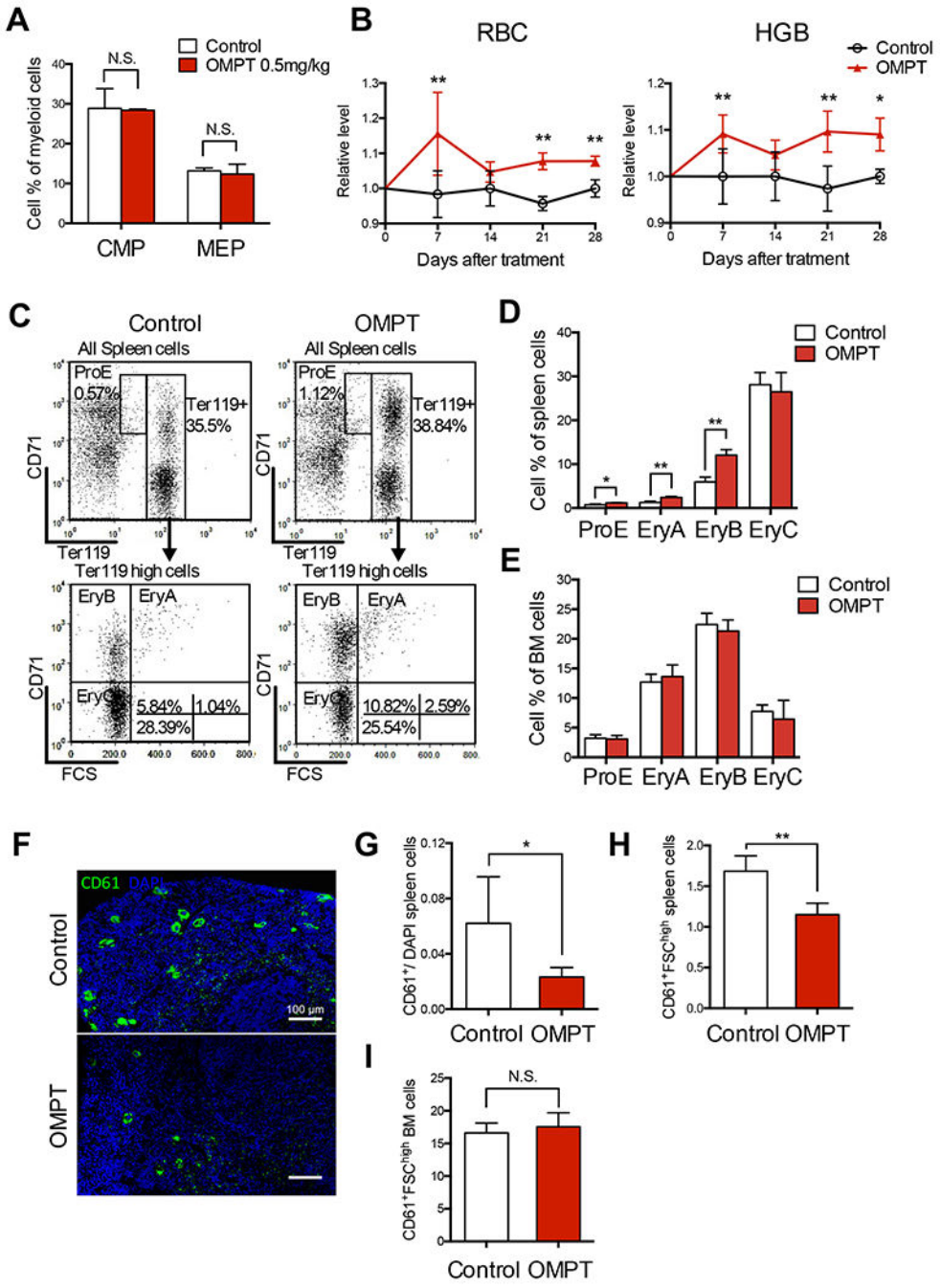

Fig. 3. LPA_3 agonist OMPT promoted erythropoiesis at the expense of megakaryopoiesis.

Wild-type BALB/*c* mice were injected every other day with either ethanol:chloroform as solvent control, or 0.5 mg/kg LPA_3 agonist OMPT to activate LPA_3. (A) No changes in frequencies of CMP and MEP among myeloid cells that were obtained from the bone marrow of wide-type BALB/*c* mice. (B) Whole blood was drained from a facial vein and analyzed by CBC continually each week for 4 weeks. Activation of LPA_3 showed persistent (for 1 month) enhancement of the average levels of hemoglobin and the number of erythrocytes in mice. (C) Representative plots showing erythroblasts in the spleen, ProE

(CD71^{high}Ter119^{intermediate}), EryA (CD71^{high}Ter119^{high} FSC^{high}), EryB (CD71^{high}Ter119^{high}FSC^{low}), and EryC (CD71^{low}Ter119^{high}FSC^{low}) after treatment with OMPT and control through flow cytometric analysis. (D) The mean frequency of erythroblast precursors in the spleen was increased after treatment with OMPT for 4 weeks, whereas (E) there was no changes shown in erythropoiesis of bone marrow (BM). (F) After 4 weeks of treatment with OMPT, spleen sections were analyzed through immunofluorescence staining with CD61, a surface marker of megakaryocytes. (G) Immunostaining showed a decreased number of megakaryocytes with a decreasing ratio of CD61/DAPI. (H) The mean megakaryocyte frequency of flow cytometry analysis showed a decreased number of CD61^{+}FSC^{high} cells, suggesting that treatment with OMPT reduced megakaryopoiesis in the spleen, (I) but no changes shown in megakaryopoiesis in the bone marrow. Each group of mice: $N \geq 3$. Histograms represent mean ± SD. *$p \leq 0.05$, **$p \leq 0.01$.

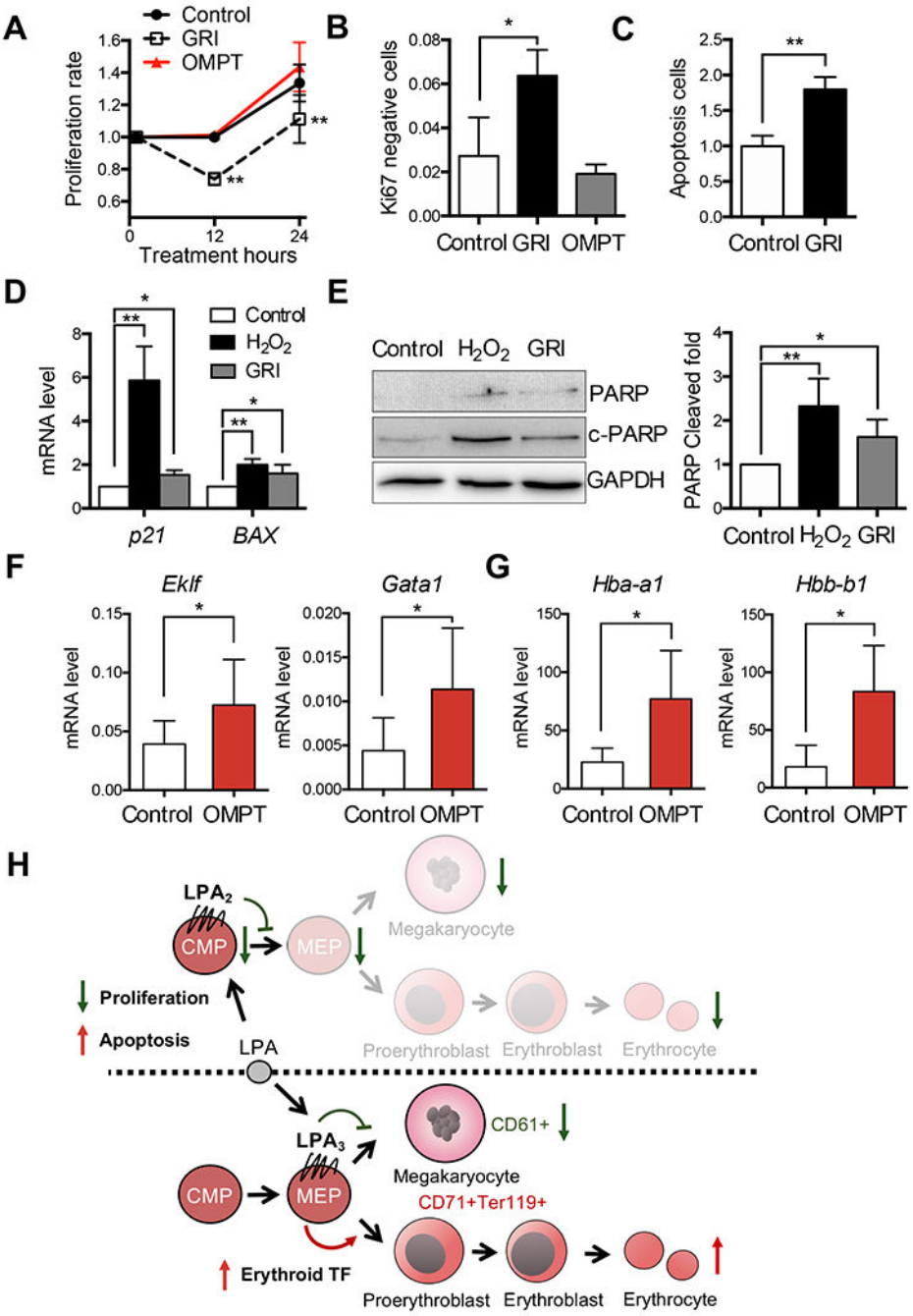

Fig. 4. Regulatory roles of LPA_2 and LPA_3 in myeloid-erythroid differentiation.

(A) K562 cells were treated with DMSO (Control), 50 nM OMPT, and 5 μM GRI for 12 and 24 h. The numbers of cells were calculated at the indicated time points, and the proliferation rates were normalized to the number of cells counted at 0 h. The mean proliferation rate was decreased after treatment with GRI for 12 h and 24 h. Treatment with OMPT had no effect on the proliferation of K562 cells. (B) Cells were treated with agonists for 12 h, followed by staining with proliferation marker, Ki67. The percentage of Ki67 negative cells was calculated. The result showed significant proliferation inhibition with GRI treatment. (C)

Live cells were treated with agonists for 12 h, followed by staining with both PE-Annexin V and 7-AAD. The population of apoptosis cells (PE-Annexin V^+ and 7-AAD^-) were calculated. The ratio of the apoptosis cells were increased after treatment with GRI relative to the control cells. (D) Cells were treated with 200 μM H_2O_2 or 5 μM GRI for 5 h. The results of real-time qPCR showed significant upregulation of p21 and BAX mRNA after treatment with GRI and H_2O_2. H_2O_2 was used as a positive control for apoptosis, and the expression level of GAPDH was used for normalization as the internal control. (E) Western blot analysis was performed to demonstrate the ratio of protein levels of full-length PARP (PARP) relative to the cleaved form PARP (c-PARP) in GRI treated K562 cells. The results showed an increase in the level of c-PARP following treatment with GRI and H_2O_2. (F) After 1 week of treatment with 0.5 mg/kg OMPT in wild-type BALB/*c* mice, the mRNA expression level in homogenized spleen lysates was analyzed with selected genes that are crucial for erythroid lineage differentiation. Eklf, Gata-1, and (G) Hba-a1/Hbb-b1 were measured using real-time qPCR. The expression level of GAPDH was used as an internal control. The expression levels of erythroid transcription factors were significantly increased in the spleen of OMPT treated mice. (H) The model shows cell type-distinct regulatory roles of LPA_2 and LPA_3 during myeloid-erythro-megakaryocytic differentiation. Histograms represent mean ± SD from three independent biological replicates. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

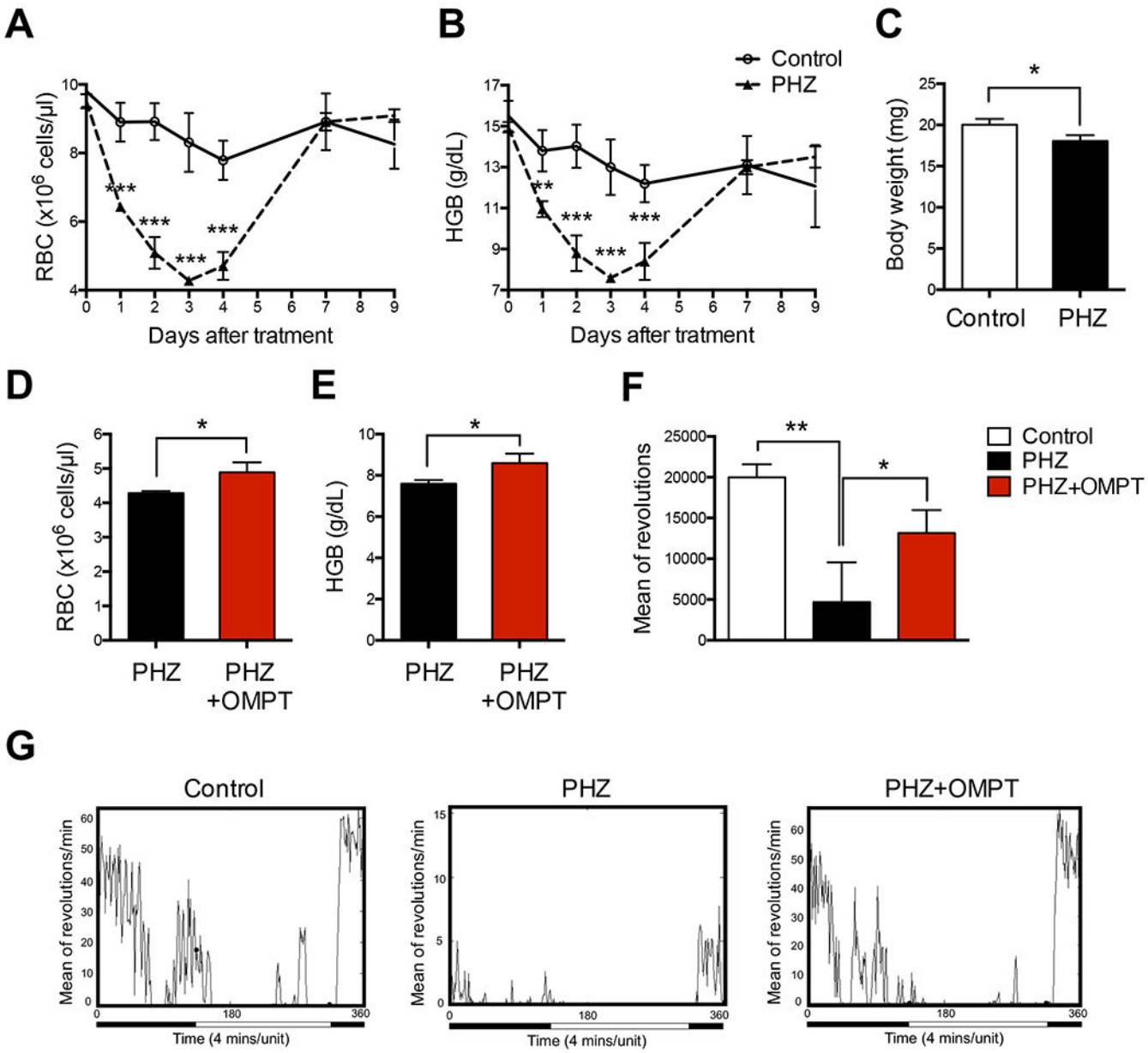

Fig. 5. Treatment of OMPT alleviated PHZ-induced hemolytic anemia.

Wild-type BALB/*c* mice were pretreated with either solvent control, ethanol:chloroform, or 0.5 mg/kg OMPT for 3 days prior to injection of 60 mg/kg PHZ at Day 0. Control or OMPT were injected daily for 7 days (Days 0–6) and blood samples were daily withdrawn for 6 time points until the number of (A) red blood cells (RBC) and concentration of (B) hemoglobin (HGB) recovered to the basal level. (C) The general parameter, body weight was damaged by PHZ. (D, E) Treatment with OMPT resulted in significantly higher levels of hemoglobin and numbers of erythrocytes compared with control between Days 2 to 4 (the most severe state of anemia). (F) Mice were housed individually in cages containing rodent exercise wheels equipped with a digital counter activated by wheel rotation. The mean number of revolutions performed each day was quantified. The exercise performance of

PHZ-induced mice was reduced compared with that observed in the control group between Days 2 to 4. (G) The counts of revolutions in PHZ-induced anemia mice were recorded. The horizontal bar below indicates the period of nighttime (black) or daytime (white). Each group of mice: $N \geq 3$. Histograms represent mean ± SD. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

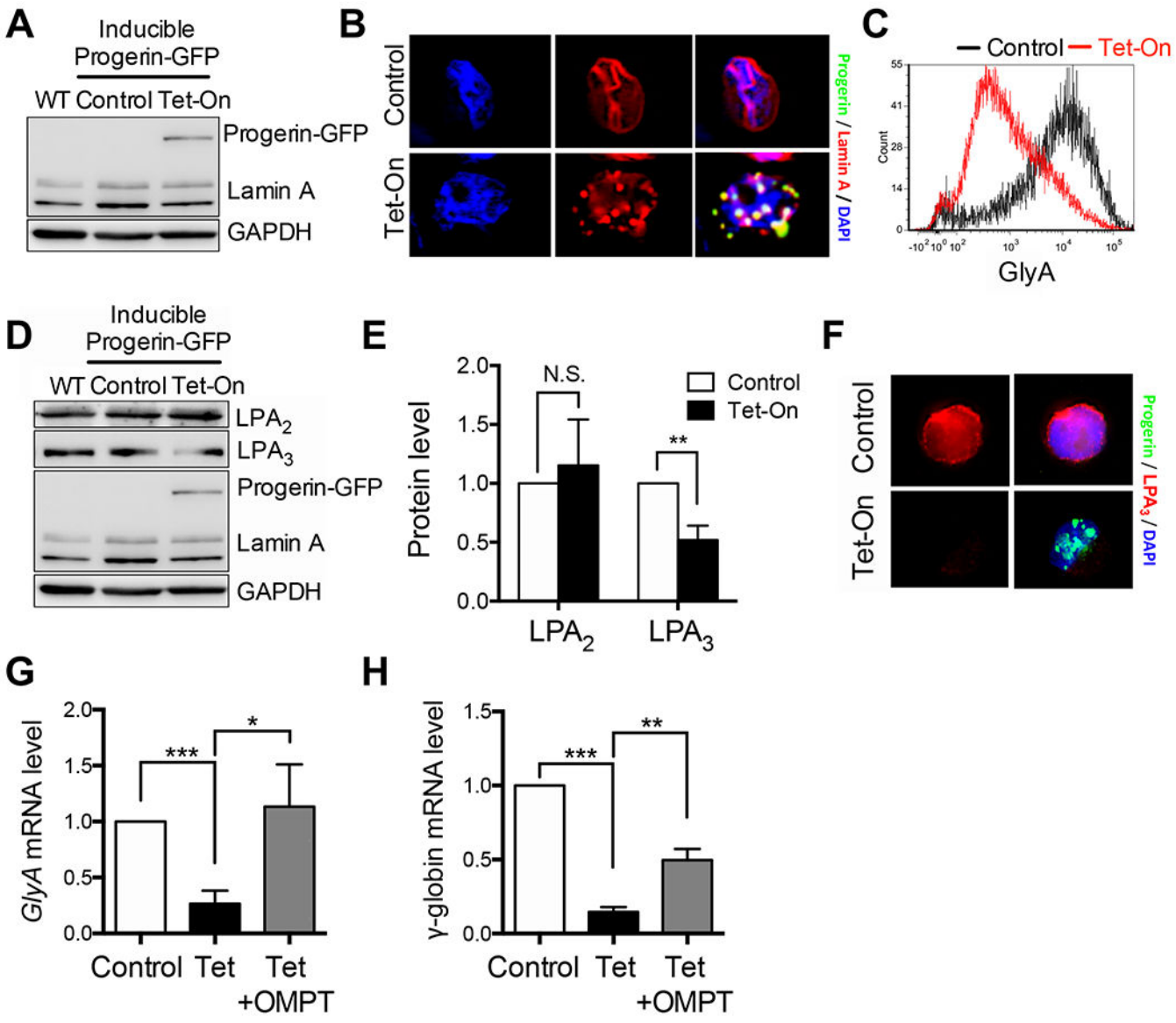

Fig. 6. The association of LPA receptors and erythropoiesis impairment in tetracycline (Tet-on) inducible HGPS K562 cell.

150-nucleotides deletion LaminA (progerin) with EGFP tagging was constructed in pcDNA5/pcDNA6 dual vector induction system to build up a Tet-on inducible HGPS K562 cell line. (A) Comparing to non-transfect (WT) and non-induced cells (Control), Tetracycline inducible HGPS cell (Tet-on) showed expression of GFP-progerin by western blot probing with Lamin A antibody. (B) Immunofluorescent staining showing the red signal with the LaminA antibody indicated the location of Lamin A and progerin, and the green signal indicated the GFP-progerin. Progerin indeed resulted in abnormal nucleus morphology. (C) Flow cytometry signal of GlyA was decreased in Tet-on HGPS K562 cell. (D) Protein level of LPA_2 and LPA_3 in WT, transfected control, and Tet-on HGPS K562 were measured by western blot. The expression level of GAPDH was used for normalization as the internal control. Over-expression of progerin decreased protein level of LPA_3,

whereas level of LPA_2 was not altered. Quantification is shown in (E). (F) Immunofluorescent staining showed decreased protein level of LPA_3. The red signal indicated the location of LPA_3, and the green signal indicated the GFP-progerin. (G,H) Tet-on induced HGPS K562 cells were co-treatment with 50 nM OMPT for 3 days incubation. The mRNA expression level of GlyA and γ-globin were measured using real-time qPCR, normalized with internal control GAPDH and relative to non-induced control cell. OMPT treatment rescued the level of erythrocytic markers, GlyA and γ-globin, in Tet-on HGPS K562 cell. Histograms represent mean ± SD from three independent biological replicates. p**≤ 0.01.