Received: 22 March 2021 | Revised: 26 July 2021 | Accepted: 30 July 2021

DOI: 10.1111/gtc.12887

ORIGINAL ARTICLE

Genes to Cells WILEY

Blockade of the interaction between BMP9 and endoglin on erythroid progenitors promotes erythropoiesis in mice

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Funding information
This study was conducted with a research fund from Kyowa Kirin Co., Ltd.

Communicated by: Shunsuke Ishii

Abstract

Bone morphogenetic protein-9 (BMP9), a member of the transforming growth factor β (TGFβ) superfamily, plays important roles in the development and maintenance of various cell lineages via complexes of type I and type II TGFβ receptors. Endoglin is a coreceptor for several TGFβ family members, including BMP9, which is highly expressed in a particular stage of differentiation in erythroid cells as well as in endothelial cells. Although the importance of the interaction between BMP9 and endoglin for endothelial development has been reported, the contribution of BMP9 to endoglin-expressing erythroid cells remains to be clarified. To address this point, we prepared an anti-BMP9 antibody that blocks the BMP9-endoglin interaction. Of note, challenge with the antibody promotes erythropoiesis in wild-type mice but not in a mouse model of renal anemia in which erythropoietin (EPO) production in the kidneys is genetically ablated. While endoglin-positive erythroid progenitors are mainly maintained as progenitors when bone marrow-derived lineage-negative and cKit-positive cells are cultured in the presence of EPO and stem cell factor, the erythroid-biased accumulation of progenitors is impeded by the presence of BMP9. Our findings uncover an unrecognized role for BMP9 in attenuating erythroid differentiation via its interaction with endoglin on erythroid progenitors.

KEYWORDS

antibody, BMP9, endoglin, erythropoiesis

1 | INTRODUCTION

The transforming growth factor β (TGFβ) superfamily is composed of more than 30 substances (Morikawa et al., 2016) that are involved in not only a variety of cellular functions in normal development and physiology but also the pathogenesis of diseases (Dituri et al., 2019; Morikawa et al., 2016). Most members of the TGFβ superfamily are categorized into three structurally related groups, the Activin/Inhibin, TGF-β and bone morphogenetic protein (BMP)/growth differentiation factor (GDF) subfamilies, based on their biological functions and phylogenetic analysis (Morikawa et al., 2016); each of these groups recruits different Smad proteins to govern characteristic signaling pathways in cellular homeostasis and disease (Sartori et al., 2014). TGFβ superfamily members

share 7 TGFβ type I (activin receptor-like kinase [ALK] 1–7) and 5 type II (activin receptor type IIA [ActRIIA], ActRIIB, BMPRII, TGFβRII and anti-Müllerian hormone receptor type II [AMHRII]) serine/threonine kinase receptors. Endoglin (CD105) is also known as a TGFβ type III receptor that modulates TGFβ signaling by forming a complex with type I and type II receptors (Cheifetz et al., 1992; Lastres et al., 1996; Zhang et al., 1996). Endoglin is primarily known to be associated with angiogenesis and vascular development and has been identified as a genetic factor for hereditary hemorrhagic telangiectasis (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999). Subsequently, endoglin expression on interstitial fibroblasts (St-Jacques et al., 1994), interstitial and peritoneal macrophages (Lastres et al., 1992; Ojeda-Fernández et al., 2016), mesenchymal stem cells (Jones et al., 2002) and osteogenic populations in common skeletal progenitor cells (Chan et al., 2013) has been reported. In particular, its expression on hematopoietic stem/precursor cells was determined to play important roles in hematopoiesis during development and adulthood (Borges et al., 2019; Meurer & Weiskirchen, 2020; Pierelli et al., 2000). In particular, endoglin expression is utilized as a marker to identify erythroid lineage cells in a certain developmental stage in combination with transferrin receptor (CD71) expression (Mori et al., 2015).

It has been determined that several members of the TGFβ superfamily play a diverse and critical array of biological roles in erythropoiesis. BMP2, BMP4 and activin A have been reported to promote erythroid differentiation of CD34-positive primary cells in vitro, although BMPs and activin A work in different ways in erythropoiesis (Detmer & Walker, 2002; Kang et al., 2012; Maguer-Satta et al., 2003). In contrast, TGF-β1 acts as a gatekeeper to prevent unneeded erythroid progenitors from proceeding to mature erythroid cells via erythropoietin (EPO) signaling; consequently, blocking TGF-β1 function promotes erythroid cell production (Di Giandomenico et al., 2020; Gao et al., 2016). GDF15 down-regulates hepatic hepcidin expression and is highly expressed in proliferating erythroblasts in patients with ineffective erythropoiesis (Tanno et al., 2007). Recent work revealed that GDF15 negatively regulates proliferating erythroid cells by stimulating apoptosis and inhibiting cell proliferation in an autocrine and/or paracrine manner (Ranjbaran et al., 2020). Thus, the TGFβ superfamily network seems to orchestrate erythroid homeostasis under steady-state and stress conditions; however, many researchers have not investigated how other TGFβ superfamily members contribute to erythroid differentiation.

BMP9, also known as growth differentiation factor 2 (GDF2), was originally identified in developing mouse livers as a secreted signaling molecule in hepatic growth and function (Song et al., 1995). Accumulating evidence supports the notion that BMP9 plays crucial roles in the maintenance and development of a variety of cell lineages, such as chondrogenic differentiation (Majumdar et al., 2001), adipogenic and osteogenic differentiation (Kang et al., 2004, 2009; Lord et al., 2010; Xiang et al., 2012), the nervous system (Lopez-Coviella et al., 2000) and endothelial progenitor cell differentiation (Kim et al., 2015). Moreover, it has been shown that BMP9 regulates various cell physiological processes, such as glucose and lipid metabolism and iron homeostasis (Caperuto et al., 2008; Chen et al., 2003; Truksa et al., 2006; Wang et al., 2020). In particular, BMP9 has been implicated in the initiation, progression and metastasis of malignant neoplasms through the regulation of a variety of cellular processes (Li et al., 2013), such as epithelial-to-mesenchymal transition, proliferation (Herrera et al., 2009), survival and motility (Herrera et al., 2013; Li et al., 2013). Thus, BMP9 may be a target for regenerative medicine and clinical medicine.

BMP9 has shown high specificity in binding and signaling for ALK1 and endoglin to promote endothelial cell quiescence (David et al., 2007, 2008; Nolan-Stevaux et al., 2012; Scharpfenecker et al., 2007; Suzuki et al., 2010; Upton et al., 2009). Because of a report showing that endoglin specifically binds BMP9 and BMP10 with high affinity (Castonguay et al., 2011), BMP9 has been thought to be the physiological ligand of the endoglin pathway in angiogenesis and vascular homeostasis. Therefore, although the involvement of BMP9 via endoglin in the maintenance of erythroid homeostasis has been assumed, the direct function of BMP9 in erythroid lineage cells remains to be elucidated. In this paper, we prepared a neutralizing antibody for BMP9, which blocks the interaction of BMP9 with endoglin, and found that the administration of the antibody stimulates erythropoiesis in mice. Our results demonstrate that BMP9 exerts an inhibitory effect on erythroid differentiation through interaction with endoglin on the surface of erythroid progenitors. Our data provide new insight into the mechanisms underlying the control of erythropoiesis by the TGFβ superfamily.

2 | RESULTS

2.1 | BMP9 binds to endoglin-expressing cells

We prepared a neutralizing antibody for the human BMP9 protein, which was isolated from BMP9 knockout mice immunized with the complex form of the human BMP9 protein (U.S. Patent No. 8,969,040, 2015). To verify whether the antibody truly neutralizes BMP9 signaling, we conducted a reporter assay utilizing an ALK1-expressing HepG2 reporter cell line (U.S. Patent No. 8,969,040, 2015), in which the luciferase protein is produced under the regulation of Smad binding motifs (Kusanagi et al., 2000). Reporter cells express luciferase activity when BMP9 binds to cell surface receptors. We cultured reporter cells with various amounts of the anti-BMP9 antibody with fixed amounts of human BMP9

protein (Figure 1a) and found that BMP9-mediated luciferase activity was attenuated by the anti-BMP9 antibody in a dose-dependent manner. The results clearly indicated that the antibody was able to neutralize BMP9-ALK1 signaling.

It has been reported that BMP9 contributes to endothelial homoeostasis through a direct interaction with endoglin on the surface of endothelial cells (Young et al., 2012). As endoglin is also known as an erythroid marker (Pronk et al., 2007), we examined the binding activity of BMP9 to endoglin-expressing cells. We prepared biotinylated BMP9 (bio-BMP9) and examined whether BMP9 directly binds to endoglin on the surface of 293F cells by flow cytometry using allophycocyanin (APC)-conjugated streptavidin. When we examined the original 293F cells, fluorescence-positive cells could not be detected (Figure 1b left panel, blue line). In contrast, when we examined 293F cells transfected with an endoglin expression vector, most of the cells became fluorescence-positive (Figure 1b right panel, blue line), indicating that BMP9 binds to endoglin in 293F cells. Of note, the fluorescence on 293F cells became undetectable after the addition of the anti-BMP9 antibody (red line). Thus, these results support the notion that the anti-BMP9 antibody eliminates BMP9 function by blocking the binding of BMP9 to its receptor endoglin.

2.2 | BMP9 binds to endoglin-positive erythroid progenitors

We next examined the expression of endoglin in bone marrow hematopoietic cells to understand its expression profile during erythropoiesis. Although endoglin is used as an erythroid

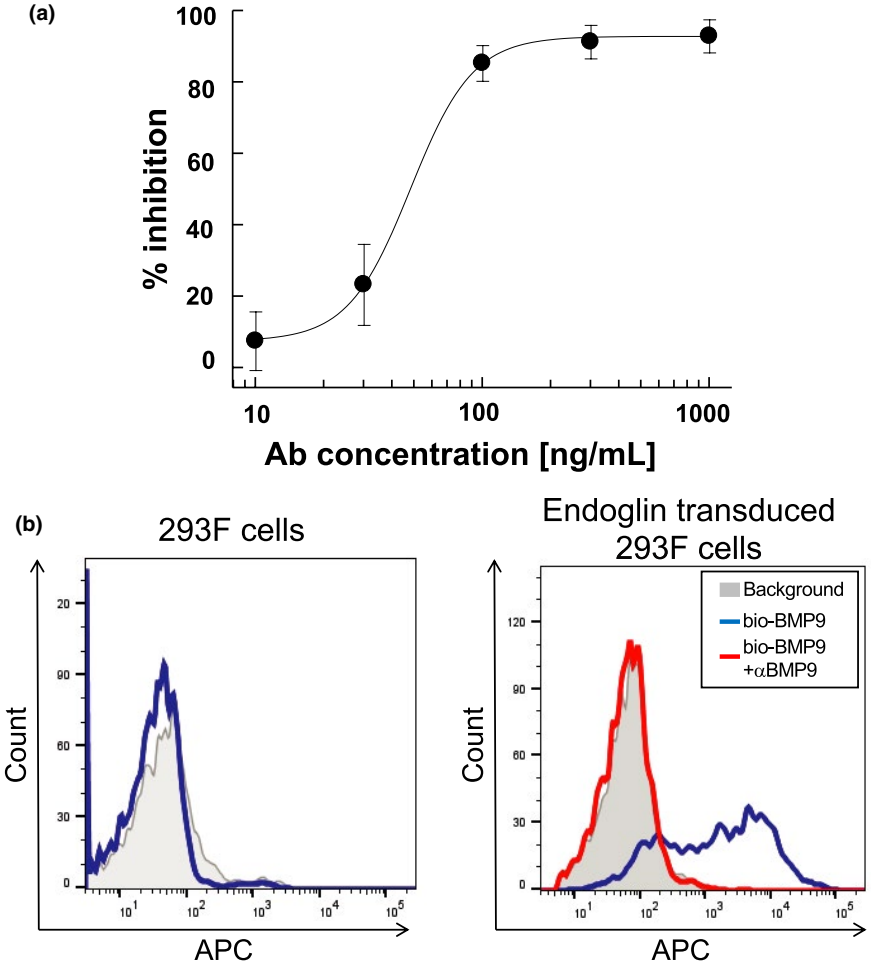

FIGURE 1 BMP9-mediated signaling is neutralized by impeding the BMP9-endoglin interaction. (a) The percent inhibition of the luciferase activity was calculated by using following formula: % inhibition = (mean of three measured data of 10 ng/ml human BMP9 without the anti-BMP9 antibody - mean of three measured data of 10 ng/ml human BMP9 with the anti-BMP9 antibody at the indicated concentrations)/(mean of three measured data of 10 ng/ml human BMP9 without the anti-BMP9 antibody - mean of six measured data of basal luciferase activity)*100. Representative data of six independent experiments are shown. (b) Flow cytometry analysis of BMP9 binding in wild-type (left) and endoglin-transduced 293F cells (right). Binding of bio-BMP9 was identified by APC-labeled streptavidin. The gray-filled histogram depicts background fluorescence in the absence of bio-BMP9. The blue and red histograms show the number of cells versus the fluorescence intensity measured in the absence and presence of the neutralizing anti-BMP9 antibody, respectively. Experiments were performed twice, and representative data are shown

marker (Pronk et al., 2007), the frequency of endoglin-positive cells is not high. We verified the endoglin-positive cells by cKit, CD71 and Ter119 expression and found that endoglin-positive cells were most enriched in the CD71(+) Ter119(−) fraction (Figure 2a).

We next examined whether BMP9 specifically binds cells expressing endogenous endoglin. We incubated bone marrow cells from mice with bio-BMP9, and BMP9-bound cells were subsequently detected with APC-labeled streptavidin. We found that lineage (Lin)(−)cKit(+) (LK) cells, in which myeloid and megakaryocyte-erythroid progenitors were enriched (Du et al., 2012), were divided into two groups according to the status of endoglin expression (Figure 2b). We further found that the level of endoglin expression was well correlated with the APC fluorescence intensity in the endoglin-positive fraction, indicating that BMP9 binding is well correlated with the level of endoglin expressed on the cell surface (Figure 2b).

It has been reported that LK cells gated on Sca1(−) IL7Rα(−)FcγRII/III(−)CD41(−) are subfractionated into committed erythroid-restricted progenitors (preCFU-E; CD150(+)endoglin(+)), more committed erythroid-restricted progenitors (CFU-E; CD150(−)endoglin(+)), bipotent premegakaryocyte erythrocyte progenitors (preMegE; CD150(+)endoglin(−)) and myeloid progenitors (preGM; CD150(−)endoglin(−)) by the combination of endoglin and CD150 expression (Figure 2c) (Pronk et al., 2007). We confirmed that the cells in the preCFU-E and CFU-E fractions were exclusively fluorescence-positive in the presence of bio-BMP9 (Figure 2d, blue lines), and the fluorescence was completely canceled by treatment with the antibody (Figure 2d, red lines). Thus, BMP9 binds endoglin on erythroid progenitors and possibly controls erythroid differentiation.

2.3 | BMP9 neutralization leads to erythropoiesis in BALB/c mice

To assess BMP9 function in adult hematopoiesis in vivo, we evaluated the blood parameters of mice treated with the anti-BMP9 antibody. We randomly assigned 8-week-old BALB/c mice into 6 groups: one, two or four weekly subcutaneous doses of either 1 mg/kg of the antibody or an equal volume of phosphate-buffered saline (PBS) (Figure 3a). Circulating blood was obtained from the abdominal aorta of mice under anesthesia at 7 days after the final injection. We found that the red blood cell (RBC) count, hemoglobin concentration (Hb) and hematocrit value were significantly increased in mice treated with a single dose of the antibody compared to the corresponding control group of mice injected with PBS by the Wilcoxon rank sum test (Figure 3b–d).

Interestingly, the reticulocyte count was not elevated at 7 days after the single-dose administration of the antibody, whereas it increased later and continued to increase with repeated administration of the antibody (Figure 3e). It has been reported that impaired BMP9-ALK1 signaling leads to increased vascular permeability (Akla et al., 2018). We surmised that the early changes in erythrocyte parameters are at least in part caused by changes in the volumes of the bodily fluid compartments.

Nonetheless, the plethoric tendency of erythrocyte parameters was sustained by repeated administration of the antibody (Figure 3b–d). We also found that the frequency of CD71(+) Ter119(+) cells was decreased, while that of CD71(−) Ter119(+) cells tended to be increased in mice that received four weekly administrations of the anti-BMP9 antibody (Figure 4). Erythroid differentiation proceeds from CD71(+) Ter119(+) proliferating erythroid cells to CD71(−)Ter119 (+) mature and small erythroblasts (Chen et al., 2009). Thus, the anti-BMP9 antibody could promote erythroid differentiation from CD71(+)Ter119(+) cells to mature CD71(−) Ter119 (+) cells in mice.

Interestingly, we found that the platelet counts were gradually decreased in mice treated with the anti-BMP9 antibody relative to the corresponding PBS control (Figure 3f). As cells carrying bipotential erythroid-megakaryocyte colony-forming capacity express a low level of endoglin in mice (Ng et al., 2012), platelet production is expected to be influenced to some extent by the anti-BMP9 antibody. In contrast, in humans, the up-regulation of endoglin expression marks erythroid lineage commitment at the erythroid-megakaryocytic lineage bifurcation step (Mori et al., 2015). We surmised that the decline in platelet count might be a characteristic phenotype in mice. We also found that the white blood cell (WBC) count was increased by sequential treatment with the antibody (Figure 3g). BMP9 may currently have unknown physiological activities in the regulation of WBCs.

2.4 | BMP9 neutralization is not effective in AnRED mice

Erythropoietin is a cytokine that promotes erythropoiesis by stimulating the proliferation and differentiation of early erythroid progenitors (Jelkmann, 2007).

TGF-β1 neutralization did not have a significant ability to stimulate erythroid differentiation of cord blood CD34-positive cells toward mature erythrocytes in the absence of EPO (Akel et al., 2003). To elucidate the relevance of EPO to the BMP9-neutralizing effect in vivo, we evaluated the effect of the anti-BMP9 antibody on an anemic model with renal EPO deficiency (AnRED) mice that lack EPO production in the kidneys. AnRED mice are chronic renal anemia model mice that are established by rescuing embryonic lethality of mice with homozygous *Epo* gene deletion with an *Epo*-expressing BAC transgene lacking a specific regulatory

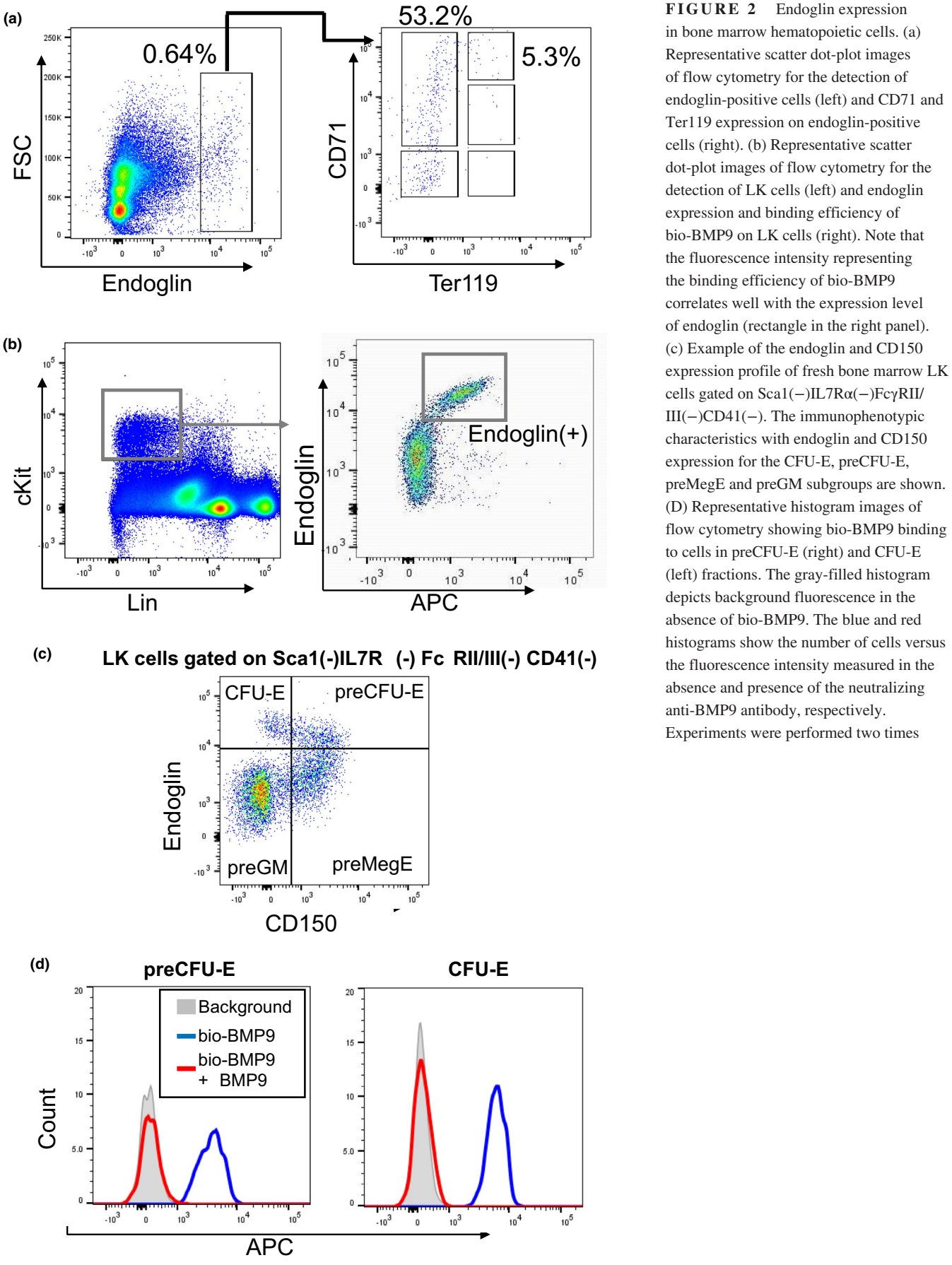

FIGURE 2 Endoglin expression in bone marrow hematopoietic cells. (a) Representative scatter dot-plot images of flow cytometry for the detection of endoglin-positive cells (left) and CD71 and Ter119 expression on endoglin-positive cells (right). (b) Representative scatter dot-plot images of flow cytometry for the detection of LK cells (left) and endoglin expression and binding efficiency of bio-BMP9 on LK cells (right). Note that the fluorescence intensity representing the binding efficiency of bio-BMP9 correlates well with the expression level of endoglin (rectangle in the right panel). (c) Example of the endoglin and CD150 expression profile of fresh bone marrow LK cells gated on Sca1(−)IL7Rα(−)FcγRII/III(−)CD41(−). The immunophenotypic characteristics with endoglin and CD150 expression for the CFU-E, preCFU-E, preMegE and preGM subgroups are shown. (D) Representative histogram images of flow cytometry showing bio-BMP9 binding to cells in preCFU-E (right) and CFU-E (left) fractions. The gray-filled histogram depicts background fluorescence in the absence of bio-BMP9. The blue and red histograms show the number of cells versus the fluorescence intensity measured in the absence and presence of the neutralizing anti-BMP9 antibody, respectively. Experiments were performed two times

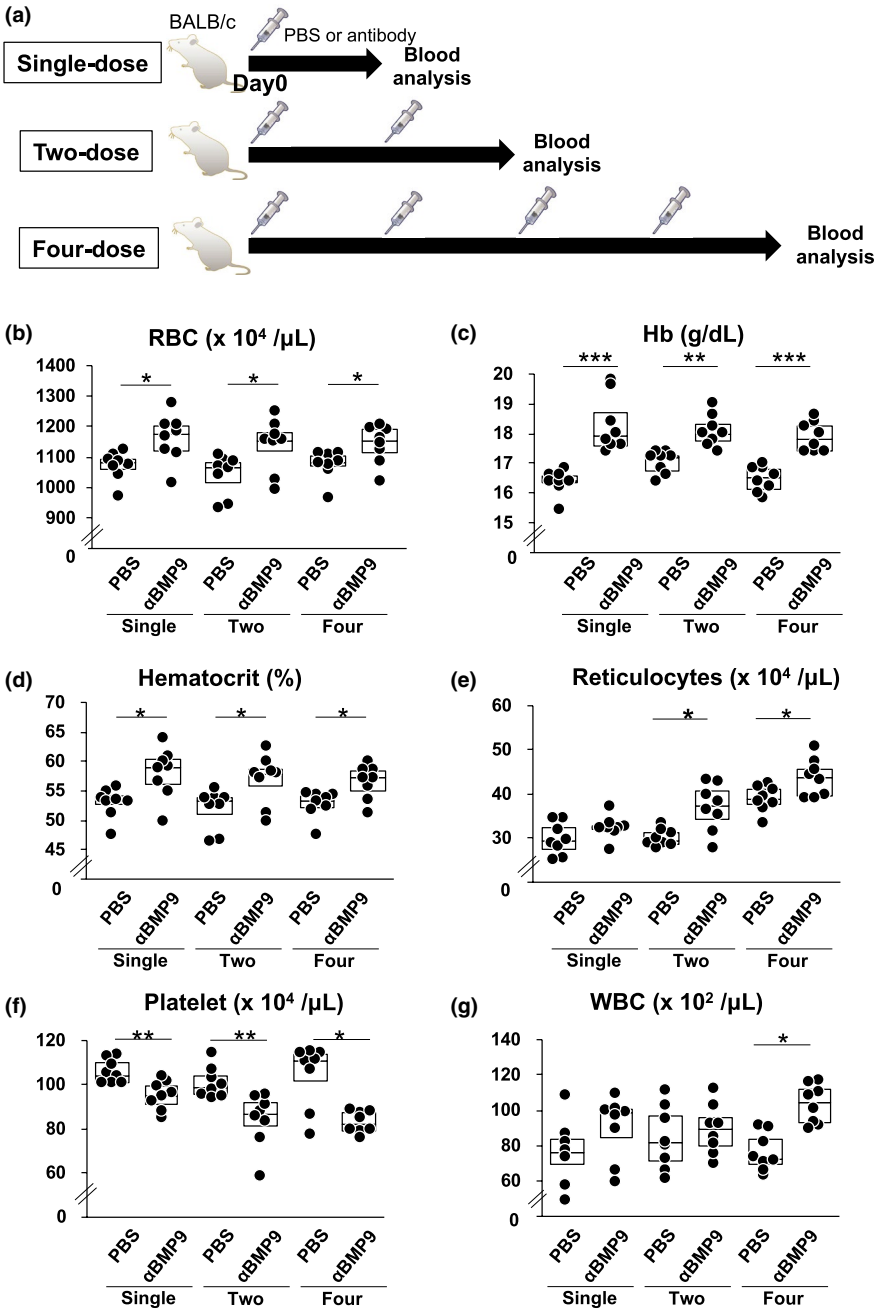

FIGURE 3 Changes in the hematopoietic parameters of BALB/c mice treated with the anti-BMP9-neutralizing antibody. (a) Schematic diagram of the experimental design. The anti-BMP9-neutralizing antibody (or PBS as a control) was subcutaneously injected into BALB/c mice weekly for a total of one, two or four times. Eight mice were used in each group. (b–g) Scatter plots and box plots showing the upper quartile, median value and lower quantile of hematopoietic parameters. The RBC count (B), Hb concentration (c), hematocrit value (d), reticulocyte count (e), platelet count (f) and WBC count (g) are shown. *$p < .05$, **$p < .01$, and ***$p < .001$ versus the corresponding control group; Wilcoxon rank sum test

region responsible for EPO production in the kidneys (Hirano et al., 2017). As the antibody was more effective on the BALB/c genetic background than on the C57BL/6 background for an unknown reason (Figure S1), AnRED mice on the C57BL/6 background were backcrossed 5 times with the BALB/c strain, and heterozygous offsprings were intercrossed to generate AnRED mice. Littermates carrying heterozygous *Epo* knockout alleles and/or transgenes were used as controls and showed no anemia, with a normal range of EPO levels (Hirano et al., 2017). Twenty-four AnRED and 14 control mice aged 11–43 weeks were used for further experiments.

The neutralizing anti-BMP9 antibody or an equal volume of PBS was injected intraperitoneally into AnRED and control mice once per week for four weeks. Seven days after the last injection, blood samples were collected from submandibular veins under anesthesia and subsequently killed to harvest bone marrow cells and kidneys. No significant change in the RBC count, Hb concentration or hematocrit value was observed after antibody treatment in either control or AnRED mice compared with the corresponding control mice by the Wilcoxon rank sum test (Figure 5a–c). AnRED mice showed severe anemia, and we could not observe improvement in anemia after anti-BMP9 antibody treatment (Figure 5a–c). Nevertheless, although no significant difference was found by Wilcoxon rank sum test, the median value of the reticulocyte count was found to be increased in the group of control mice treated with the antibody compared

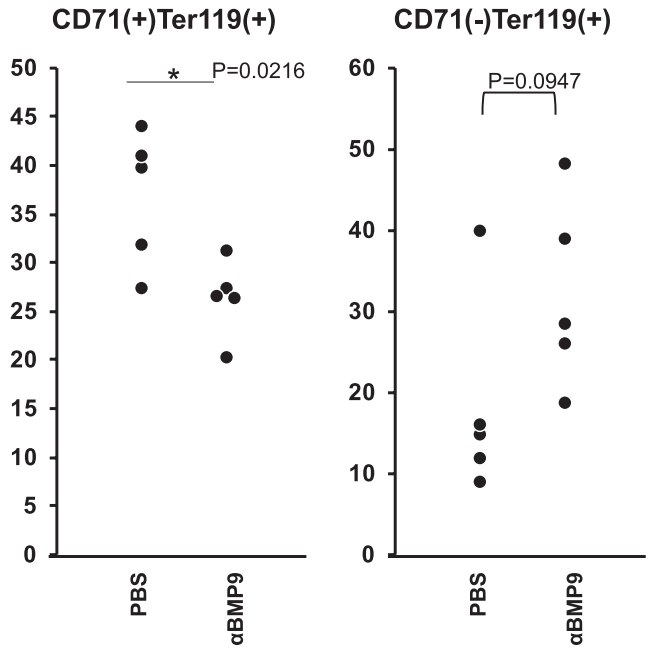

FIGURE 4 Flow cytometry analysis of erythroid lineage cells in BALB/c mice. The frequencies of CD71(+)Ter119(+) (left) and CD71(−)Ter119(+) (right) cells in mice that received four weekly administrations of the anti-BMP9 antibody are shown. *p*-values versus the corresponding vehicle group by Wilcoxon rank sum test are indicated

to the group of parental control mice ($p < .02$), suggesting that backcrossing 5 times could partially overcome the differences between the BALB/c and C57BL/6 strains (Figure 5d). The median reticulocyte count in the AnRED group did not show any significant difference.

Good agreement with the findings obtained in BALB/c mice, the frequency of CD71(+)Ter119(+) cells in the bone marrow was decreased, while that of CD71(−)Ter119(+) cells was elevated in control mice after treatment with the antibody. However, this change was not clear in AnRED mice (Figure 5e,f). These findings indicate that the anti-BMP9 antibody could promote erythroid differentiation in the control mice but not in AnRED mice.

2.5 | BMP9 neutralization does not elevate EPO production in the kidneys

AnRED mice have less responsiveness to the anti-BMP9 antibody. We analyzed green fluorescence protein (*Gfp*) reporter gene activity in AnRED mice. As the *Epo* knockout allele was constructed by insertion of *Gfp* cDNA into the *Epo* gene and the *Gfp* gene was transcribed from the *Epo* promoter (Figure 6a), the actual transcriptional condition of the *Epo* gene could be monitored by *Gfp* reporter gene activity in AnRED mice (Hirano et al., 2017; Yamazaki et al., 2021). We used littermates carrying the heterozygous *Epo* knockout/*Gfp* knockin allele as controls. We found that *Gfp* expression under *Epo* gene regulation was not changed by anti-BMP9 antibody treatment in the control mice (Figure 6b). A trace level of *Gfp* expression was detected in only one of 7 control mice and was not detected in the remaining 6 mice, presumably because EPO production may not be accelerated under steady-state conditions in the control mice. As *Gfp* expression under *Epo* gene regulation is accelerated in AnRED mice due to anemia, it should be appreciated that the level of *Gfp* expression was generally increased in AnRED mice compared with that in control mice regardless of whether anti-BMP9 antibody was administered. We found that the anti-BMP9 antibody did not activate *Gfp* expression in AnRED mice (Figure 6b). We further confirmed that the serum EPO level and expression of the *Epo* gene in the kidneys of BALB/c mice were not influenced by anti-BMP9 antibody treatment (Figure S2). Thus, the neutralizing BMP9 signal does not activate *Epo* gene expression in the kidneys at steady-state and even under anemic conditions.

It is interesting to note that the expression of the *Gdf2* gene (encoding BMP9) was significantly decreased in the livers of steady-state AnRED mice. We also found that *Gdf2* gene expression was not influenced by administration of the anti-BMP9 antibody regardless of the genotype (Figure 6c). We envisage that *Gdf2* gene expression might be restrained in AnRED mice to avoid worsening the anemic situation. Overall, we envisage that BMP9 neutralization is less effective for promoting erythropoiesis in AnRED mice, probably at least in part, due to reduced circulating BMP9 levels.

2.6 | BMP9 hampers EPO/SCF-mediated expansion of primary erythroid progenitors

We next analyzed whether BMP9 directly influences hematopoietic progenitor cells. To this end, we developed a strategy of in vitro erythroid differentiation from primary mouse bone marrow LK cells. Consistent with our result showing that cells in the CFU-E and preCFU-E fractions are bound to BMP9 (Figure 2d), we speculated that those cells may be physiologically influenced by BMP9.

We incubated 1.8×10^5 LK cells in StemPro-34 medium supplemented with stem cell factor (SCF) and EPO at concentrations of 20 ng/ml and 10 ng/ml, respectively, in the absence or presence of BMP9 at concentrations of either 10 or 50 ng/ml. After 72 hr of incubation, the cells were analyzed by flow cytometry. The surviving cells were decreased to approximately one-third in number during ex vivo culturing, and BMP9 treatment worsened survival in a dose-dependent manner (Figure 7a).

We then analyzed the differentiation status of the surviving cells. Most cells were differentiated toward Lin-positive mature cells by 72 hr of culture in the presence of SCF and

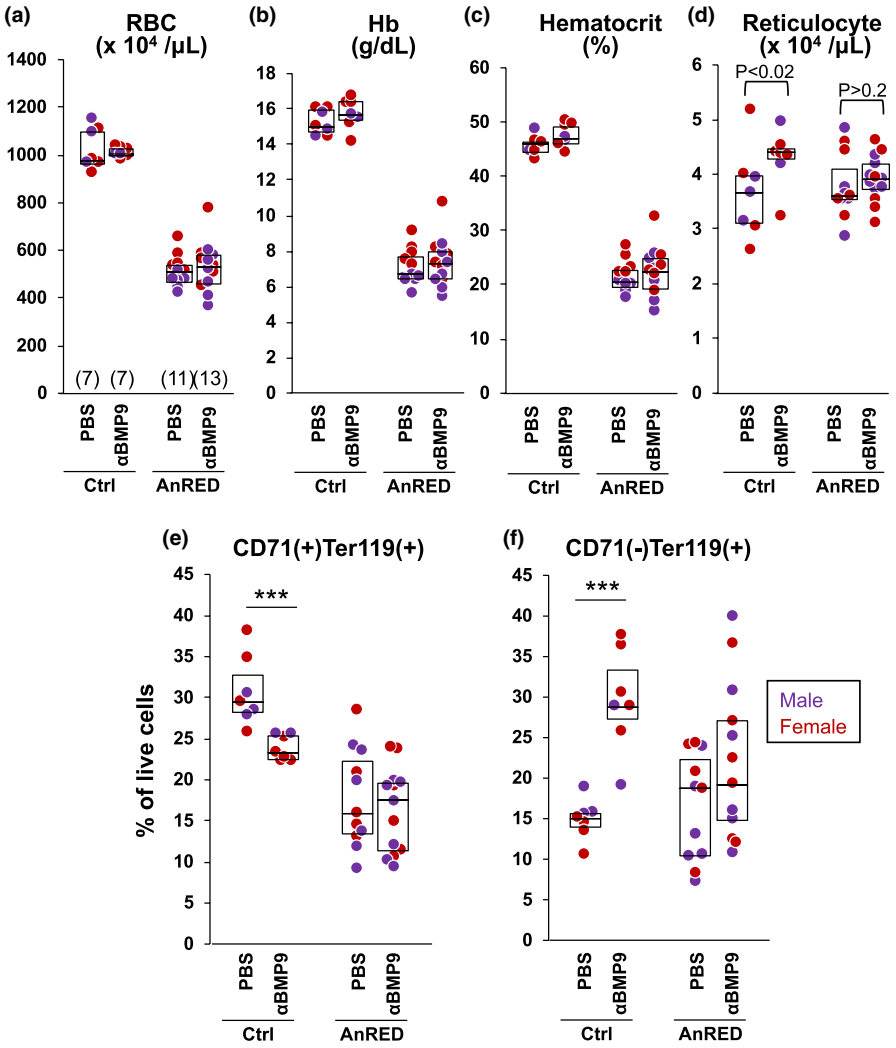

FIGURE 5 The anti-BMP9-neutralizing antibody does not work in AnRED mice. (a–d) Scatter plots and box plots showing the upper quartile, median value and lower quantile of hematopoietic parameters. The RBC count (a), Hb concentration (b), hematocrit value (c) and reticulocyte count (d) are shown. (e, f) Scatter plots and box plots showing the upper quartile, median value and lower quantile of the erythroblast proportion in the bone marrow. The frequencies of CD71(+) Ter119(+) (e) and CD71(−)Ter119(+) (f) cells are shown. Red and purple dots indicate female and male mice, respectively. The number of mice used in each group is shown in parentheses in (a). ***$p < .001$ versus the corresponding vehicle group; Wilcoxon rank sum test. p-values for median test are described in (d)

EPO, while cells that retained the features of LK cells were present (Figure 7b). The absolute number of LK cells gated on Sca1(−)IL7Rα(−)FcγRII/III(−)CD41(−) was significantly reduced by BMP9 treatment (Figure 7c). Furthermore, most of the Lin(−)Sca1(−)IL7Rα(−)FcγRII/III(−)cKit(+)CD41(−) cells were endoglin-positive after cultivation with SCF and EPO, and these cells were defined as either CFU-E or preCFU-E (Figure 7d left), suggesting that erythroid progenitors were mainly sustained under culture conditions with EPO and SCF. Notably, the frequencies of CFU-E and preCFU-E were significantly decreased by BMP9 treatment (Figure 7d right and 7e). In stark contrast, the frequencies of preMegE and preGM were increased (Figure 7f). These findings indicate that BMP9 disturbs the SCF/EPO-mediated function of sustaining erythroid progenitors.

3 | DISCUSSION

BMP9 is a member of the TGFβ superfamily and is subcategorized into BMP/GDF subfamilies along with BMP2 and BMP4 based on phylogenetic analysis and sequence similarities. Given that BMP9 is one of the most physiological ligands of endoglin in endothelial cells (Alt et al., 2012; Castonguay et al., 2011), we hypothesized that BMP9 may act as a regulator of erythropoiesis in endoglin-positive erythroid cells. To address this hypothesis, in this study, we prepared an anti-BMP9 antibody that interferes with the binding of BMP9 to endoglin and discovered that the antibody enhanced erythropoiesis in mice. We also found that BMP9 specifically bound to endoglin-expressing hematopoietic cells and disturbed the expansion of primary erythroid precursors in the EPO/SCF-mediated in vitro differentiation system. We conclude that BMP9 is a principal ligand of endoglin signaling that controls the excess production of erythroid progenitors. Thus, BMP9 represses erythroid differentiation through its interaction with endoglin, which counteracts the function of BMP2 and BMP4 in the erythroid lineage.

Endoglin is a transmembrane glycoprotein that is located on the cell surface and functions as a coreceptor for several members of the TGFβ superfamily (Cheifetz et al., 1992; Lastres et al., 1996; Zhang et al., 1996). In endothelial cells, endoglin plays an important role in balancing the TGFβ signaling pathway by regulating the formation of the

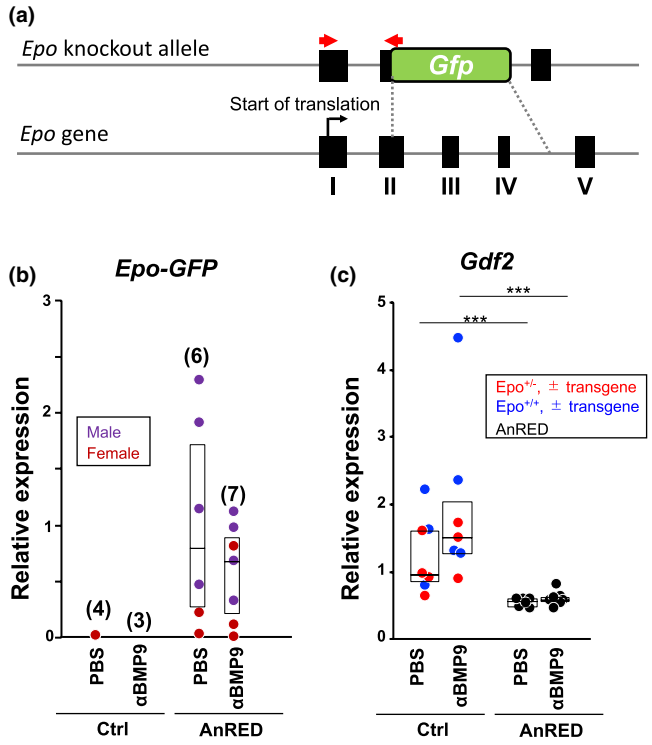

FIGURE 6 The anti-BMP9 antibody does not activate *Epo* gene expression in the kidney. (a) The structure of the mouse *Epo* knockout allele. The exon/intron structure of *Epo* gene is shown. Relative positions of the PCR primers used for detection of Epo gene expression are depicted by red arrows. (b) Quantitative analysis of *Gfp* expression under regulation of the *Epo* gene. The *Y*-axis shows the relative expression of *Gfp* compared to *Hprt*. *Gfp* expression was not determined in six out of 7 control mice. Red and purple dots indicate female and male mice, respectively. The numbers of mice analyzed are shown in parentheses. (c) Scatter plots and box plots showing the upper quartile, median value and lower quantile of *Gdf2* expression in the livers. The *Y*-axis shows the relative expression of *Gdf2* compared to *Hprt*. Red, blue and black dots indicate mice with heterozygous *Epo* gene deletion with or without *Epo*-expressing BAC transgene, mice wild type for *Epo* gene locus with or without *Epo*-expressing BAC transgene and AnRED mice, respectively. ***$p < .005$ versus the corresponding control group; Wilcoxon rank sum test

heterotetrameric receptor complex of TGFβ type I and type II receptors (Pomeraniec et al., 2015). TGF-β1, BMP2 and BMP4 promote the hematopoietic commitment of endoglin-positive mesodermal cells in an early developmental stage (Borges et al., 2012), indicating that endoglin-regulated erythroid differentiation certainly exists. However, the function of endoglin in erythropoiesis remains controversial, with one study reporting that endoglin-depleted bone marrow hematopoietic stem cells acquired more clonogenic capacity of burst-forming unit-erythroid (BFU-E) (Moody et al., 2007) and other reports showing that BFU-E formation was significantly impaired in embryonic stem (ES) cells and yolk sacs derived from endoglin-knockout embryos (Borges et al., 2012; Zhang et al., 2011). Notably, the reduced erythroid clonogenic capacity of endoglin-knockout ES cells was rescued by overexpression of ALK1 (Zhang et al., 2011), suggesting that loss of endoglin could be compensated by overactivation of the TGFβ superfamily pathway. Indeed, conditional depletion of endoglin did not interfere with erythropoiesis in mice (Borges et al., 2019), probably due to the compensatory effect of the TGFβ superfamily pathway that is not involved in endoglin function in vivo. We surmised that the roles of endoglin in erythropoiesis seem to be limited to enhancing the TGFβ superfamily pathway via the physiological expression level of the endoglin ligand in animals.

Notably, BMP9 signaling-specific inhibition of endoglin function successfully promoted erythropoiesis in mice. To the best of our knowledge, this is the first observation that shows the discrepancy in the hematopoietic phenotype between pan-endoglin dysfunction and single ligand-specific endoglin dysfunction. The importance of BMP9 in endoglin signaling in the vascular system has been well documented. A specific antibody for endoglin, which interferes with BMP9 binding to endoglin but does not alter TGF-β1/endoglin signaling, blocks endothelial tube formation (Nolan-Stevaux et al., 2012). We showed here that immunopharmacological inhibition of BMP9/endoglin function could not be compensated under physiological conditions in mice, unlike the eradication of pan-endoglin function, as previously reported (Borges et al., 2019). We imagined that specific disruption of the BMP9-endoglin interaction may disharmonize the TGFβ superfamily pathway through endoglin of erythroid progenitors, presumably by disrupting the balance between BMP9 and TGF-β1-mediated ALK1/Smad1/5/8 and TGF-β1-mediated ALK5/Smad2/3 pathways (Lebrin et al., 2004; Pomeraniec et al., 2015).

The second important observation is that induction of erythropoiesis after neutralizing BMP9/endoglin signaling is not observed in AnRED mice, which are severely anemic mice due to EPO deficiency (Hirano et al., 2017). One plausible explanation for this observation is that BMP9 signaling has already been restrained in AnRED mice due to severe anemia. Previous work demonstrated that blockade of TGF-β1 signaling increases the pool of erythroid progenitors independent of EPO; however, abnormally expanded erythroid progenitors are prone to apoptosis and cannot differentiate into mature erythrocytes in the absence of EPO (Di Giandomenico et al., 2020). Although current data have not yet clarified how EPO is involved in BMP9/endoglin-mediated erythropoiesis, we envisage that EPO is required for the appropriate function of BMP9 in maintaining erythroid homeostasis in vivo.

To date, several TGFβ ligand trap agents are in clinical trials (Abou-Alfa et al., 2019; Cappellini et al., 2019, 2020; Strauss et al., 2020) or may be in a development stage, aiming for therapeutic effects by modifying TGFβ signaling. The TGFβ ligand trap is a soluble recombinant extracellular

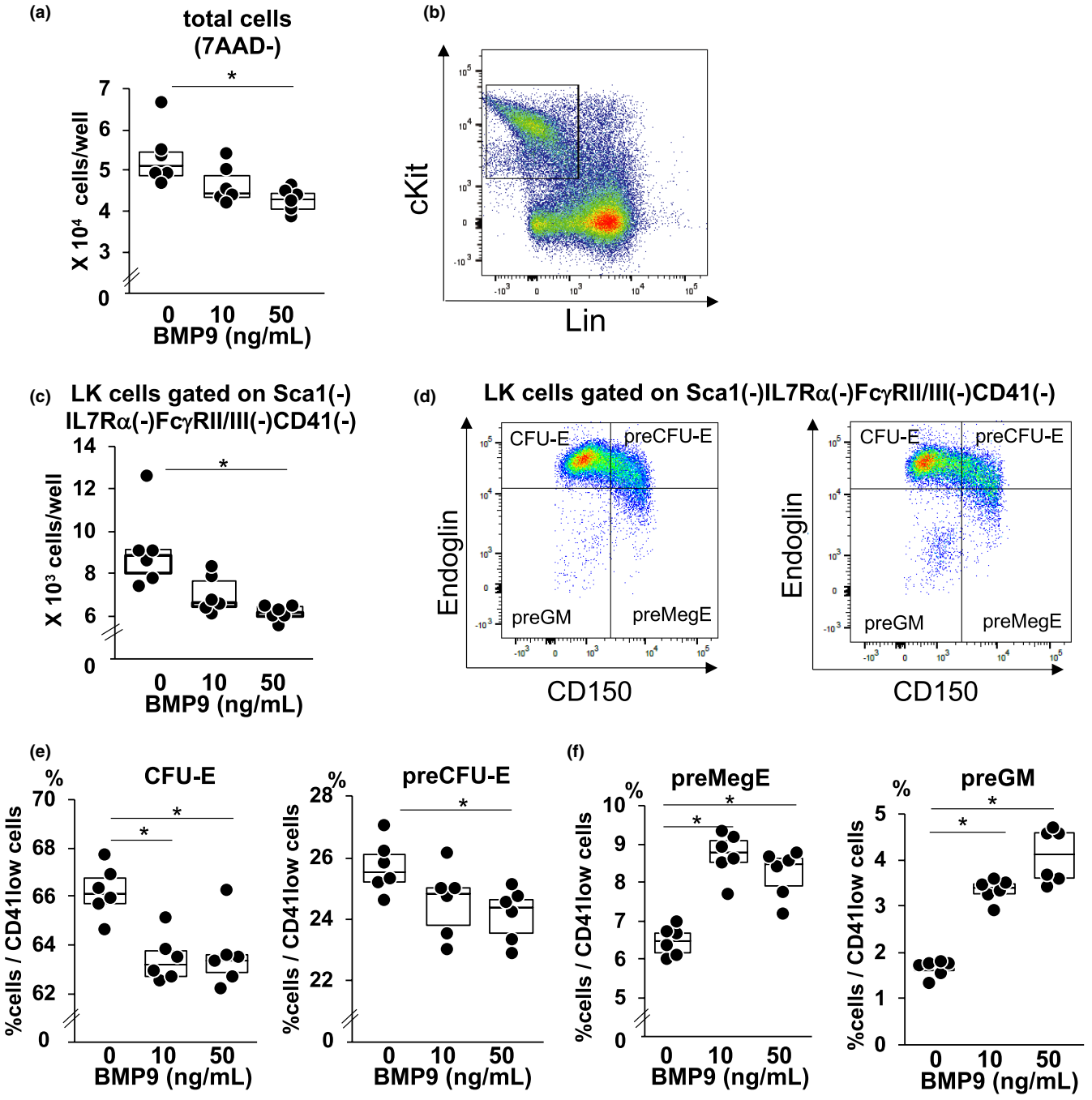

FIGURE 7 BMP9 attenuates EPO/SCF-mediated expansion of erythroid progenitors. (a) Number of surviving cells after culturing for 72 hr with EPO and SCF. Dead cells were determined using 7-AAD staining. $n = 6$ in each group. (b) Representative scatter plot image of the expression of cKit and Lin markers on surviving cells after culture for 72 hr with EPO and SCF. (c) Number of surviving cells retaining the features of LK cells gated on Sca1(−)IL7RαFcγRII/III(−)CD41(−) after a 72-hr culture. $n = 6$ in each group. (d) Representative endoglin and CD150 expression profile of surviving cultured cells treated with EPO and SCF with (right) or without (left) 50 ng/ml BMP9 gated on Lin(−)cKit(+)Sca1(−)IL7Rα(−)FcγRII/III(−))CD41(−). (e and f) Comparison of the frequencies of CFU-E and preCFU-E (e) and preMegE and preGM (f) cells gated on the Lin(−)cKit(+)Sca1(−)IL7Rα(−)FcγRII/III(−))CD41(−) fraction. Results obtained after culturing the cells for 72 hr with EPO and SCF in the absence or presence of BMP9. *$p < .05$ versus the absence of BMP9 by the Steel test. The representative dot plots and box plots showing the upper quartile, median value and lower quantile of three independent experiments are shown in (a), (c), (e) and (f)

domain of the TGFβ receptor of interest, which is fused with the human immunoglobulin Fc region (Komesli et al., 1998). This chimeric molecule can effectively trap the ligand and work as a TGFβ antagonist (Komesli et al., 1998). In particular, ActRIIA-Fc and ActRIIB-Fc, which contain an extracellular domain from one of two types of activin type II receptors, ActRIIA and ActRIIB, respectively, are expected to be influential erythropoiesis-stimulating agents that promote

late-stage erythroid differentiation and maturation in patients with anemia (Cappellini et al., 2019, 2020), suggesting that erythropoiesis may be stimulated by trapping TGFβ ligands that specifically interact with activin type II receptors. Upton and colleagues have shown that ActRII and BMPRII have redundant functions in Smad1/5 phosphorylation mediated by BMP9/ALK1, while ActRII plays greater roles in Smad2 phosphorylation in BMP9/ALK1 signaling in pulmonary endothelial cells (Upton et al., 2009). An antagonistic effect of ActRIIA-Fc and ActRIIB-Fc against BMP9 may contribute to the efficacy of these drugs.

It has been reported that endoglin-positive erythroblasts are increased in patients with myelodysplastic syndrome (MDS), while only a few cells appear in healthy controls (Della Porta et al., 2006; Xu et al., 2012). Furthermore, the expression level of endoglin has been reported to be correlated with the severity of erythroid dysplasia in MDS cases (Della Porta et al., 2006). It is well known that ineffective erythropoiesis is one of the common pathogenic features of MDS, which may arise via differentiation blockade and induced apoptosis in hematopoietic progenitor cells (Claessens et al., 2002). As BMP9 is a strong ligand for endoglin (Castonguay et al., 2011), it is plausible that inadequate erythropoiesis due to imbalanced BMP9/endoglin signaling is partly responsible for the pathogenesis of MDS. Because BMP9-endoglin signaling is vital for a variety of biological processes, such as maintaining endothelial quiescence and promoting osteogenic differentiation (David et al., 2008; Kang et al., 2004; Xiang et al., 2012), blockade of BMP9 function in the body may lead to systemic changes. Indeed, we observed an increase in RBC count unaccompanied by changes in reticulocyte count in mice treated with the anti-BMP9 antibody, probably via increased vascular permeability. We also observed that the mice treated with the anti-BMP9 antibody showed thrombocytopenia, which might be relevant to the endoglin expressed in murine megakaryocyte progenitors. Nonetheless, we envisage that BMP9 neutralization may be a therapeutic strategy targeting MDS cells expressing endoglin at pathologically high levels.

4 | EXPERIMENTAL PROCEDURES

4.1 | Animal studies

BALB/c and C57BL/6N mice were purchased from Charles River Laboratories, Japan, and maintained under pathogen-free conditions at Kyowa Kirin Co., Ltd. AnRED mice were maintained at Tohoku University. Hematopoietic indices were examined using an Advia 120 Automated Hematology Analyzer (Bayer) or Hemocytometer (Nihon Koden). For measuring serum erythropoietin concentration, Quantikine Mouse/rat Erythropoietin Immunoassay (R&D Systems) was used according to the manufacturer's protocol. Serum samples were diluted twice with the provided buffer prior to the assay. The absorbance was recorded at 450 nm wavelength with a reference at 570 nm using an ELISA plate reader (Parkin Elmer). All experiments were performed in accordance with the Standards for Proper Conduct of Animal Experiments at Kyowa Kirin Co., Ltd., under the approval of the company's Institutional Animal Care and Use Committee or the Regulations for Animal Experiments and Related Activities of Tohoku University. Tokyo Research Park of Kyowa Kirin Co., Ltd., is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

4.2 | Preparation of the anti-BMP9 antibody

The anti-BMP9 antibody is a mouse monoclonal antibody prepared in BMP9 knockout mice immunized with the complex form of the recombinant human BMP9 protein (W.O. Patent No. 2010/126169, 2010) and has been patented (U.S. Patent No. 8,969,040, 2015). Briefly, hybridomas were obtained by fusing the spleen cells of the immunized mouse and Sp2/o mouse myeloma cells (ATCC ID: CRL1581) with polyethylene glycol and maintained in DMEM (Invitrogen) containing 10% FBS, 50 μmol/L β-mercaptoethanol, 50 μg/ml insulin, 10 ng/ml IL-6 and HAT media supplement (Sigma). The first screening of hybridoma cells was performed by enzyme-linked immune sorbent assay (ELISA). Microplates for ELISAs were prepared by coating with 1 μg/ml human BMP9 recombinant protein (R&D Systems) in 50 mmol/L $NaHCO_3$ buffer (Wako) for 18 hr at 4°C and subsequent blocking with SuperBlock (Thermo Fisher Scientific) for 1 hr at room temperature. Commercially available anti-BMP9 antibody (R&D Systems) and hybridoma culture medium were used as positive and negative controls, respectively. The selected hybridomas were monocloned by the limiting dilution method, and subsequently, a second ELISA screening was performed to obtain clonal lines of hybridomas secreting highly reactive monoclonal antibodies to BMP9. A hybridoma clone producing the neutralizing antibody against BMP9 was selected using the reporter assay described below. Upon the use of the antibody, the selected hybridoma clone was suspended in e-RDF medium (Kyokuto Pharmaceutical Industrial) containing 1% Ultra-Low IgG FBS (GIBCO), 5 μg/ml transferrin, 5 μg/ml insulin, 10 mmol/L ethanolamine and 25 nmol/L sodium selenite. After cultivating the hybridoma cells for 6–8 days, the culture supernatant was filtered using an open column packed with Protein G Sepharose 4 Fast Flow (GE Healthcare) and sterilized using a 0.22 μm filter. The concentration of purified antibody was determined by measuring the absorbance at a wavelength of 280 nm.

4.3 | Reporter assay

The ALK1-expressing HepG2 reporter cell line, in which the luciferase protein is produced under the regulation of the Smad binding motif GCCGCCGC repeated 12 times (Kusanagi et al., 2000; U.S. Patent No. 8,969,040, 2015), was suspended in DMEM (Invitrogen) containing 10% FBS in a 96-well plate at a density of 1.5 x 10^4/well. After 24 hr of incubation, the cells were washed with serum-free DMEM and then reincubated with 10 ng/ml human BMP9 recombinant protein (R&D Systems) together with anti-BMP9 antibody at concentrations of 0, 10, 30, 100, 300 or 1,000 ng/ml in DMEM containing 0.1% bovine serum albumin (BSA). Six hours later, a chemiluminescent reagent (Promega) was added to determine luciferase activity. DMEM containing 0.1% BSA was used as a negative control.

4.4 | Preparation of endoglin-transfected cells

Full-length human endoglin (Ref-Seq ID: NM_000118.3) cDNA was amplified from human lung cDNA (Clontech, cat# 637206) using the primer set ENG-Fw (CTAGCCTCGAGAATTCACCATGGACCGCGGC ACGCTCCCTCTG) and ENG-Rv (TAAAGGGAAG CGGCCGCTCACTGTGGGGGCCTGGGGTACTCAC) and subsequently cloned into the pCI-neo vector (Promega). The pCI-neo-endoglin plasmid DNA and 293fectin (Thermo Fischer Scientific) were reacted according to the manufacturer's protocol, and the complexes were added to FreeStyle 293F cells (293F cells, Thermo Fischer Scientific) at 1×10^6 cells/ml. The 293F cells were then cultured in FreeStyle 293F Expression medium (Thermo Fischer Scientific) for 72 hr.

4.5 | Measurement of BMP9-binding activity

To prepare a bio-BMP9 solution reagent, 100 μg of human recombinant complexed BMP9 (W.O. Patent No. 2010/126169, 2010) was biotinylated using biotin-AC_5-OSu (biotin, DOJINDO); subsequently, the buffer of the reaction solution was replaced with 0.5 ml of PBS using Amicon Ultra (0.5 ml, 10 K, Millipore). For the competitive assay, the bio-BMP9 solution was preincubated with the anti-BMP9 antibody at a concentration of 10 μg/ml for 15 min at room temperature. The cells were incubated with one-twentieth volume of bio-BMP9 with or without preincubation with the anti-BMP9 antibody at 4°C for 30 min and washed twice with ice-cold PBS. Cells bound to bio-BMP9 were detected by APC-labeled streptavidin (BD Biosciences, cat# 554067) using a flow cytometer (FACSCanto II, BD Biosciences).

4.6 | Flow cytometry analyses

Bone marrow cell culture was performed using StemPro-34 medium (Thermo Fisher Scientific) with 10 ng/ml mouse EPO (R&D Systems) and 20 ng/ml mouse SCF (R&D Systems) for 3 days. LK cells were prepared by a two-step strategy of depleting Lin-positive cells from bone marrow cells using a Lineage Cell Depletion Kit (Miltenyi, cat# 130-090-858) followed by isolating cKit-positive cells using cKit-tagged magnetic beads (Miltenyi, cat# 130-091-224) according to the manufacturer's protocols. Fresh bone marrow cells, cultured cells and isolated LK cells were suspended in autoMACS Running Buffer (Miltenyi Biotec) or PBS containing BSA, EDTA and azide, incubated with an antibody against FITC-conjugated (BioLegend, cat# 101306) or unlabeled (BD Biosciences, cat# 553142) FcγRII/III for 30 min on ice and further incubated with antibodies against FITC-conjugated Lin (BD Biosciences, cat# 340546), FITC-conjugated Sca1 (BioLegend, cat# 108106), FITC-conjugated IL7Rα (BioLegend, cat# 135018), APC/Cy7-conjugated cKit (BD Biosciences, cat# 105825), BV421-conjugated CD150 (BD Biosciences, cat# 562811), PE/Cy7-conjugated CD41 (BioLegend, cat# 133916), PE-conjugated endoglin (CD105) (Miltenyi Biotec, cat# 130-092-924) for 15 min on ice. Antibodies against APC-conjugated CD71 (BioLegend, cat# 11319) and BV510-conjugated Ter119 (BioLegend, cat# 116237) were used occasionally. The cells were washed twice with autoMACS Running Buffer or PBS, and samples were analyzed on a FACSCanto II or FACSVerse. Dead cells in the samples were excluded by costaining with 7-AAD (BD Biosciences). Absolute numbers of live and dead cells among cultured cells were determined with CountBright Absolute Counting Beads (Thermo Fisher Scientific).

4.7 | Quantitative reverse transcription PCR

The protocol for quantitative reverse transcription polymerase chain reaction (qRT-PCR) was described previously. Briefly, total tissue RNA from kidneys and livers was extracted using ISOGEN (NIPPON GENE), and cDNA was subsequently synthesized with ReverTra Ace® qPCR RT Master Mix (TOYOBO). qRT-PCR was performed using an ABI7300, ABI7500, QuantStudio 6, QuantStudio 12k Flex system or StepOnePlus system (Applied Biosystems) with target-specific primers (GFPEpo-Fw: GAAGACTTGCAGCGTGGACA, GFPEpo-Rv: GGTGGATCCTAAAGCAGCAG; Gdf2-Fw: AG ATACACAACGGACAAATCGTC, Gdf2-Rv: GGCAGCT GTCGATATAGCATC) and Power SYBR green PCR master mix (Applied Biosystems) or Thunderbird qPCR mix (TOYOBO). The expression level of *Epo* was determined with primers and probes (Epo-Fw: CATCTGCGACAGTCGA GTTCTG, Epo-Rv: CACAACCCATCGTGACATTTTC,

Epo-probe: GTACATCTTAGAGGCCAAGGAG) and qPCR Mastermix (NIPPON GENE). The expression levels of *Hprt* determined with primers (Hprt-Fw: GTTGGATA CAGGCCAGACTTTGT, Hprt-Rv: CCACAGGACTAGAA CACCTGC) were used as internal standards.

4.8 | Statistical analysis

The results obtained from in vivo experiments were compared by the Wilcoxon rank sum test or median test for between-group comparisons, and responses to multiple BMP9 doses in liquid culture of bone marrow cells were statistically analyzed by the Steel test for multiple comparisons using SAS9.4 or JMPpro software (SAS Institute). Differences between experimental groups were considered to be significant for values of $p < .05$.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Yuji Yamazaki for helpful discussions, Dr. Shinya Ogawa for plasmid construction, and Waka Haruyama, Yuki Tanbo, Hiroe Toki, Kimika Iwai and Tomoko Haba for helping collect *in vivo* data.

CONFLICT OF INTEREST

Ayami Yamaguchi, Kiyoshi Shimizu, Hiroyuki Ariyama, Kengo Yamawaki and Kenji Nagao are employees of Kyowa Kirin Co., Ltd.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Yamaguchi, A., Hirano, I., Narusawa, S., Shimizu, K., Ariyama, H., Yamawaki, K., Nagao, K., Yamamoto, M., & Shimizu, R. (2021). Blockade of the interaction between BMP9 and endoglin on erythroid progenitors promotes erythropoiesis in mice. *Genes to Cells*, 26, 782–797. https://doi.org/10.1111/gtc.12887