Molecules and Cells

Overexpression of COMP-Angiopoietin-1 in *K14*-Expressing Cells Impairs Hematopoiesis and Disturbs Erythrocyte Maturation

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Numerous studies highlight the potential benefits potentials of supplemental cartilage oligomeric matrix protein-angiopoietin-1 (COMP-Ang1) through improved angiogenic effects. However, our recent findings show that excessive overexpression of COMP-Ang1 induces an impaired bone marrow (BM) microenvironment and senescence of hematopoietic stem cells (HSCs). Here, we investigated the underlying mechanisms of how excessive COMP-Ang1 affects the function of BM-conserved stem cells and hematopoiesis using *K14-Cre;inducible-COMP-Ang1-transgenic* mice. Excessive COMP-Ang1 induced peripheral egression and senescence of BM HSCs and mesenchymal stem cells (MSCs). Excessive COMP-Ang1 also caused abnormal hematopoiesis along with skewed differentiation of HSCs toward myeloid lineage rather than lymphoid lineage. Especially, excessive COMP-Ang1 disturbed late-stage erythroblast maturation, followed by decreased expression of stromal cell-derived factor 1 (SDF-1) and globin transcription factor 1 (GATA-1) and increased levels of superoxide anion and p-p38 kinase. However, transplantation with the mutant-derived BM cells or treatment with *rh*COMP-Ang1 protein did not alter the frequency or GATA-1 expression of erythroblasts in recipient mice or in cultured BM cells. Together, our findings suggest that excessive COMP-Ang1 impairs the functions of BM HSCs and MSCs and hematopoietic processes, eventually leading to abnormal erythropoiesis via imbalanced SDF-1/CXCR4 axis and GATA-1 expression rather than Ang1/Tie2 signaling axis alterations.

Keywords: bone marrow-conserved stem cells, cartilage oligomeric matrix protein-angiopoietin-1, globin transcription factor 1, stromal cell-derived factor 1/CXCR4 signaling axis

INTRODUCTION

Angiogenesis is linked with hematopoiesis. Angiopoietin-1 (Ang1), a dominant ligand for long-term repopulating activity of hematopoietic stem cells (HSCs), plays crucial roles not only in vascular and hematopoietic development but also in the maintenance of HSCs in a quiescent state in the bone marrow (BM) niche (Arai et al., 2004; Joo et al., 2011; Suda et al., 2000; Takakura et al., 2000). Thus, in addition to angiogenesis, the Ang1/Tie2 signaling axis is critical for adhesion of long-term HSCs to the BM niche and maintenance of these HSCs in the BM.

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Supplemental cartilage oligomeric matrix protein (COMP)-Ang1 suppresses vascular inflammation, leakage, and ischemic side effects, improves cell survival, and enhances blood vessel remodeling and formation (Koh, 2013; Lee et al., 2014; Youn et al., 2011). Administration of COMP-Ang1 by adenoviral vector induced long-lasting vascular enlargement and increased blood flow better than supplementation with recombinant human (*rh*) COMP-Ang1 protein (Cho et al., 2005). This suggests that long-term and sustained administration of Ang1 protects against microvascular regression-associated diseases more efficiently than does its short-term administration. However, considerable evidence highlights that the Ang1/Tie2 signaling axis differentially regulates hematopoietic development, repopulation of long-term HSCs, and vasculogenesis depending on the amount of Ang1 (Cho et al., 2004; Ikushima et al., 2013). Our recent findings also support that transgenic overexpression of COMP-Ang1 in *K14*-expressing cells induces excessive and abnormal angiogenesis, disrupts the HSC pool in the BM niches, and causes peripheral circulation and senescence of BM HSCs (Kook et al., 2018). Taken as a whole, investigating the exact impacts of systemic and excessive COMP-Ang1 on the fate of BM HSCs and mesenchymal stem cells (MSCs) and hematopoiesis will be necessary.

In this study, we examined the underlying mechanisms of how genetic overexpression of COMP-Ang1 affects BM retention, senescence of HSCs and MSCs, and hematopoietic processes using *K14-Cre;inducible-COMP-Ang1-transgenic* mice (*K14-Cre* mutants) and their wild type (WT) littermates. We also explored whether senescence of BM HSCs or the Ang1/Tie2 signaling axis is directly associated with excessive COMP-Ang1-mediated abnormal hematopoiesis. Our current findings demonstrate that excessive COMP-Ang1 induces impaired retention and function of BM HSCs and MSCs and disturbs erythrocyte maturation at the late stage, and these results are associated with dysregulation of a signaling axis related to stromal cell-derived factor 1 (SDF-1)/CXCR4 and globin transcription factor 1 (GATA-1) rather than the Ang1/Tie2 signaling axis.

MATERIALS AND METHODS

Study approval

This study was carried out in strict accordance with the recommendations in the Guide for Animal Care and Use of Jeonbuk National University. Before experiments, all procedures were approved by the University Committee on Ethics in the Care and Use of Laboratory Animals (CBU2014-00055) according to the ARRIVE guidelines.

Animals and treatment

All animals were cared for based on the guidelines of the Animal Care Committee of Jeonbuk National University. We generated *K14-Cre* mutants by crossing *K14-Cre* mice with *inducible-COMP-Ang1-transgenic* mice that were backcrossed with the C57BL6 strain for more than 3 generations (Dassule et al., 2000; Hato et al., 2009). The *Cre* mice and *R26R* reporter mice were purchased from the Jackson Laboratory (USA) (Soriano, 1999). Mouse offspring were genotyped by PCR analysis according to the methods described previously (Dassule et al., 2000; Hato et al., 2009) and used in all experiments regardless of gender. Before or after the experimental use, weights of body, thymus, and spleen in the mutants and WT littermates were measured. To perform transplantation assays, C57BL/6 (B6) mice (3 weeks old) were supplied by Orient Bio (Korea). To explore direct effect of COMP-Ang1 on erythrocyte frequency and GATA-1 expression, BM cells (2×10^6 cells) derived from B6 mice (3 weeks old) were exposed to various concentrations (0-1,000 ng/ml) of *rh*COMP-Ang1 protein (Enzo Life Science, USA) for 24 h.

Flow cytometry

Cells from BM, spleen, and peripheral blood were collected from mutants, WT littermates, or B6 mice before treatment with red blood cell (RBC) lysis buffer (Sigma-Aldrich, USA) for 15 min on ice. Here BM cells were harvested by flushing the femur and tibia with phosphate-buffered saline (PBS) using a syringe, without crushing bones or treating with collagenase. After washing with PBS, frequencies of BM- and peripheral blood-conserved cells were analyzed using a flow cytometer (BD Calibur or BD Aria; BD Biosciences, USA) installed in the Center for University-Wide Research Facilities (CURF) at Jeonbuk National University. Populations of these cells were sequentially gated using FlowJo software (FlowJo, USA). In this study, populations of $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells and $CD150^+CD48^-LSK$ cells were phenotypically identified using the following antibodies: lineage markers PE-Cy7-conjugated anti-CD3 (cat.#552774), anti-B220 (CD45R, cat.#552772), anti-CD11b (cat.#552850), anti-Gr-1 (cat.#552958), or anti-TER-119 (cat.#557853) (all of these markers were from BD Biosciences); FITC-conjugated anti-Sca-1 (cat.#557405; BD Biosciences) or PE-conjugated anti-Sca-1 (cat.#553108; BD Biosciences); APC-conjugated anti-c-Kit (cat.#553356; BD Biosciences); perCP/Cy5.5-conjugated anti-CD150 (cat.#46-1502; eBioscience, USA); and APC-Cy7conjugated anti-CD48 (cat.#561826; BD Biosciences). Populations of $Lin^-Sca\text{-}1^+CD29^+CD105^+$ cells were phenotypically identified as BM-derived MSCs using the same PE-Cy7-conjugated lineages as used for identification of hematopoietic cells: APC-Cy7-conjugated Sca-1, PE- or FITC-conjugated CD29, and APC-conjugated CD105. For further characterization of MSCs, PE-CF594-conjugated CD44 (cat.#562464; BD Biosciences) and perCP/Cy5.5-conjugated vascular cell adhesion molecule 1 (VCAM-1/CD106) (cat.#562464; BioLegend, USA) antibodies were also used. Senescence-associated-β-galactosidase (SA-β-gal) activity in LSK, $CD150^+CD48^-LSK$, and $Lin^-Sca\text{-}1^+CD29^+CD105^+$ cells that had already been incubated with the cell surface markers were analyzed with C_{12}FDG (cat.#I2904; Molecular Probes, USA). Alternatively, hematopoietic progenitor cells (HPCs) including granulocyte-monocyte progenitors, common myeloid progenitors, megakaryocyte-erythroid progenitors, and common lymphoid progenitors were defined using PE-conjugated anti-FcR (BD Biosciences), perCP/Cy5.5-conjugated anti-CD34 (BioLegend), or PE-conjugated anti-IL-7R (BD Biosciences) as a basis for LSK cell markers. Erythroblasts at four stages in the BM, peripheral blood, or spleen were discriminatively gated with PE- or FITC-conjugated anti-CD71 (BD Biosciences) and PE-Cy7-conjugated

anti-TER-119 (BD Biosciences) antibodies. Myeloid cells were evaluated with PE-Cy7-conjugated anti-CD11b antibody in BM cells of mutants and WT littermates. Mitochondrial superoxide anion levels were analyzed by flow cytometry after staining cells with MitoSOX™ Red reagent (cat.#M36008; Invitrogen, USA). The levels of GATA-1 and p-p38 kinase were determined with PE-conjugated (Cell Signaling Technology, USA) and Alexa Fluor 488-conjugated (cat.#sc-1661; Santa Cruz Biotechnology, USA) antibodies, respectively, after fixation and permeabilization.

Visualization of blood vessels in the hind limb

To optimize the contrast between blood vessels and surrounding soft tissue, contrast agent Microfil® MV-122 (FlowTech, USA) was administered into the aorta of mutants and WT littermates prior to imaging. Systemic perfusion was started by anesthetizing these animals and then the left heart chamber was punctured in the apex of the left ventricle after opening the chest. Perfusion was started by flushing the circulatory system with 10 ml of 0.9% NaCl and 200 U/ml heparin at 37°C. Thereafter, the manual perfusion was performed with yellow Microfil® (MV-122) at a rate of 3 ml/min. After the left ventricle and right atrium were ligated, mice were placed at 4°C for 24 h and then the knee joints were excised and fixed in 10% formalin for additional 24 h. Femurs were decalcified for two days in Decalcifying Solution-Lite (Sigma-Aldrich). After washing with PBS, tissue samples were scanned with a Skyscan 1076 (Skyscan; Bruker, Belgium) at a pixel size of 9 μm. Images were reconstructed and analyzed using the CtVox software (Bruker).

Annexin V and propidium iodide (PI) staining

BM-derived stem and progenitor cells in mutants and WT littermates were harvested from the tibia and femur by flushing with PBS using a syringe. These cells were treated with RBC lysis buffer before washing with PBS. Cells were treated with antibodies specific to LSK and $CD150^{+}CD48^{-}$LSK cells, or HPCs. Cells were incubated with aqueous buffered solution of FITC-labeled Annexin V (200 ng/ml) and PI (300 ng/ml) at room temperature for 20 min. The frequency of these cells was analyzed using a flow cytometer and the scatter signals of Annexin V- and/or PI-positive cells were evaluated after sequentially gating cell populations using the FlowJo software.

Blood test and colony forming cell (CFC) assay

Peripheral blood samples were isolated from mutants and WT littermates and collected into Vacutainer plastic tubes coated with K_2EDTA. An automated complete blood cell counter (Sysmex XE-2100; TOA Medical Electronics, Japan) was used to measure the levels of RBCs (number/μl), hemoglobin (Hb, g/dl), hematocrit (Hct, %), and mean corpuscular volume (MCV, fl). For the pre-B CFC assay, whole BM cells (2×10^5 cells per dish) were divided into 35 mm dishes with MethoCult™ M3630 (Stem Cell Technologies, Canada). After 7 days, the number of colonies formed was counted. For colony-forming unit (CFU) assays, whole BM (3×10^4 cells per dish), peripheral blood (1×10^6 cells per dish), and spleen (0.5×10^6 cells per dish) cells were incubated in 35 mm dishes with MethoCult® GF M3434 (Stem Cell Technologies). After 12 days, the numbers of CFU-granulocyte/macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) colonies were counted using standard criteria. For colony forming unit-erythroid (CFU-E) assay, whole BM cells (2×10^5 cells per dish) from mutants and WT littermates were plated into 35 mm dishes with MethoCult™ M3334 (Stem Cell Technologies). After 14 days of incubation, colonies formed were counted under optic microscopic observation (Carl Zeiss, Germany).

Immunohistochemistry

Immunohistochemistry (IHC) assays were performed using the Histostain Plus Rabbit Primary kit (Zymed Laboratories, USA) according to the manufacturer's instruction. Hind limbs dissected from mutants or WT littermates were fixed in 4% paraformaldehyde at 4°C for 12 h. After rinsing with PBS, specimens were decalcified in 10% EDTA for 4 weeks, dehydrated, embedded in paraffin solution, and sectioned at a thickness of 5 μm. Dried slides were incubated at 60°C for 15 min before treatment with xylene I and II for 10 min each. Thereafter, slides were hydrated through a descending series of ethanol concentration (70%-100%) followed by treatment with 3% hydrogen peroxide. Slides that included the trabecular zone were incubated with rabbit-anti-SDF-1 (cat.#sc-28876, 1:50; Santa Cruz Biotechnology), mouse-anti-CD31 (cat.#ab9498; Abcam, UK), anti-Ang1 (cat.#ab8451; Abcam), or with anti-vascular endothelial growth factor (VEGF) (cat.#BS2853; Bioworld Technology, USA) according to the manufacturer's instructions. After counterstaining with Mayer's hematoxylin (Sigma-Aldrich), slides were observed using a microscope linked with a camera and image processing software (Carl Zeiss).

Western blotting and ELISA

Equal amounts (20 μg/sample) of protein extract obtained from BM cells of mutants and WT littermates were run on 10% to 12% SDS-PAGE followed by blotting onto polyvinyl difluoride membranes. Blots were probed with primary antibodies specific to rabbit-anti-polyclonal SDF-1 (cat.#sc-28876, 1:200; Santa Cruz Biotechnology), rabbit-anti-osteopontin (cat.#ab8448; Abcam), rabbit-anti-runt-related transcription factor 2 (Runx2) (cat.#BS2831; Bioworld Technology, USA), rabbit-anti-osterix (cat.#ab94744; Abcam), or mouse-anti-monoclonal β-actin (cat.#sc-81178, 1:200; Santa Cruz Biotechnology) at 4°C. Membranes were washed and exposed to horseradish peroxidase-conjugated rabbit-anti IgG or mouse-anti Ig. Immunoreactive bands were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology) before exposure to X-ray film (Eastman Kodak, USA). To evaluate the level of SDF-1 in BM supernatants or cultured BM cells, ELISA was performed using an SDF-1 mouse ELISA kit (Abcam) following the manufacturers' instructions. The levels of inflammatory cytokines such as interleukin (IL)-1α, IL-6, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α in BM supernatants of mutants and WT littermates were measured using Multi-Analyte ELISArray Kits according to the manufacturer's instructions (Qiagen Sciences, Germany).

Osteoblast differentiation and mineralization assay

To determine osteogenic capacity of the mutant and WT littermate-derived MSCs, these cells were isolated at 2 weeks of age and seeded onto 24-well culture plates (10^5 cells/well). The non-adherent cells were removed the next day, and adherent cells were cultured in osteogenic differentiating medium (α-minimum essential medium containing 5% fetal bovine serum, 50 μM ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerophosphate). Cells were stained at 21 days post-incubation with Alizarin red for 5 min after fixation in 4% paraformaldehyde for 30 min. The degree of mineralization was observed under a light microscope. Alizarin red-stained cells were also treated with 10% acetylpyridinum chloride for 20 min and the amount of red dye was quantified by measuring the absorbance at 405 nm.

Transplantation assay

To investigate whether senescence of BM HSCs is associated with abnormal erythropoiesis and altered GATA-1 expression, BM cells (2×10^6 cells) isolated from mutants and WT littermates (CD45.2) were transplanted by tail vein injection into conditioned recipients (CD45.1/2) that were lethally irradiated 12 to 24 h before transplantation. The numbers of BM erythroblasts, circulating blood cells, and GATA-1-positive erythroblasts were assessed in the recipient mice at 5 months post-transplantation. The frequencies of HSCs positive to MitoSox, C_{12}FDG, and p-p38 kinase in CD45.2-expressing BM cells in the recipients were also determined using flow cytometry at 5 months post-transplantation.

Statistical analyses

All data are expressed as the mean ± SD and were analyzed using SPSS (ver. 12.0; SPSS, USA). Differences between two

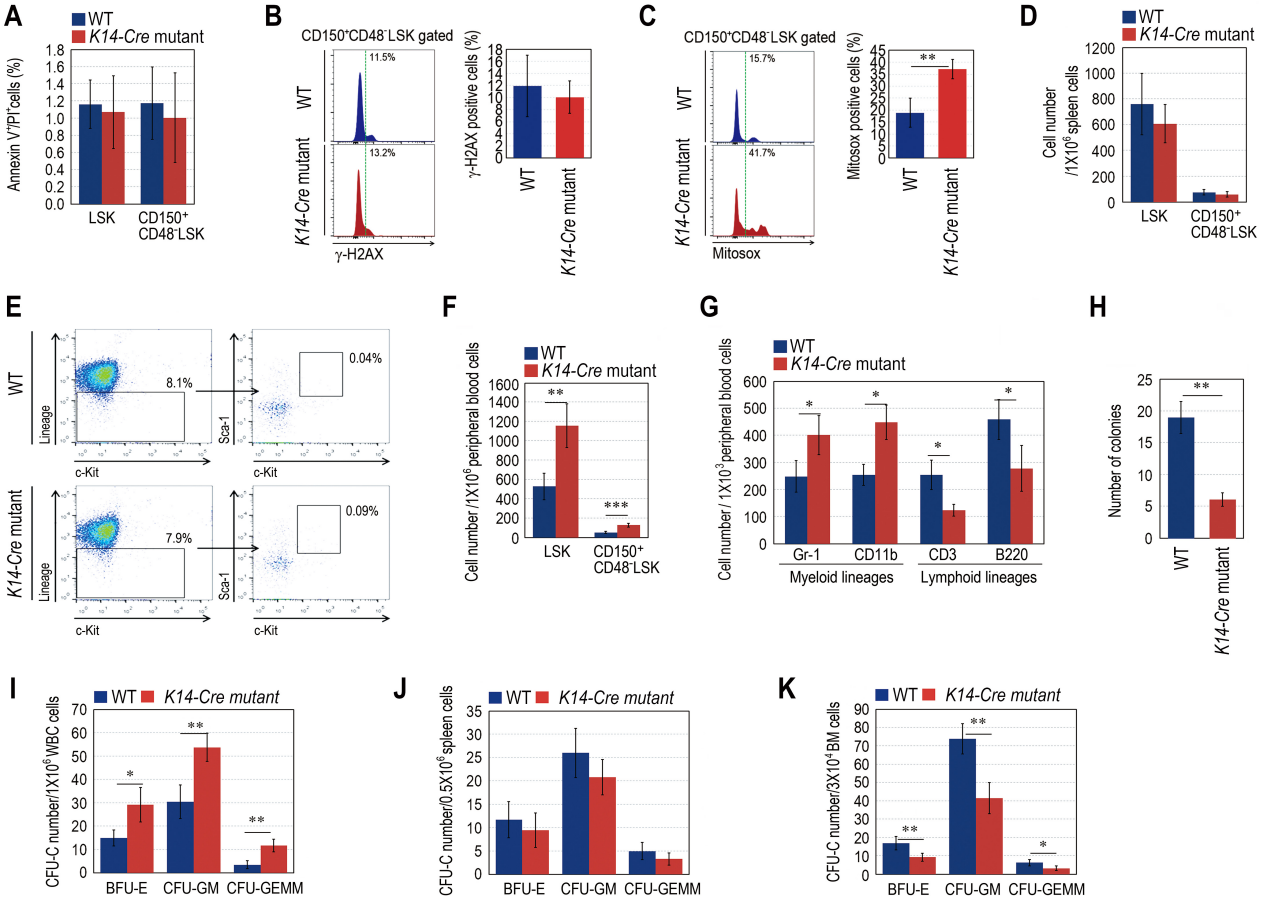

Fig. 1. Overexpression of COMP-Ang1 in *K14*-expressing cells increases peripheral circulation of HSCs along with skewing toward a myeloid lineage of progenitor cells. LSK and CD150$^+$CD48$^-$LSK cells (%) positive to (A) Annexin V/PI, (B) γ-H2AX, or (C) MitoSox in BM of the *K14-Cre* mutants and WT littermates (n = 6). Flow cytometric analysis showing the numbers of LSK and CD150$^+$CD48$^-$LSK cells conserved in (D) spleen or (E and F) peripheral blood (n = 6). (G) Numbers of circulating myeloid lineage (Gr-1, granulocytes; CD11b, myeloid cells) and lymphoid lineage cells (CD3, T cells; B220, B cells) the mutants and WT littermates (n = 10). (H) Number of colonies formed by the mutant- or WT-derived BM cells (2×10^5 cells) after 7 days of incubation in MethoCult™ M3630. A representative result is shown for three independent experiments. CFU-C numbers of BFU-E, CFU-GM, and CFU-GEMM conserved in (I) peripheral blood, (J) spleen, or (K) BM of the mutants and WT littermates, where the progenitor cells were incubated for 12 days in methylcellulose-based reagents. Representative data are shown for three independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ by two-tailed Student's *t*-test.

groups were analyzed by a Student's *t*-test. One-way ANOVA followed by the Scheffe's multiple range test was used for multiple comparisons among more than two groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Genetic overexpression of COMP-Ang1 in *K14*-expressing cells triggers peripheral egression of BM HSCs along with preferable skewing toward a myeloid lineage

Initially, we compared various phenotypes in regard to genetic overexpression of COMP-Ang1 with those of WT littermates. Similar to our previous study (Kook et al., 2018) and with other reports (Suri et al., 1998; Thurston et al., 1999), the *K14-Cre* mutants exhibited excessive angiogenic phenotypes such as redder skin and more branched and enlarged vessels around the hind limbs compared with their WT littermates (Supplementary Figs. S1A and S1B). The mutants also revealed smaller body size and lower weights of body, thymus, and spleen (Supplementary Figs. S1C-S1E). IHC assays showed that expression levels of CD31, Ang1, and VEGF in the BM of the mutants were greater than those of WT littermates (Supplementary Figs. S1F-S1H). As the overexpression of COMP-Ang1 in *K14*-expressing cells impaired BM retention and induced senescence of HSCs (Kook et al., 2018), we next investigated how excessive COMP-Ang1 affects peripheral egression of BM HSCs and their lineage differentiation. The BM decrease in $CD150^+CD48^-$LSK cells of the *K14-Cre* mutants was not due to an increase in cell death, as demonstrated by Annexin V and PI staining (Fig. 1A). While the number of γ-H2AX-positive $CD150^+CD48^-$LSK cells in BM of the *K14-Cre* mutants was comparable to that of WT littermates (Fig. 1B), the mutants showed a larger number of MitoSox-positive $CD150^+CD48^-$LSK cells (Fig. 1C). Significantly

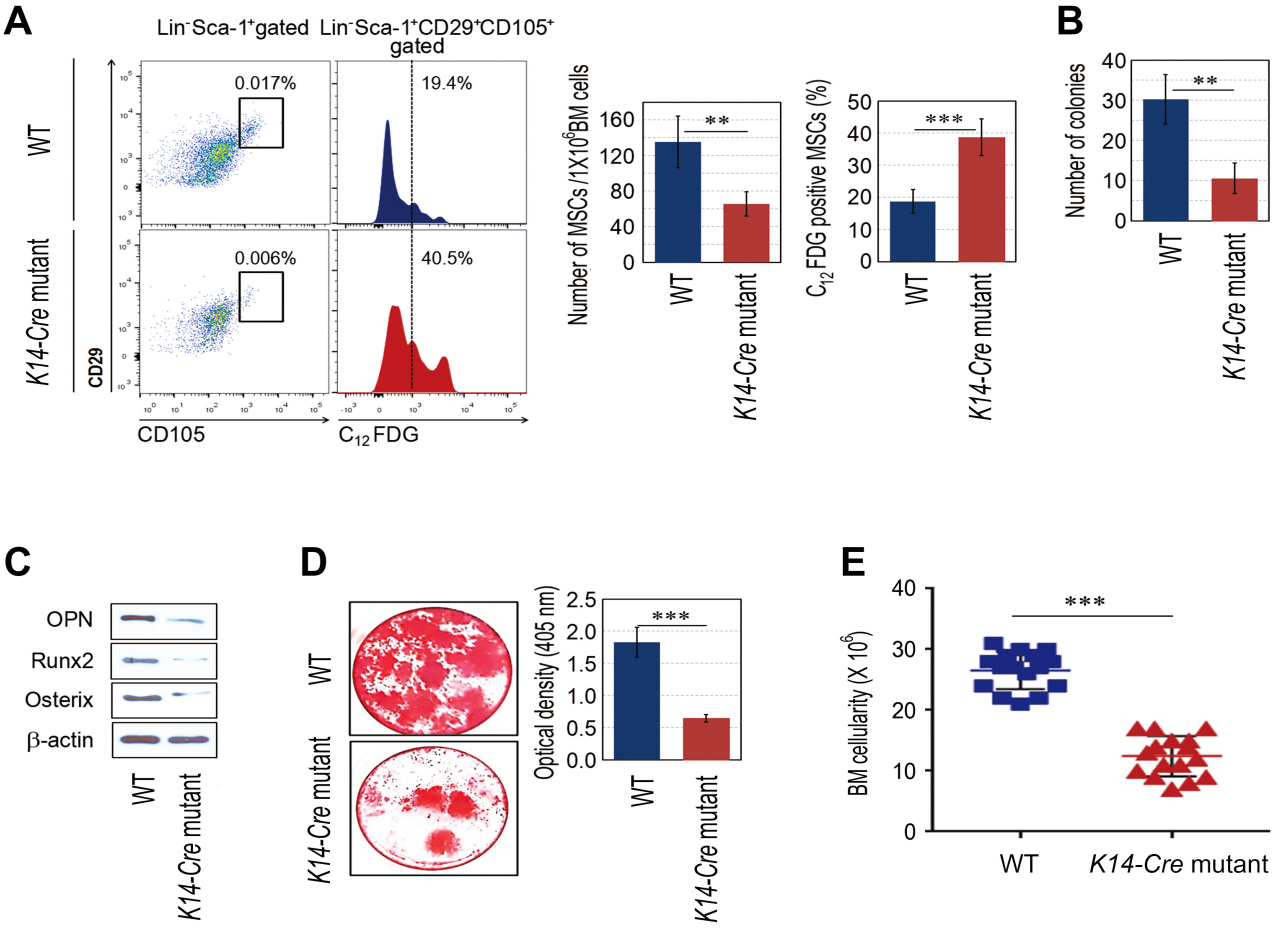

Fig. 2. Overexpression of COMP-Ang1 in *K14*-expressing cells impairs retention and induces senescence of BM MSCs and diminishes their functions with decreased BM cellularity. (A) Numbers of BM MSCs and C_{12}FDG-positive BM MSCs were determined by flow cytometry (n = 10). (B) A representative result from three independent experiments showing the colonies formed by the mutant- and WT-derived BM MSCs. (C and D) The *K14-Cre* mutant- and WT-derived BM MSCs were incubated in osteoblast differentiating medium. After 5 days of incubation, the levels of osteogenic markers were determined by western blotting, while the degree of mineralization in the cells were evaluated by Alizarin red staining followed by quantification of the red dye by spectrophotometer at 21 days post-incubation (n = 6). (E) Whole BM cellularity of the mutants and WT littermates was determined (n = 15). $^{**}P < 0.01$ and $^{***}P < 0.001$ by two-tailed Student's *t*-test.

higher numbers of LSK ($P < 0.01$) and $CD150^+CD48^-$LSK cells ($P < 0.001$) were detected in peripheral blood, but not in the spleen, of the *K14-Cre* mutants compared with WT littermates (Figs. 1D-1F). The *K14-Cre* mutants displayed preferable skewing toward a myeloid lineage rather than a lymphoid lineage in peripheral blood (Fig. 1G). BM cells of the *K14-Cre* mutants also showed fewer cells in pre-B colony formation compared to BM cells from WT littermates (Fig. 1H). When CFU-C numbers of BFU-E, CFU-GM, and CFU-GEMM were determined, the *K14-Cre* mutants exhibited higher numbers in peripheral blood (Fig. 1I), unchanged numbers in the spleen (Fig. 1J), and reduced numbers of these cells in BM cells (Fig. 1K) compared with those of WT littermates. These results indicate that excessive COMP-Ang1 impairs retention of BM HSCs and HPCs and causes abnormal hematopoiesis.

Excessive COMP-Ang1 also impairs retention and induces senescence of BM MSCs and diminishes their clonogenic and osteogenic potentials

We explored whether genetic overexpression of COMP-Ang1 in *K14*-expressing cells dysregulates the maintenance and function of BM MSCs. To support that $Lin^-Sca\text{-}1^+C\text{-}D29^+CD105^+$ gated cells are specific to MSCs, we determined expression levels of CD44 and VCAM-1 in these cells. More than 99% and 57% of the gated cells expressed CD44 and VCAM-1, respectively, indicating phenotypical gating and analysis of MSC-specific cells (Supplementary Fig. S2). Compared with WT littermates, the *K14-Cre* mutants revealed

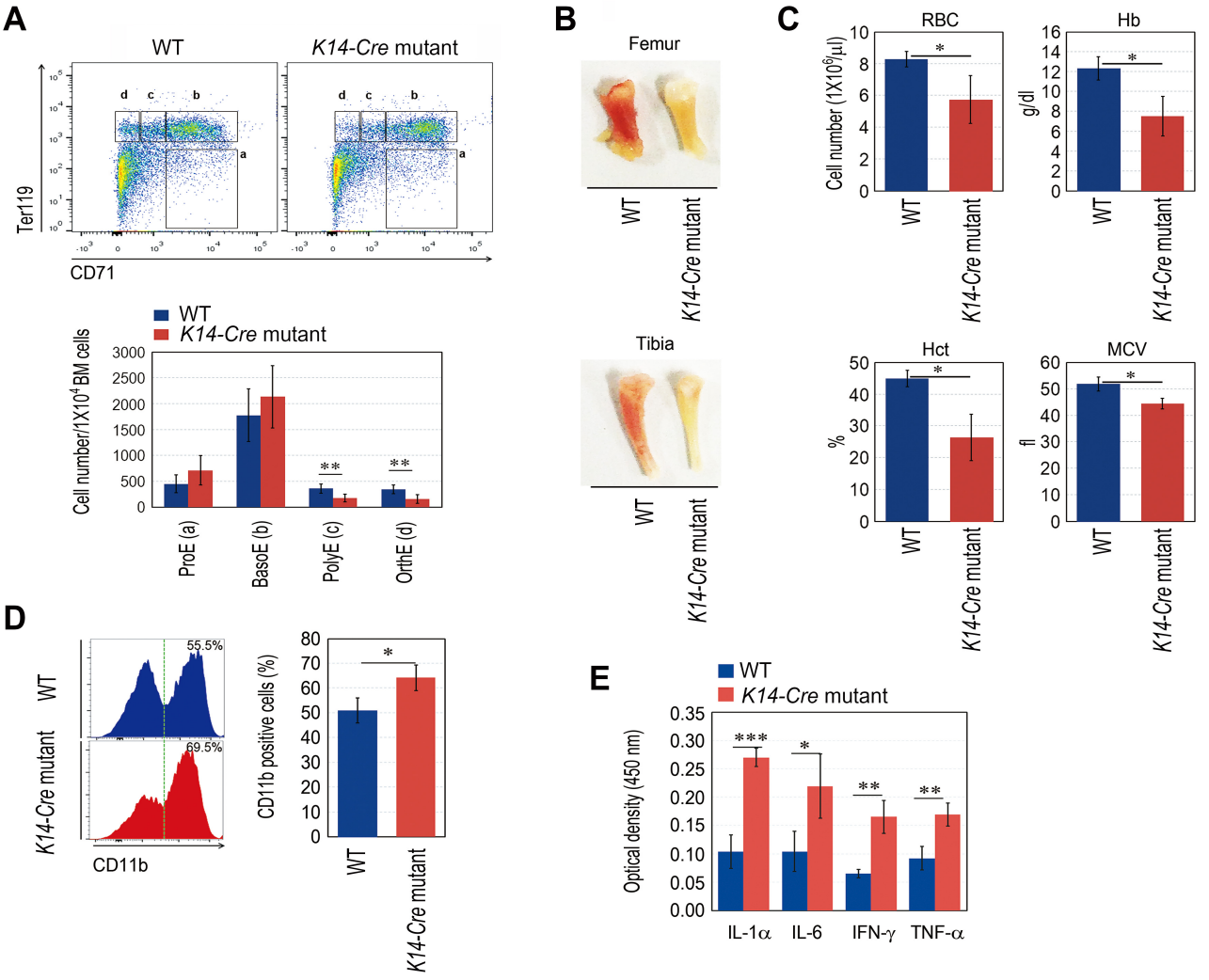

Fig. 3. Overexpression of COMP-Ang1 in *K14*-expressing cells disturbs erythroblast maturation and increases the level of inflammatory cytokines. (A) Phenotypical identification of the mutant- and WT-derived BM erythroblasts in four distinct stages (a, ProE; b, BasoE; c, PolyE; d, OrthE) (n = 12). (B) Photographs showing femur and tibia of the mutants and WT littermates. (C) Circulating levels of RBC, Hb, Hct, and MCV were measured using an automated complete blood cell counter (n = 6). (D) BM-conserved CD11b-positive cells (%) in the mutants and WT littermates (n = 5). (E) Levels of IL-1α, IL-6, IFN-γ, and TNF-α in BM supernatant of the mutants and WT littermates were determined using an ELISA kit. Representative data are shown for three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by two-tailed Student's *t*-test.

a decreased frequency of MSCs along with their increased senescence (Fig. 2A). BM MSCs of the *K14-Cre* mutants also showed lower clonogenic activity (Fig. 2B) as well as decreased osteoblast-specific marker expression (Fig. 2C) and mineralization (Fig. 2D) compared to those of WT littermates. In addition, overexpression of COMP-Ang1 in *K14*-expressing cells significantly decreased ($P < 0.001$) BM cellularity (Fig. 2E). These results indicate that, in addition to BM HSCs, excessive COMP-Ang1 impairs retention and induces senescence of BM MSCs, while also reducing their clonogenic and osteogenic potentials.

Excessive COMP-Ang1 disturbs maturation of erythroblasts at late stages

As the *K14-Cre* mutants revealed a preferable skewing toward myeloid lineage in their peripheral blood, we examined how excessive COMP-Ang1 affects erythropoietic differentiation. Overexpression of COMP-Ang1 in *K14*-expressing cells did not change the frequency of BM-conserved HPCs, the percentage of Annexin V^+/PI^+ cells in HPCs, or the colony formation of BM erythroid precursors (Supplementary Fig. S3). The levels of Annexin V^+/PI^- or Annexin V^-/PI^+ cells in HPCs also did not differ between the mutants and WT (data not shown). To explore whether overexpression of COMP-Ang1 in *K14*-expressing cells disturbs the maturation process by which erythroblasts differentiate into erythrocytes, we discriminated four stages of BM-conserved erythroblasts by measuring their frequencies via combinatorial conjugation with cell surface expression markers, Ter119 and CD71. The phenotypically defined gating schemes revealed that excessive COMP-Ang1 decreased the numbers of polychromatophilic (PolyE) and orthochromatophilic erythroblasts (OrthE), but not of proerythroblasts (ProE) or basophilic erythroblasts (BasoE) (Fig. 3A). The reduced PolyE and OrthE numbers correlated with the color of the BM, which was less red in the femurs and tibias of the mutants compared with those of WT littermates (Fig. 3B). The *K14-Cre* mutants also exhibited significantly lower levels ($P < 0.05$) of RBCs, Hb, Hct, and MCV in peripheral blood than did their WT littermates (Fig. 3C). In addition, excessive COMP-Ang1 increased the number of BM myeloid cells and production of inflammatory cytokines in the BM supernatants (Figs. 3D and 3E). These results suggest that overexpression of COMP-Ang1 in *K14*-expressing cells impairs erythropoietic differentiation at late stages and hinders production of mature erythrocytes while inducing inflammatory responses.

Excessive COMP-Ang1 inhibits the induction of BM-derived SDF-1

SDF-1 is known to play crucial roles in maintaining HSCs via induction of the SDF-1/CXCR4 signaling axis (Katayama et al., 2006; Zhang et al., 2003). We next investigated how overexpression of COMP-Ang1 in *K14*-expressing cells affects the induction of SDF-1 in BM and BM supernatants. IHC assays showed that SDF-1 levels in trabecular and cortical zones of the *K14-Cre* mutants were lower than those of WT litter-

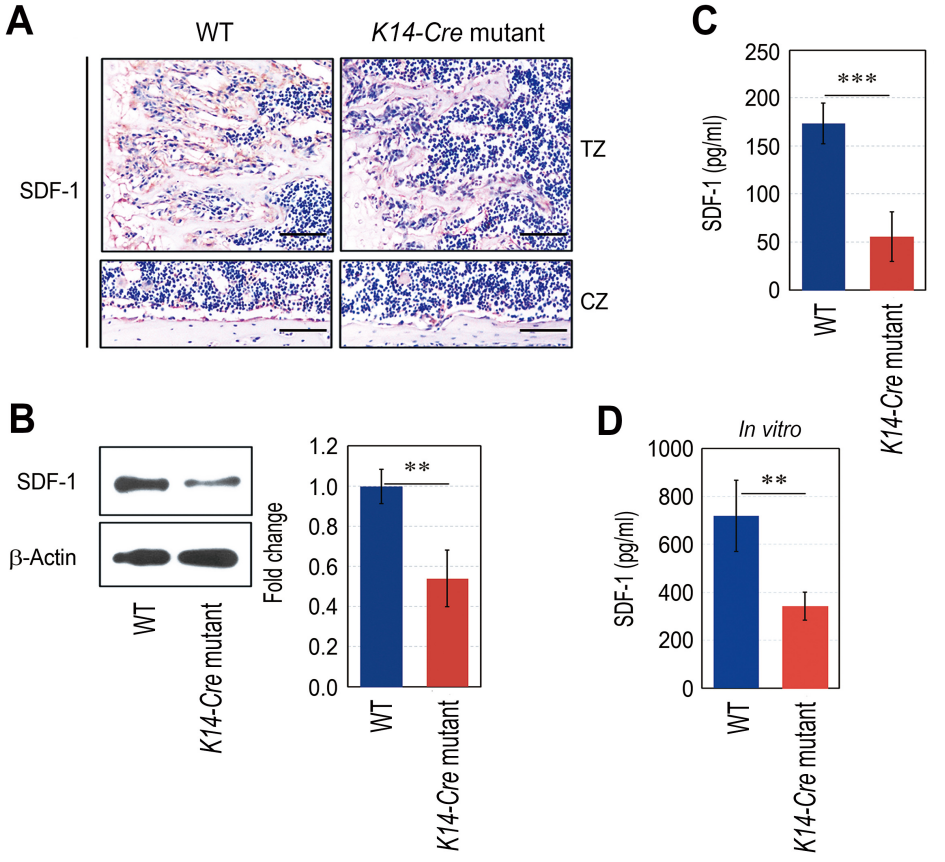

Fig. 4. Overexpression of COMP-Ang1 in *K14*-expressing cells reduces BM levels of SDF-1. (A) IHC assay exhibiting lower SDF-1 expression in the trabecular zone (TZ) and cortical zone (CZ) of the *K14-Cre* mutants compared with WT littermates. Scale bars represent 100 μm. (B and C) Level of SDF-1 protein in BM cells and BM supernatants of the mutant and WT littermates was determined by western blotting and ELISA, respectively (n = 4). (D) SDF-1 level detected in cultures of BM cells by ELISA (n = 4). $**P < 0.01$ and $***P < 0.001$ by two-tailed Student's *t*-test.

mates (Fig. 4A). The results from immunoblot assays (Fig. 4B) and ELISA (Fig. 4C) supported that SDF-1 levels in the BM were significantly diminished under excessive COMP-Ang1. BM cells isolated from the *K14-Cre* mutants also produced less SDF-1 compared to those from WT littermates (Fig. 4D). These results indicate that an impaired SDF-1/CXCR4 axis is associated with peripheral egression and senescence of BM HSCs under excessive COMP-Ang1.

Senescence of BM HSCs and the Ang1/Tie2 signaling axis are not directly associated with excessive COMP-Ang1-related disturbances in erythrocyte maturation

To examine whether excessive COMP-Ang1-mediated senescence of HSCs is associated with defects in erythroblast maturation, we transplanted LSK cells derived from the *K14-Cre* mutants and WT littermates (CD45.2) into conditioned recipients (CD45.1/2). The frequencies of BM erythroblasts in the recipients transplanted with the mutant-derived LSK cells were comparable to those transplanted with WT littermate-derived cells (Fig. 5A). No changes in numbers of circulating RBCs, Hb, Hct, or MCV were observed between the recipients transplanted with the mutant- or WT-derived LSK cells (Fig. 5B). The numbers of erythroblasts positive to GATA-1, which plays essential roles in the differentiation and maturation of erythroid cells into erythrocytes, in the recipients transplanted with the mutant-derived LSK cells were also similar to those transplanted with WT littermate-derived cells (Fig. 5C). However, significantly higher numbers of HSCs positive for MitoSox, C_{12}FDG, or p-p38 kinase in CD45.2-expressing BM cells were found in the recipients transplanted

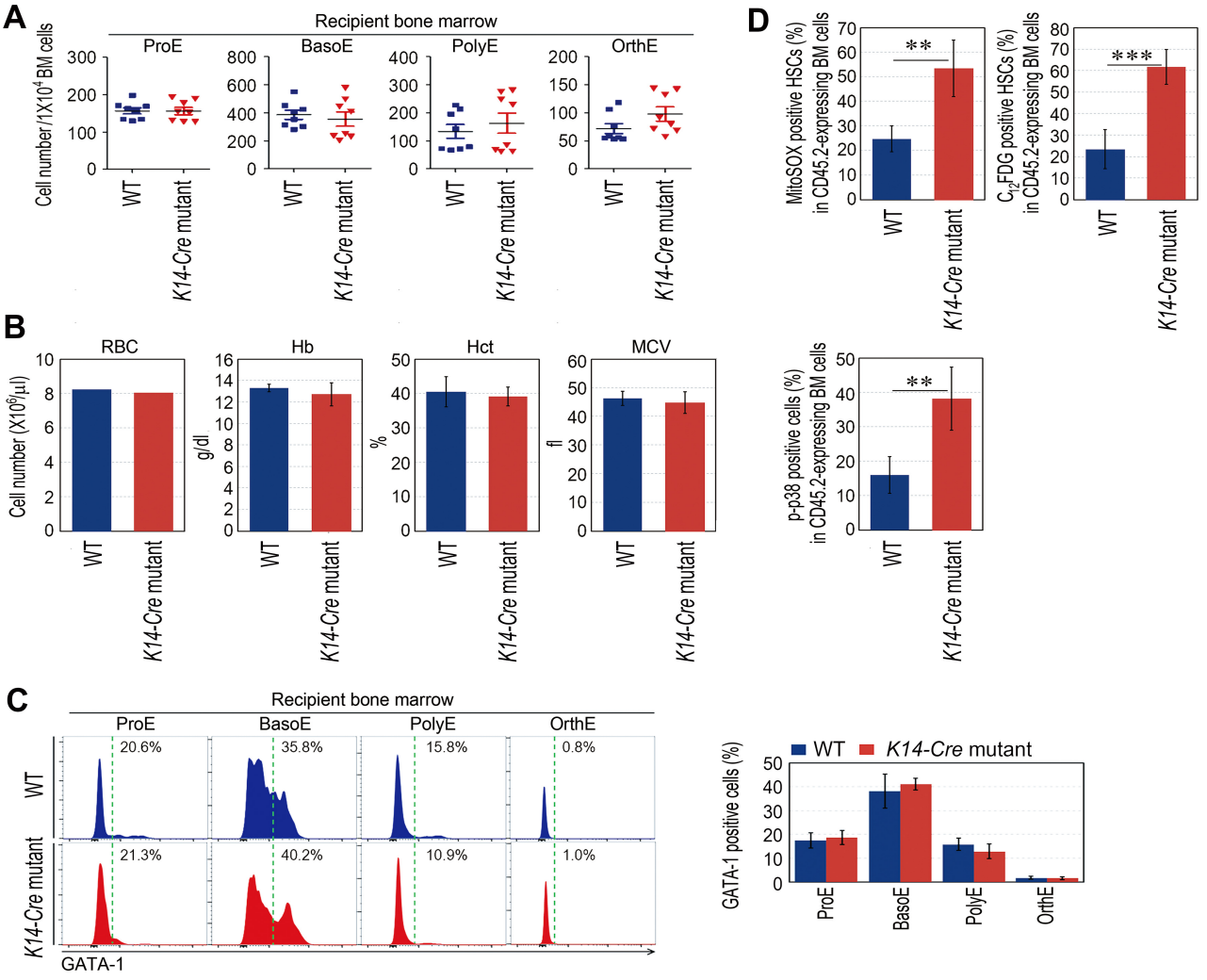

Fig. 5. Transplantation with the mutant-derived BM cells induces senescence of BM HSCs, but does not affect the numbers of BM and circulating erythroblasts or their GATA-1 expression in the recipients. BM cells (2×10^6 cells) isolated from the mutants and WT littermates (CD45.2) were transplanted into conditioned recipients (CD45.1/2). (A) Frequencies of BM erythroblasts and (B) the levels of RBC, Hb, Hct, and MCV in peripheral blood of the recipients were determined after 5 months post-transplantation by flow cytometric analysis (n = 8). At the same time, (C) GATA-1 expression in BM erythroblasts and (D) the numbers of HSCs positive for MitoSox, C_{12}FDG, or p-p38 kinase in CD45.2-expressing BM cells of the recipients were determined by flow cytometric analysis (n = 8). $^{**}P < 0.01$ and $^{***}P < 0.001$ by two-tailed Student's *t*-test.

with BM cells of the *K14-Cre* mutants compared to those of WT littermates (Fig. 5D). These results suggest that defected erythropoietic differentiation under excessive COMP-Ang1 is not directly associated with senescence of BM HSCs.

As the Ang1/Tie2 signaling axis plays important roles in maintaining the quiescence and survival of HSCs, we subsequently explored whether the COMP-Ang1/Tie2 signaling is related to the GATA-1 expression in and frequency of erythroblasts using B6-derived BM cells. B6 mouse-derived BM erythroblasts expressed Tie2 at various levels depending on maturation stages (Supplementary Fig. S4). When *rh*COMP-Ang1 was exogenously added at various concentrations (0-1,000 ng/ml), neither the frequencies of nor the GATA-1 expression in BM erythroblasts at four stages were

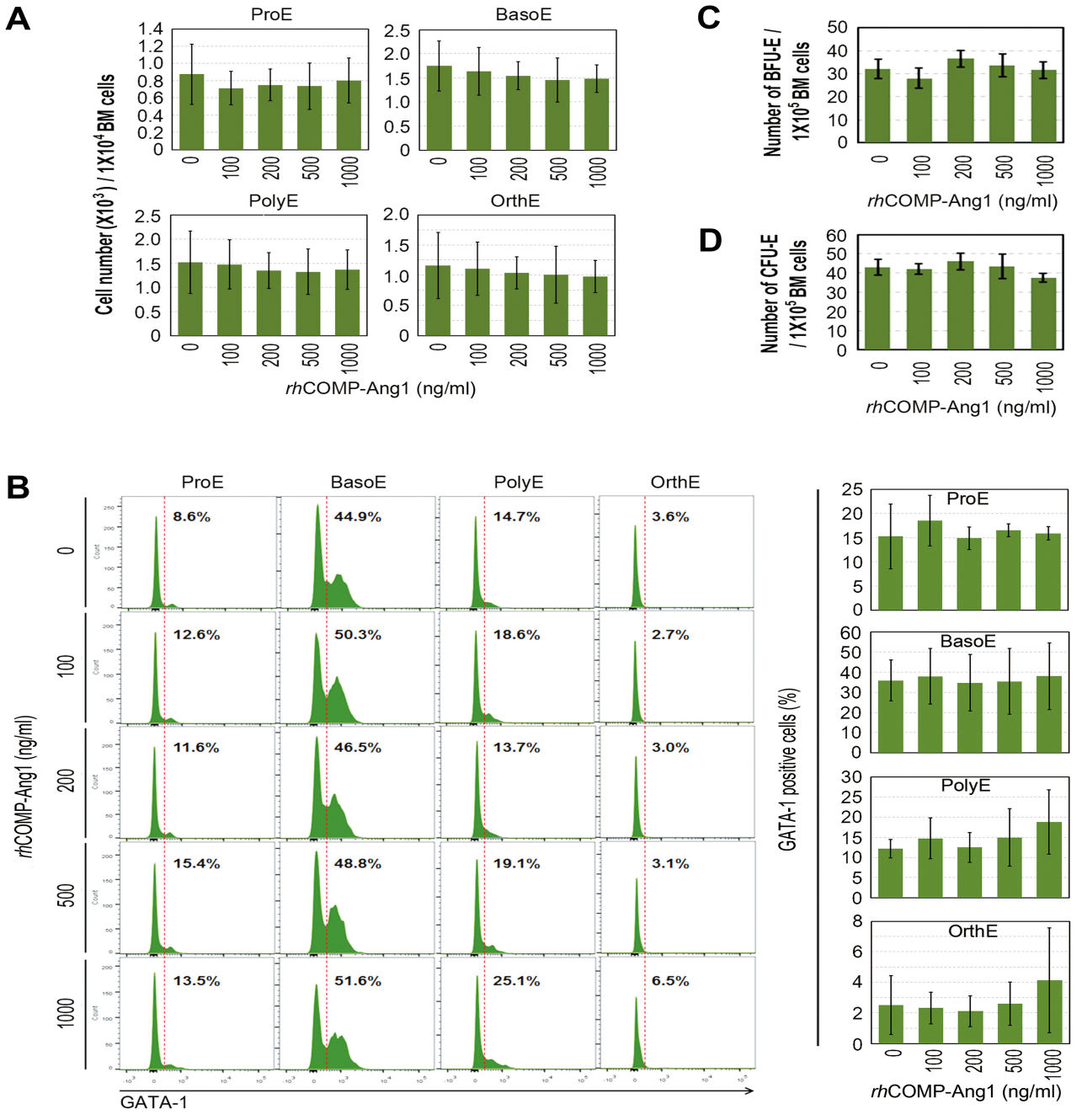

Fig. 6. Treatment with *rh*COMP-Ang1 does not change not only the frequency of, or GATA-1 expression in, BM erythroblasts but also clonogenic activity of BM-derived progenitor cells. BM cells isolated from B6 mice (3 weeks old) were incubated with various concentrations (0-1,000 ng/ml) of *rh*COMP-Ang1 for 24 h and (A) the total frequency of and (B) GATA-1-positive cells (%) of BM erythroblasts at four stages were analyzed using flow cytometry. (C and D) BM cells (2×10^5 cells) collected from B6 mice (3 weeks old) were incubated with MethoCult M3334 in the presence of *rh*COMP-Ang1 (0-1,000 ng/ml) and, after 14 days of incubation, numbers of BFU-E and CFU-E were measured.

changed (Figs. 6A and 6B). Similarly, neither the numbers of BFU-E (Fig. 6C) nor of CFU-E (Fig. 6D) in B6 mice-derived BM cells were altered by treating these cells with *rh*COMP-Ang1. These results suggest that like HSC senescence, the impaired maturation of BM erythroblasts under excessive COMP-Ang1 is not directly associated with COMP-Ang1/Tie2 signaling.

Peripheral circulation and impaired maturation of erythroblasts are associated with an alteration in CXCR4 and GATA-1 expression

As the SDF-1/CXCR4 axis and GATA-1 play important roles in hematopoiesis and erythrocyte maturation, we further examined how excessive COMP-Ang1 affects the expression of CXCR4 and GATA-1 in BM-derived or circulating erythroblasts. Overexpression of COMP-Ang1 in *K14*-expressing cells did not alter BM frequency of GATA-1-positive HPCs (Fig. 7A), but decreased the numbers of GATA-1-positive BM erythroblasts while increasing these cells positive to MitoSox or p-p38 kinase (Figs. 7B-7D; Supplementary Figs. S5A-S5D). Dissimilar to BM erythroblasts (Fig. 7E), circulating erythroblasts in the *K14-Cre* mutants displayed significantly greater expression of GATA-1 and CXCR4 at four stages compared with those in WT littermates (Figs. 7F and 7G; Supplementary Figs. S5E-S5G). Excessive COMP-Ang1 also increased the numbers of polyE and OrthE, but not of ProE or BasoE, in peripheral blood (Fig. 7H). These findings indicate that abnormal peripheral circulation and erythroblast maturation under excessive COMP-Ang1 is in part associated with dysregulated expression of CXCR4 and GATA-1.

DISCUSSION

BM niches play a pivotal role in HSCs by producing various cellular factors, in which the SDF-1/CXCR4 signaling axis exerts important roles for maintenance of the HSC pool, as well as for regulation of HSC mobilization and homing (Kata-

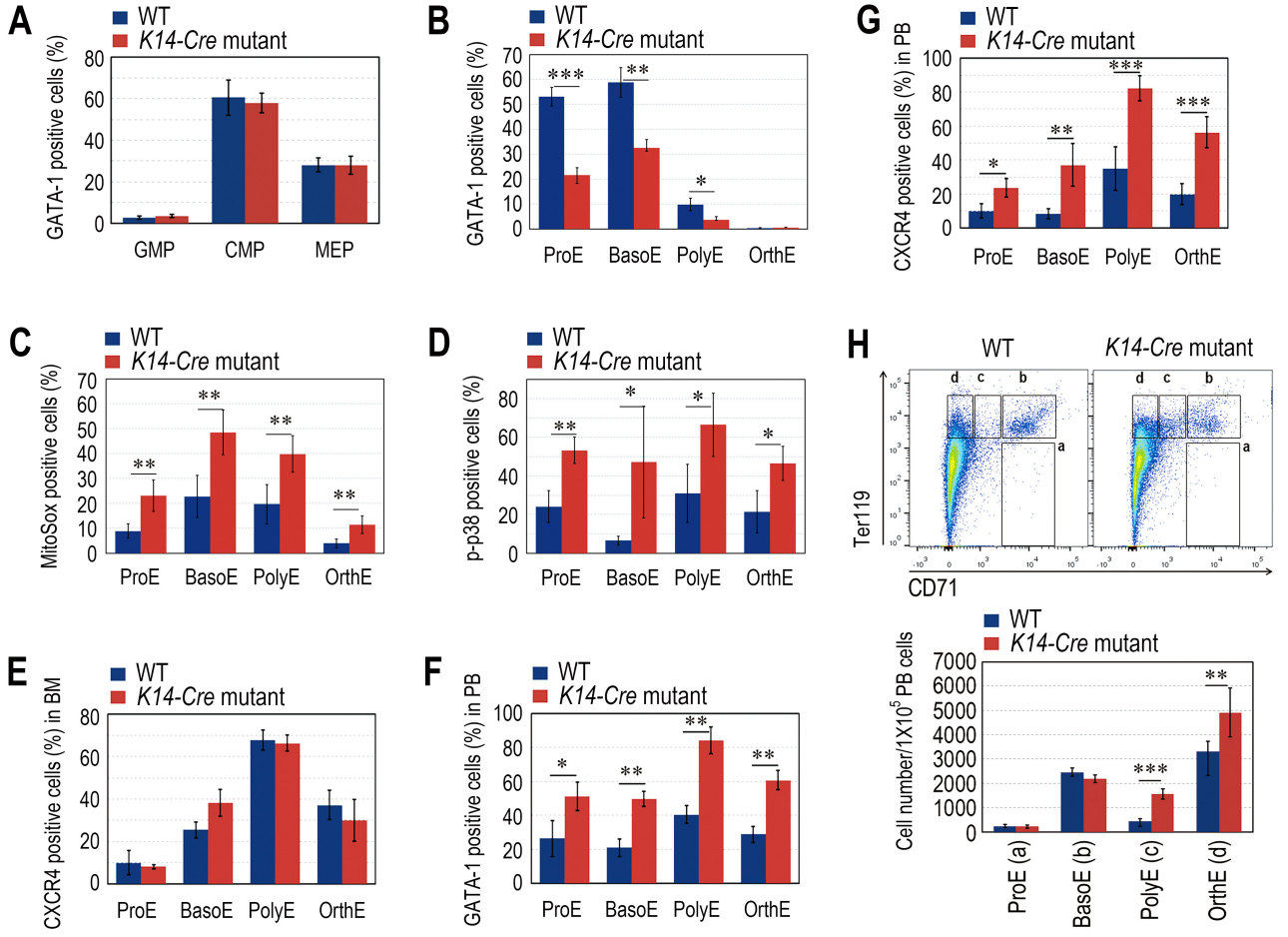

Fig. 7. Impaired erythropoiesis under excessive COMP-Ang1 is associated with dysregulation of the SDF-1/CXCR4 axis and GATA-1 expression along with increased oxidative stress. GATA-1 positive cells (%) in (A) HPCs and (B) BM erythroblasts of the *K14-Cre* mutants and WT littermates were determined by flow cytometric analysis (n = 6). Levels of (C) cellular superoxide anion and (D) p-p38 kinase in the mutant- and WT-derived BM erythroblasts were measured by staining these cells with MitoSOX™ Red reagent or with Alexa Flour 488-conjugated p-p38 kinase antibody (n = 6). (E) BM-conserved erythroblasts (%) positive to CXCR4 and circulating erythroblasts (%) positive to (F) GATA-1 or (G) CXCR4 were determined using flow cytometry (n = 6). (H) Flow cytometric analysis showing the number of circulating erythroblasts in the mutants and WT littermates (n = 8). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by two-tailed Student's *t*-test.

yama et al., 2006; Sugiyama et al., 2006; Zhang et al., 2003). Our results indicate that overexpression of COMP-Ang1 in *K14*-expressing cells diminishes the level of BM-derived SDF-1 and leads to impaired BM retention and senescence of HSCs. Together with our previous report (Kook et al., 2018), the current findings also imply that SDF-1 loss in BM and the impaired BM microenvironment synergistically contribute to senescence induction and peripheral egression of BM HSCs.

Osteoblasts are associated with maintenance of HSCs in the BM microenvironment, where angiogenesis is coupled with osteogenesis, which is essential for homeostatic bone renewal and regenerative fracture healing (Kusumbe et al., 2014). We previously found that mutants with *Smad4* conditionally knocked out in *Col1a1*-expressing cells exhibited decreased SDF-1 induction and increased HSC senescence-associated phenotypes in the BM compared with their WT littermates (Kook et al., 2016). Thus, it is postulated that overexpression of COMP-Ang1 under the *K14-Cre* promoter negatively affects osteoblast function and/or osteoblast interaction with osteoclasts, eventually impairing the BM microenvironment and SDF-1 induction. Additionally, different stem/progenitor cells occupy distinct niches in BM, where the perivascular niche and endosteal niche contribute to HSCs and early lymphoid progenitors, respectively (Ding and Morrison, 2013). Perivascular stromal cells are thought to be the predominant cells to express SDF-1 in BM compared with other BM cells such as endothelial cells, osteoblasts, and some hematopoietic cells (Ding and Morrison, 2013). Together, we consider that SDF-1 levels in the BM are inversely correlated with the amount of COMP-Ang1 generated, and that excessive COMP-Ang1, impaired BM niches, and increased senescence of MSCs may also synergistically contribute to SDF-1 loss in the BM.

Erythropoiesis occurs mainly in BM and an abnormal BM microenvironment contributes to hematopoietic dysfunction in Fanconi anemia (Kertesz et al., 2004; Zhou et al., 2017). HSC senescence is connected to erythrocyte defect-induced anemia, and activation of the Ang1/Tie2 signaling axis is crucial for maintaining the quiescence and survival of HSCs (Arai et al., 2004; Zhang et al., 2007). However, our results indicate that the impaired maturation of erythroblasts under excessive COMP-Ang1 is not directly associated with senescence of BM HSCs or the Ang1/Tie2 signaling axis (Brindle et al., 2006). Rather, our results support that in addition to GATA-1, the SDF-1/CXCR4 signaling axis is responsible for impaired BM retention and induction of HSC senescence, as well as for disturbed erythropoiesis. GATA-1 plays essential roles in the differentiation and maturation of erythroid cells into erythrocytes (Ferreira et al., 2005; Pevny et al., 1991; 1995; Yu et al., 2002). The regulatory roles of GATA-1 on erythropoiesis are closely related to its potential to control erythroid differentiation and erythroblast formation by inducing the expression of erythroid genes and by cooperating with erythropoietin (Ferreira et al., 2005; Pevny et al., 1991). GATA-1 is downregulated via an autonomous cell signaling mechanism during the terminal stages of erythropoiesis (Ferreira et al., 2005). Inflammatory responses repress GATA-1 expression via mediation by p38 kinase activation in erythroid cells (Bibikova et al., 2014). As erythroid differentiation involves production of reactive oxygen species, efficient antioxidant defense systems are required to limit persistent and severe oxidative stress in erythropoiesis (Matte and de Franceschi, 2019). Hypoxia is also capable of increasing BM numbers of HSCs and HPCs in mice. This condition decreases oxidative stress and increases GATA-1 expression, resulting in increased erythropoiesis and enhanced HSC engraftment (Chen et al., 2016). Overall, a balanced expression of GATA-1 is critical for terminal erythroid differentiation and sequential formation of erythroblasts; thus its overexpression can inhibit terminal differentiation and maturation of these cells (de Thonel et al., 2010; Ferreira et al., 2005; Pevny et al., 1991; Whyatt et al., 1997; 2000). Collectively, our present findings indicate that defective erythroblast maturation during overexpression of COMP-Ang1 is accompanied by a dysregulation of the SDF-1/CXCR4 signaling axis in the BM along with imbalanced GATA-1 expression in erythroblasts rather than in HPCs. These data also suggest that the impaired erythropoietic differentiation is in part associated with enhanced oxidative stress in erythroblasts.

MSCs play crucial roles as a stem cell niche in BM by expressing higher levels of signaling molecules involved in HSC maintenance (Boulais and Frenette, 2015; Ehninger and Trumpp, 2011). Macrophages are part of BM niches, and loss of macrophages is associated with egression of BM HSCs into the peripheral blood and spleen. Our current findings indicate that myeloid cells under excessive angiogenesis or COMP-Ang1 contribute to the production of cellular reactive oxygen species and inflammatory cytokines, thereby negatively affecting hematopoietic differentiation. Our findings also indicate that excessive COMP-Ang1 or angiogenesis is associated with impaired function and senescence induction of not only HSCs but also BM MSCs, through which the BM microenvironment, SDF-1 production, and body weight will be also affected. Furthermore, our findings show no hematopoietic changes after the transplantation of mutant-derived cells, indicating that excessive angiogenesis or COMP-Ang1 expression impairs the functions of BM-conserved stem cells in developmental stages and hampers normal growth and bone mass accrual.

In summary, our previous and current findings highlight that excessive angiogenesis or COMP-Ang1 expression induces senescence and peripheral egression of BM HSCs and MSCs, thereby leading to abnormal hematopoiesis and bone mass accrual. This study also indicates that impairment of the SDF-1/CXCR4 signaling axis and imbalanced GATA-1 expression are associated with excessive COMP-Ang1-mediated disturbance of erythroblast maturation and their peripheral circulation. Although our experimental system is artificial due to the fact that Ang1 from the skin does not prominently regulate hematopoiesis and because Ang1 secreted from BM HSCs and HPCs is critical for the regeneration of their niche (Zhou et al., 2015), our findings suggest that appropriate activation of Ang1/Tie2 signaling and/or a suitable level of Ang1 is required not only for vasculogenesis, angiogenesis, and hematopoiesis, but also for the functions of BM-conserved stem cells.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

S.H.K., E.S.C., and J.C.L. conceived and designed the experiments. H.J.S., M.H.K., G.B., J.W.H., S.B.P., S.H.K., and H.S.S. performed the experiments. J.W.H., M.H.K., and E.S.C. generated transgenic mice. H.J.S., M.H.K., G.B., S.H.K., E.S.C., and J.C.L. analyzed the data. H.J.S., G.B., S.H.K., E.S.C., and J.C.L. contributed reagents/materials/analysis tools. H.J.S., M.H.K., G.B., E.S.C., S.H.K., and J.C.L. wrote the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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