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Enhanced Angpt1/Tie2 signaling affects the differentiation and long-term repopulation ability of hematopoietic stem cells

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ABSTRACT

Angiopoietin-1 (Angpt1) signaling via the Tie2 receptor regulates vascular and hematopoietic systems. To investigate the role of Angpt1-Tie2 signaling in hematopoiesis, we prepared conditionally inducible transgenic (*Tg*) mice expressing a genetically engineered Angpt1, cartridge oligomeric matrix protein (COMP)-Angpt1. The effects of COMP-Angpt1 overexpression in osteoblasts on hematopoiesis were then investigated by crossing COMP-Angpt1 *Tg* mice with Col1a1-Cre *Tg* mice. Interestingly, peripheral blood analyses showed that 4 week (wk)-old (but not 8 wk-old) Col1a1-Cre+/COMP-Angpt1+ mice had a lower percentage of circulating B cells and a higher percentage of myeloid cells than Col1a1-Cre−/COMP-Angpt1+ (control) mice. Although there were no significant differences in the immunophenotypic hematopoietic stem and progenitor cell (HSPC) populations between Col1a1-Cre+/COMP-Angpt1+ and control mice, lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells isolated from 8 wk-old Col1a1-Cre+/COMP-Angpt1+ mice showed better long-term bone marrow reconstitution ability. These data indicate that Angpt1-Tie2 signaling affects the differentiation capacity of hematopoietic lineages during development and increases the stem cell activity of HSCs.

1. Introduction

Angiopoietin-1 (Angpt1) signaling via the Tie2 receptor contributes to both angiogenesis and hematopoiesis throughout development and in adulthood [1,2]. Four angiopoietins (Angpt1–4) have been identified as ligands of Tie2 [3–8], and Angpt1/Tie2 signaling has been widely studied. Moreover, both Angpt1- and Tie2-deficient mice die at mid-gestation due to vascular remodeling defects in midgestation [7,9,10]. Those embryos also have defects in heart. On the other hand, a conditional knockout of Angpt1 confirmed that Angpt1 is crucial for regulating both number and diameter of developing vessels but is dispensable for angiogenesis after E13.5 [11].

We previously reported that Angpt1-Tie2 signaling is critical not only for angiogenesis, but also for hematopoiesis [12,13]. Angpt1-Tie2 signaling between long-term hematopoietic stem cells (LT-HSCs) and the bone marrow (BM) niche facilitates the adhesion of LT-HSCs to the niche and contributes to their maintenance. It is reported that systemic administration, or skin-specific overexpression, of Angpt1 results in the formation of larger, more numerous and more highly branched vessels; however, these vessels show reduced permeability, possibly due to the aberrantly promoted integration of endothelial cells during vasculogenesis [14–16]. Unfortunately, no gain-of-function studies have been conducted to address the endpoints of Angpt1-Tie2 signaling in hematopoiesis.

In this study, we overexpressed COMP-Angpt1, a potent chimeric Angpt1 [17,18], in a transgenic mouse model in which COMP-Angpt1 expression was promoted by Cre-induced loxP recombination [19]. Using this system, we generated CAG-Cre+/COMP-Angpt1+, Mx1-Cre+/COMP-Angpt1+, and Col1a1-Cre+/COMP-Angpt1+ mice and investigated the role of Angpt1-Tie2 signaling in regulating hematopoiesis. We show that CAG-Cre+/COMP-Angpt1+ mice were embryonic lethal, with vascular malformations, whereas Mx1-Cre+/COMP-Angpt1+ mice were 70% lethal after poly I:C

Abbreviations: Angpt1, angiopoietin1; Tie2, tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2.

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injection. Hematopoietic analyses performed on peripheral blood (PB) from Col1a1-Cre+/COMP-Angpt1+ mice also showed that Angpt1 overexpression in osteoblasts induced lineage bias. On the other hand, HSCs isolated from Col1a1-Cre+/COMP-Angpt1+ mice displayed a more quiescent population and showed better long-term reconstitution (LTR) activity than the control. These data suggest that Angpt1-Tie2 signaling is crucial for the regulation of hematopoietic lineage differentiation and the function of LT-HSCs.

2. Methods

2.1. Mice

C57BL/6 mice were purchased from Japan SLC. Conditional *Tg* mice were created by expressing a potent form of Angpt1 protein (COMP-Angpt1) under the control of Cre recombinase [19]. Col1a1-Cre, CAG-Cre and Mx1-Cre *Tg* mice were used for COMP-Angpt1 overexpression studies. 300 μg poly I:C (Sigma–Aldrich) was injected intraperitoneally (three times every other day) to induce Mx1-Cre expression at 5–19 wk-of-age. Animals were cared for in accordance with guidelines prescribed by the Keio University School of Medicine, and an internal ethical review board approved all of the experiments involving animals.

2.2. Antibodies

The antibodies (Abs) used in this study are listed in Supplementary data.

2.3. Cell preparation and analyses

Methods describing the preparation of BM cells and the immunostaining of all other cells for flow cytometric analysis have been published previously [20]. Procedure for the Ki67 staining was described previously [21]. Fluorescently-stained cells were sorted and analyzed using FACSVantage DIVA and FACSAria flow cytometers (BD Biosciences).

2.4. Real time quantitative PCR (Q-PCR) array analysis

RNA was obtained from LSK cells isolated from Col1a1-Cre−/COMP-Angpt1+ and Col1a1-Cre+/COMP-Angpt1+ mice. RNA was reverse transcribed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Procedures for Q-PCR array and data analyses have been described previously [22]. The *Taq*Man Gene Expression Assay Mixes are listed in Supplementary Table 1.

2.5. Immunoblot analysis

To estimate the amount of COMP-Angpt1 protein in mouse serum, PB was harvested from control and Mx1-Cre+/COMP-Angpt1+ mice at 5–9 wk-of-age. After injection of poly I:C (300 μg, Sigma–Aldrich), sera were collected and subsequently analyzed by SDS–PAGE and immunoblotting with a monoclonal anti-FLAG antibody (clone M1, 1:1000, Sigma–Aldrich).

2.6. Immunohistochemical analysis of whole-mount embryos

Immunohistochemical analysis of whole-mount embryos was performed with an anti-PECAM monoclonal Ab as previously described [23].

2.7. BM transplantation assay

LSK cells (3×10^3) were isolated from Col1a1-Cre−/COMP-Angpt1+ (control) or Col1a1-Cre+/COMP-Angpt1+ mice and transplanted into lethally-irradiated recipient mice with $Ly5.1^+$ competitor cells (2×10^5). PB was analyzed each month (for up to 4 months) after BMT, and the percentage of $Ly5.2^+$ cells was recorded. The lineage differentiation activity of donor-derived PB cells was analyzed 4 months after BMT.

2.8. Statistical analysis

Statistically significant differences between the groups were determined using a two-tailed Student's *t*-test.

3. Results

3.1. Angpt1 overexpression in osteoblasts affects the differentiation of hematopoietic cells in young mice

To investigate the endpoints of Angpt1 overexpression on the regulation of hematopoiesis, we prepared osteoblast specific COMP-Angpt1 overexpressing mice by crossing COMP-Angpt1 *Tg* mice with Col1a1-Cre *Tg* mice (Col1a1-Cre+/COMP-Angpt1+ *Tg*). While Col1a1-Cre+/COMP-Angpt1+ *Tg* mice weighed less when they were 4 wk-old, the number of white blood cells in the PB and the total number of mononuclear cells (MNCs) within the BM of Col1a1-Cre+/COMP-Angpt1+ mice was comparable with that in age-matched control mice (Fig. 1A–C). Eight week-old Col1a1-Cre+/COMP-Angpt1+ *Tg* mice continued to weigh less and had fewer MNCs in BM compared to the control mouse (Supplementary Fig. S1A–C). Hematopoietic lineage (T, B, and myeloid cells) analyses of the PB revealed that 4 wk-old Col1a1-Cre+/COMP-Angpt1+ *Tg* mice showed a higher percentage of myeloid cells (47.7 ± 10.7% in *Tg* versus 33.6 ± 6.1% in control mice) and a lower percentage of B cells (27.0 ± 7.7% in *Tg* versus 44.8 ± 6.7% in control mice) than Col1a1-Cre−/COMP-Angpt1+ (control) mice (Fig. 1D). This lineage bias was resolved in 8 wk-old mice, in which the frequency of each lineage was comparable between *Tg* and control mice (27.9 ± 2.9% for T cells, 34.4 ± 6.1% for B cells, and 26.9 ± 4.9% for myeloid cells in *Tg* versus 26.3 ± 2.5% for T cells, 41.0 ± 5.5% for B cells, and 23.7 ± 5.7% for myeloid cells in control mice) (Fig. 1E). These results clearly illustrate that hematopoietic differentiation during development can be transiently affected by Angpt1-Tie2.

3.2. Overexpression of Angpt1 in whole body affected the abnormal vascular morphology and induced embryonic lethality

We had hoped to analyze the hematopoietic phenotype of the CAG-Cre+/COMP-Angpt1+ mice; however, CAG-Cre+/COMP-Angpt1+ *Tg* mice were embryonic lethal due to abnormal vasculogenesis, concomitant with the presence of small bodies (Supplementary Fig. S2A). Thus, we had tried to analyze the effects of COMP-Angpt1 overexpression on hematopoiesis in Mx1-Cre mice after confirming the production of COMP-Angpt1 protein (Supplementary Fig. S2B). However, fatalities were noted in approximately 70% (15 out of 21) of Mx1-Cre+/COMP-Angpt1+ mice several days after poly I:C injection. Interestingly, the increase of adipose tissue within the BM, which is characteristic in normal aged BM, was inhibited significantly in 9 month-old COMP-Angpt1-expressing mice compared with that in control mice (Supplementary Fig. S2C).

3.3. Effects of Angpt1 overexpression in osteoblasts on the frequency of HSPCs in the BM

We next examined the percentage of LSK cells within the Lin^- fraction and the percentage of $LSKCD34^-$ cells within the LSK fraction in Col1a1-Cre+/COMP-Angpt1+ *Tg* and control mice. The

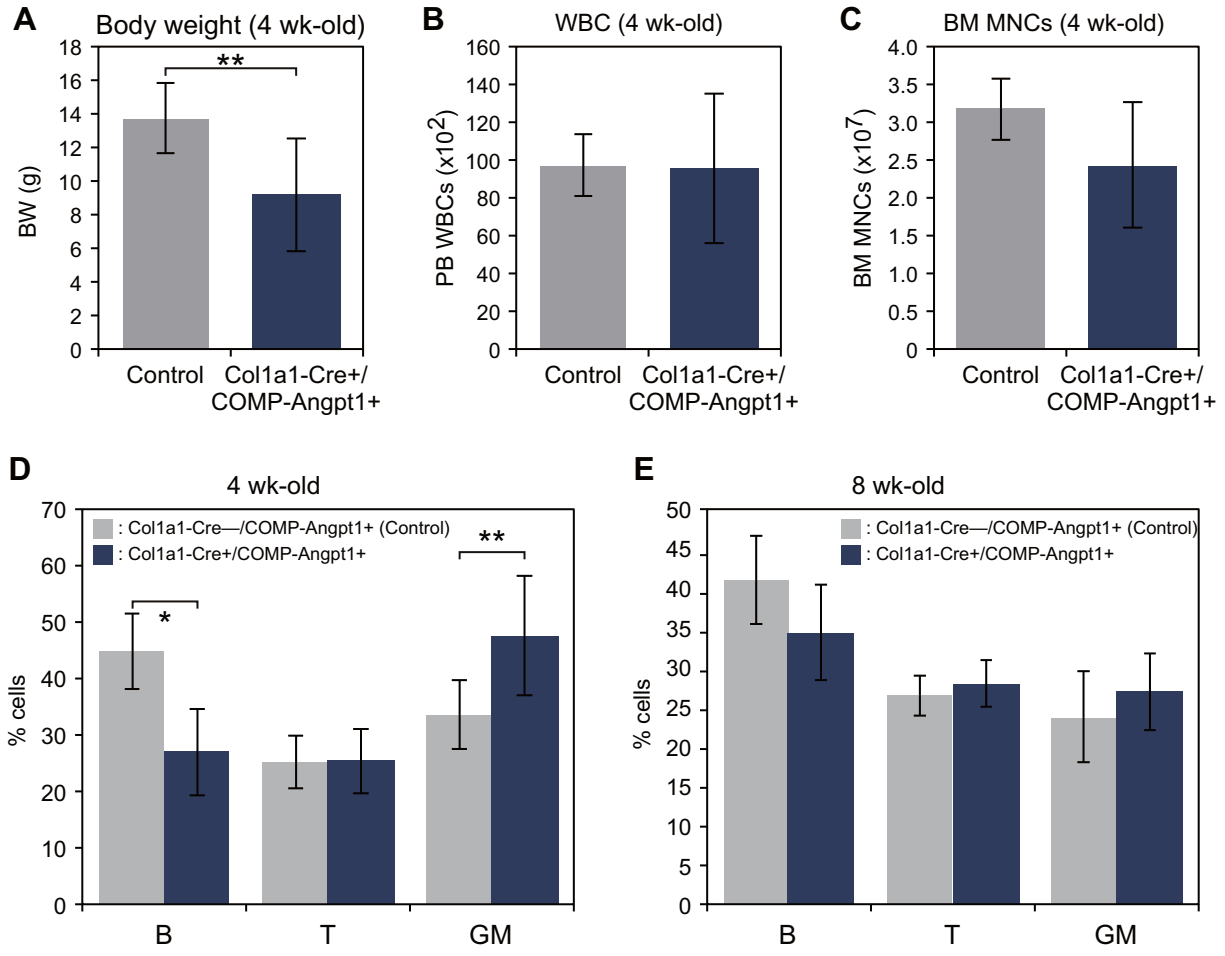

Fig. 1. Body weight, number of PB WBCs, BM MNCs and lineage differentiation in Col1a1-Cre+/COMP-Angpt1+ mice. (**A**) Body weight (BW) of Col1a1-Cre+ or −/COMP-Angpt1+ mice at 4 wk of age. Data represent means ± SD ($n = 5$/group; $^{**}p < 0.05$). Col1a1-Cre+/COMP-Angpt1+ mice weighed less than age-matched control mice. Number of PB WBCs (**B**) and BM MNCs (**C**) in control or *Tg* mice at 4 wk of age. Data represent means ± SD ($n = 5$/group). The percentage of B (B220), T (CD4/CD8) and myeloid (Mac-1/Gr-1, GM) cell lineages in PB from 4 (D) and 8 (E) wk-old Col1a1-Cre−/COMP-Angpt1+ (control) and Col1a1-Cre+/COMP-Angpt1+ mice. Data represent the mean ± SD ($n = 5$/group; $^{*}p < 0.05$, $^{**}p < 0.01$).

percentage of LSK cells within the Lin^- fraction was similar in *Tg* and control mice of both ages (Fig. 2A and C). Moreover, there was no significant difference in the percentage of $LSKCD34^-$ cells between Col1a1-Cre+/COMP-Angpt1+ and control mice at both 4 (15.0 ± 4.4% in *Tg* versus 11.1 ± 4.3% in control mice) and 8 (14.8 ± 1.3% in *Tg* versus 13.5 ± 2.1% in control mice) wk-of-age (Fig. 2B and D). On the other hand, the number of $Ki67^-$ cells in the LSK fraction increased in 8 wk-old *Tg* mice (Fig. 2E).

3.4. Effects of Angpt1 overexpression on gene expression in HSPCs

LSK cells were isolated from Col1a1-Cre+/COMP-Angpt1+ *Tg* and control mice and the expression of several HSPC markers (i.e., cell adhesion molecules, cell cycle regulators, and transcription factors) was analyzed. Q-PCR array analysis revealed that LSK cells from Col1a1-Cre+/COMP-Angpt1+ mice showed higher expression levels of *Cdkn1a*, *Cdkn1c*, *Hoxb4*, *Foxo4*, *Vcam1*, *Cd44*, *Cdh2* and *Ctnnd1* (Fig. 3). The increased expression of Cdk inhibitors and *Foxo4* indicated that Angpt1 overexpression resulted in increased LSK cell quiescence. These results also suggest that upregulation of *Hoxb4* may have increased self-renewal activity, whereas upregulation of *Vcam1*, *Cd44*, *Cdh2* and *Ctnnd1* may have increased LSK engraftment during BM transplantation (BMT).

3.5. COMP-Angpt1 overexpression in osteoblasts enhanced the long-term reconstitution activity of HSCs

Finally, we examined the effects of COMP-Angpt1 overexpression in osteoblasts on the LTR activity of HSCs. We previously reported that LTR activity was maintained after adding Angpt1 into LSK-SP cell cultures *in vitro* [24]. In addition, we found that Angpt1 overexpression upregulated the expression of *Cdkn1a*, *Cdkn1c*, *Hoxb4*, *Foxo4*, *Vcam1*, *Cd44*, *Cdh2* and *Ctnnd1* in LSK cells. Thus, we expected that Angpt1 overexpression would enhance the LTR activity of HSCs *in vivo*. We transplanted LSK cells (3×10^3 cells/mouse) isolated from the BM of Col1a1-Cre+/COMP-Angpt1+ and control mice into lethally-irradiated recipient mice. Compared with LSK cells from control mice, LSK cells from Col1a1-Cre+/COMP-Angpt1+ *Tg* mice showed greater engraftment 4 months after BMT. The frequency of donor-derived cells in mice transplanted with *Tg* LSK and control LSK cells was 65.75 ± 9.6% and 42.8 ± 10.3%, respectively (Fig. 4A). Analyses of B, T, and myeloid cells, as well as erythroblasts and megakaryocytes, in donor-derived PB 4 months after BMT showed that *Tg* LSK cells had normal lineage differentiation ability compared with control LSK cells (Fig. 4B), indicating that the lineage bias observed in PB from Col1a1-Cre+/COMP-Angpt1+ mice was due to the overexpression

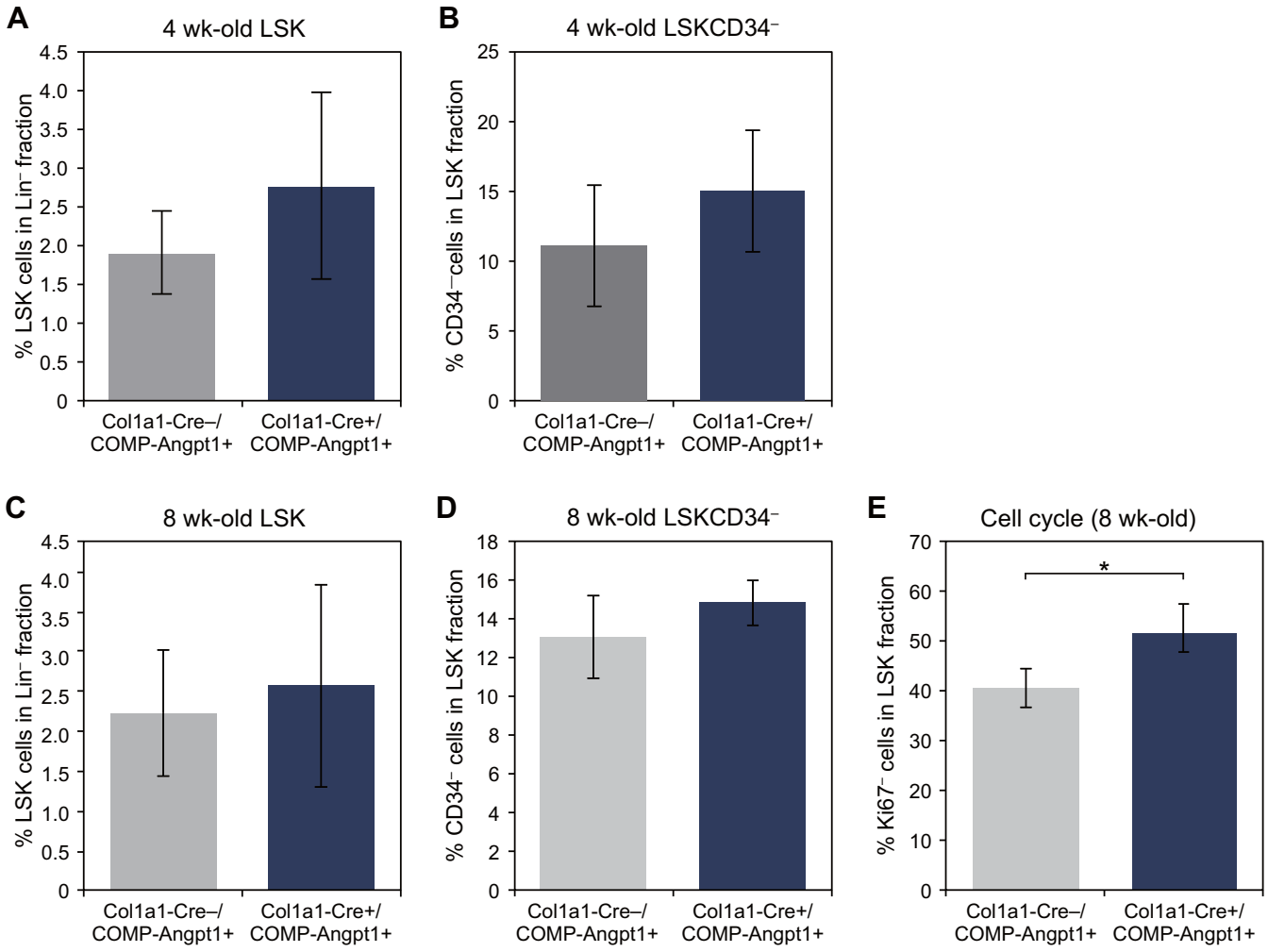

Fig. 2. Frequency of HSPCs in Col1a1-Cre+/COMP-Angpt1+ mice.The percentage of LSK cells within the Lin^- fraction (A) and $LSKCD34^-$ cells within the LSK fraction (B) derived from 4 wk-old Col1a1-Cre−/COMP-Angpt1+ (control) and Col1a1-Cre+/COMP-Angpt1+ mice. Data represent the mean ± SD (n = 5/group). The percentage of LSK cells within the Lin^- fraction (C) and $LSKCD34^-$ cells within the LSK fraction (D) derived from 8 wk-old Col1a1-Cre−/COMP-Angpt1+ (control) and Col1a1-Cre+/COMP-Angpt1+ mice. Data represent the mean ± SD (n = 9 for control mice; n = 7 for *Tg* mice). (E) The percentage of $Ki67^-$ cells within the LSK fraction derived from 8 wk-old control or Col1a1-Cre+/COMP-Angpt1+ mice. Data represent the mean ± SD (n = 3/group; $^*p < 0.05$).

of COMP-Angpt1 and not an intrinsic factor within the hematopoietic cells. These data illustrate that Angpt1-Tie2 signaling increased the LTR activity of HSCs *in vivo*.

4. Discussion

Angpt1-Tie2 signaling plays a crucial role in vascular morphogenesis, homeostasis and hematopoiesis [2]. We developed inducible *Tg* mice in which COMP-Angpt1 was overexpressed under the control of Cre-induced loxP recombination [19]. In the present study, we mated inducible COMP-Angpt1 *Tg* mice with Col1a1-Cre *Tg* mice and analyzed the role of Angpt1-Tie2 signaling in the regulation of HSCs.

PB analysis of Col1a1-Cre+/COMP-Angpt1+ *Tg* mice revealed a significant decrease in B cells and a significant increase in myeloid cells in 4 wk-old *Tg* mice compared with control mice. This lineage bias was not seen in 8 wk-old mice. These data indicate that Angpt1-Tie2 signaling affects the differentiation of HSPCs during postnatal BM development. Osteoblasts are involved in the commitment and differentiation of B cell progenitor cells, and B cell progenitors use the osteoblastic niche [25]. Col1a1-specific COMP-Angpt1 overexpression may affect the function of osteoblasts. Furthermore, HSPCs in the BM of 4 wk-old mice are actively cycling and proliferating [20], and the cell cycle state of HSPCs may be involved in lineage bias within the osteoblastic niche. Analysis of gene expression related to lineage differentiation in Col1a1-Cre+/COMP-Angpt1+ *Tg* HSCs would be interesting as it would identify the mechanism behind lineage bias.

In the present study, gene expression analyses revealed that Col1a1-Cre+/COMP-Angpt1+ *Tg* LSK cells showed higher expression of cell cycle- and adhesion-related genes. These data suggest that Angpt1/Tie2 signaling may induce quiescence in HSPCs, or increase the percentage of quiescent LT-HSCs, within the LSK cell fraction. Although the frequency of immunophenotypic LT-HSCs was similar in Col1a1-Cre+/COMP-Angpt1+ *Tg* and control mice, we found an increased frequency of $Ki67^-$ cells within the LSK fraction derived from 8 wk-old Col1a1-Cre+/COMP-Angpt1+ *Tg* mice. BMT assays showed that overexpression of Angpt1 in osteoblasts enhanced the LTR activity of HSCs. Moreover, Angpt1 overexpression induced the upregulation of *Hoxb4*, which may increase self-renewal activity; and upregulation of *Vcam1*, *Cd44*, *Cdh2*, and *Ctnnd1* may enhance the engraftment of LSK cells after BMT. It would be interesting to determine whether Tie2-Angpt1

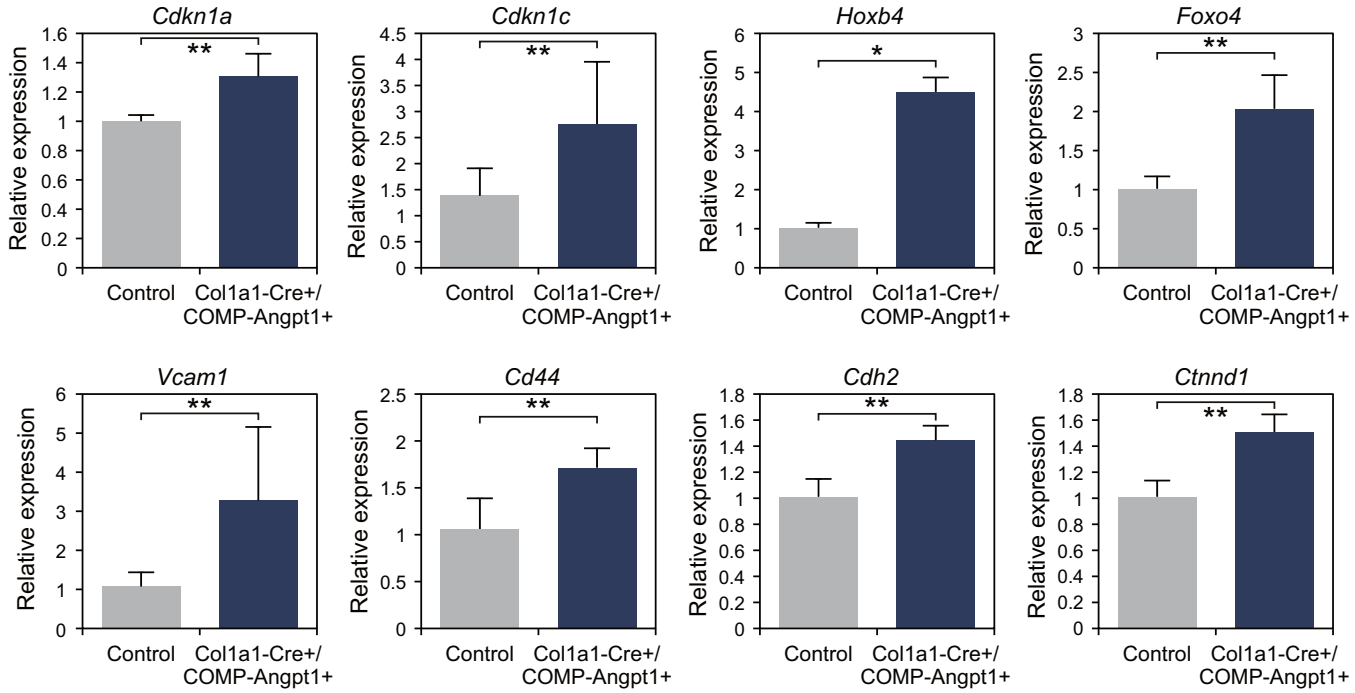

Fig. 3. Gene expression analysis in LSK cells derived from 8 wk-old Col1a1-Cre/COMP-Angpt1 mice. Relative expression levels of *Cdkn1a*, *Cdkn1c*, *Hoxb4*, *Foxo4*, *Vcam1*, *Cd44*, *Cdh2* and *Ctnnd1* in LSK cells isolated from 8 wk-old Col1a1-Cre–/COMP-Angpt1+ (control) and 8 wk-old Col1a1-Cre+/COMP-Angpt1+ mice. Data represent the mean ± SD (n = 3/group; $^{**}p < 0.05$, $^{*}p < 0.01$).

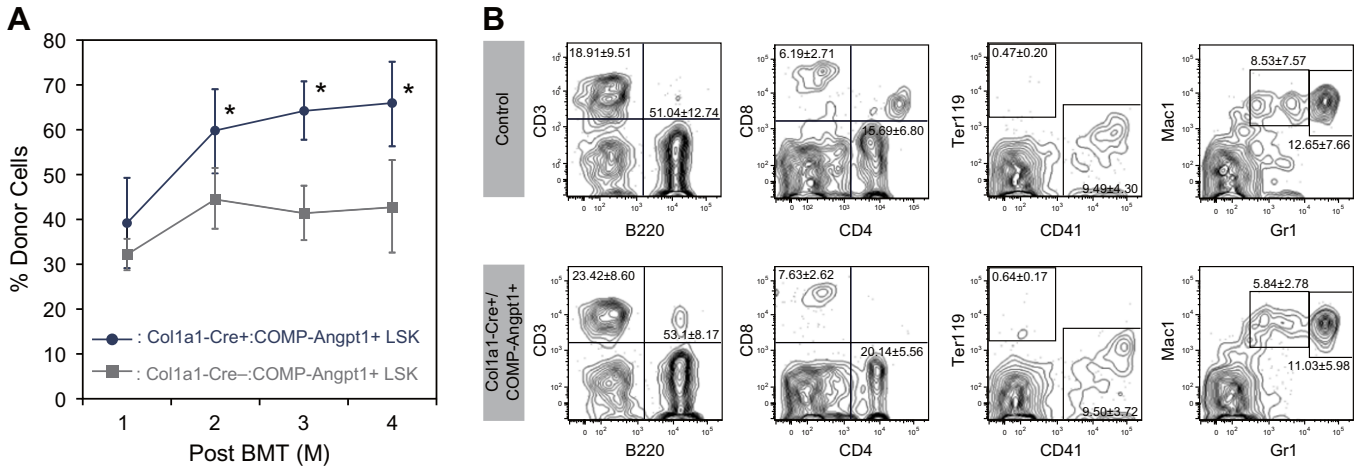

Fig. 4. BMT analysis with LSK cells from 8 wk-old Col1a1-Cre/COMP-Angpt1 mice. (A) LSK cells (3×10^3 cells/mouse) isolated from either 8 wk-old Col1a1-Cre–/COMP-Angpt1+ (control) or 8 wk-old Col1a1-Cre+/COMP-Angpt1+ mice were transplanted into lethally-irradiated recipient mice along with competitor Ly5.1$^+$ MNCs. PB chimerism of donor-derived (Ly5.2$^+$) cells was analyzed each month (M) after BMT. LTR capacity was higher in cells derived from Col1a1-Cre+/COMP-Angpt1+ *Tg* mice than in the control. Data represent the mean ± SD (n = 5/group; $^{*}p < 0.05$), (B) Lineage differentiation activity of donor-derived PB cells 4 months post-BMT. Data represent the mean ± SD (n = 5/group). Representative FACS plots are shown.

signaling increases the LTR capacity of HSCs or increases the quantity of HSCs with LTR capacity in the BM. As there was a tendency for Col1a1-Cre+/COMP-Angpt1+ *Tg* LSK cells to contain more LSKCD34$^-$ cells, we anticipate that the latter is more likely.

Angpt-like proteins affect adipogenesis [26]. In this study, we found that overexpression of Angpt1 induced by crossing Mx1-Cre mice and poly I:C injection resulted in a reduction in adipose tissue in the BM of older mice. This finding suggests that Angpt1 may affect mesenchymal cells by inhibiting adipogenesis, which is critical for the maintenance of the BM niche during aging. Angpt1 reportedly binds to integrins [27,28]. Therefore, it is possible that Angpt1 regulates the differentiation of mesenchymal stem and progenitor cells via integrins.

The present study showed that the dose of Angpt1 within the osteoblastic niche is important for hematopoietic lineage differentiation. In addition, we showed that Angpt1-Tie2 signaling increased the quiescence and LTR activity of HSCs. However, overexpression of Anglt1 in CAG-Cre or Mx1-Cre mice induced abnormal vascular development and/or morphology, indicating that a proper amount of Angpt1/Tie2 signaling is crucial for vasculo/angiogenesis. In

conclusion, the COMP-Angpt1-overexpressing mouse is an effective model for studying Angpt1-Tie2 signaling *in vivo*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.002.

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