Enforced Expression of BMI-1 in Postnatal Human CD34+ Cells Promotes Erythroid Differentiation

Gabsang Lee, Ph.D., D.V.M.[1], Byung Soo Kim, Ph.D., M.D.[2], Jae-hung Shieh[3] and Malcolm AS Moore[4]

[1]Craniomaxillofacial Life Science 21, College of Dentistry, Seoul National University, [2]Division of Hematology/Oncology, Korea University Medical Center, Seoul, Korea, [3]Cell Biology Program, Memorial Sloan Kettering Cancer Center, [4]Cell Biology Program, Memorial Sloan Kettering Cancer Center

Background: The Polycomb-group gene Bmi-1 is known to be a molecular regulator of self-renewal of normal and leukemic stem cells and be involved in various aspects of cellular proliferation, differentiation, and survival.
Methods: This study evaluated the effects of overexpression of Bmi-1 on human cord blood CD34+ cells. Bmi-1 was introduced into CD34+ cells through lentivirus transduction. Bmi-1 expressing CD34+ cells were applied to colony forming assay, stromal co-culture, and cytokine-stimulatied culture.
Results: Ectopic expression of Bmi-1 resulted in the increased number of erythroid colonies in primary and secondary colony forming assay in an erythropoietin dependent manner. In stromal co-culture, Bmi-1-expressing postnatal hematopoietic stem cells seemed to lose the ability of self-renewal, as determined by week 5 cobblestone area-forming cell assay and by week 5 secondary colony assay. In cytokine-stimulated suspension culture of Bmi-1-transduced CD34+ cells, we observed increased erythropoiesis marked by Glycophorin A expression.
Conclusion: Our data suggest that ectopic expression of Bmi-1 in human hematopoietic stem/progenitor cells may result in the differentiation to the erythroid lineage rather than promoting self-renewal. ***(Korean J Hematol 2007;42:241-249.)***

Key Words: Bmi-1, Erythroid differentiation, CD34+ cell

INTRODUCTION

Polycomb group (PcG) genes are known to be epigenetic gene silencers that preserve transcription patterns to maintain cell identity, especially in stem cells.[1,2] Among PcG genes, Bmi-1 is one of widely studied genes in field of neurology, hematology, and oncology. Bmi-1 has a profound effect on mouse neural stem cells (NSCs) determined by reduced frequency of primary and secondary neurosphere formation at embryonic and postnatal stages.[3,4] In contrast, lentiviral-delivered Bmi-1 shRNA *in vitro* and *in vivo* impaired embryonic and adult NSCs proliferation and self-renewal.[5] In postnatal Bmi-1$^{-/-}$ mice, hematopoietic stem cells (HSCs) showed the loss of self-renewal, although the number of embryonic Bmi-1$^{-/-}$ HSC in fetal liver seemed to be normal.[6] Over-expression of Bmi-1 in murine HSC resulted in the

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교신저자 : 이갑상, 서울시 종로구 연건동 275-1번지
㉾ 110-749, 서울대학교 치과대학 BK 치의학생명과학사업단
Tel: 02-740-8663, Fax: 02-740-8665
E-mail: gabsang@gmail.com

Correspondence to : Gabsang Lee, Ph.D., D.V.M.
Craniomaxillofacial Life Science 21, College of Dentistry, Seoul National University
275-1, Yeongeon-dong, Jongno-gu, Seoul 110-749, Korea
Tel: +82-2-740-8663, Fax: +82-2-740-8665
E-mail: csuh@amc.seoul.kr

increase of self-renewal determined by multipotent colony forming assay and *in vivo* repopulation studies.[7] Moreover, Bmi-1 in murine leukemic stem/progenitor cells was a important factor for proliferation and survival, which can lead to leukemia.[8,9] The expression of Bmi-1 in human brain tumors, such as medulloblastoma and glioblastoma was upregulated.[10-12] The roles of Bmi-1 in NSCs, HSCs, and neoplastic cells was explained by Bmi-1-mediated suppression of p16 INK4a/ARF or p21-Rb cell cycle regulatory pathway.[3-6,13,14] Other study showed that Bmi-1 in HSC might be involved in repressing differentiation-related gene expression, such as C/EBPα below the level of biological significance.[15] Understanding the cellular and molecular mechanisms of Bmi-1 can provide an insight on how stem cells can regulate self-renewal and proliferation. Here we used lentiviral transduction to define the effects of Bmi-1 overexpression on human cord blood (CB)-derived CD34+ cells.

MATERIAL AND METHODS

1. Production of GFP control (FUEG) and Bmi-1-GFP (FUEG-Bmi-1) lentivirus

For FUEG construct, GFP gene (driven by an EF1α promoter) was cloned into the backbone of the FUGW lentiviral vector after deletion of the GFP sequence (termed FUEG for GFP only). Human Bmi-1 cDNA was cloned from K562 and ligated into the FUEG after the ubiquitin promoter (Named as FUEG-Bmi-1) (Fig. 1A). Lentivirus was produced by transient transfection with 20μg of each lentivirus vector, 10μg pVSVG, and 15μg pΔ8.9 into the 293T cell line using the calcium-phosphate method. Serum-free Quality Biological serum-free-60 medium (QBSF; Quality Biological, Gaithersburg, MD) was used for production of virus-containing supernatants. After filtration through a 0.45-m filter (Costar, Cambridge, MA), viral supernatant was concentrated with Amicon filters (Milipore, Billerica, MA) and stored at −80°C.

2. Isolation of CD34+ cells from human CB and transduction with lentivirus

Human umbilical CB from healthy full-term pregnancies was kindly provided by the Cord Blood Bank subdivision of the New York Blood Bank. CD34+ cells were enriched from the ficoll-seperated mononuclear CB cells using the MiniMACS CD34+ isolation kit (Miltenyi Biotech, Auburn, CA). CB-derived CD34+ cells were pre-stimulated for 4 hours in QBSF supplemented with c-Kit ligand (KL, 100ng/mL), Flt3 ligand (FL, 100ng/mL), and thrombopoietin (Tpo, 100ng/mL). KL and Tpo were provided by Peprotech (Rocky Hill, NJ); FL was provided by Imclone Systems (New York, NY). Pre-stimulated CD34+ cells were incubated in lentivirus-containing medium with two rounds of transduction (first round for 12 hours and, subsequently second round for 8 hours) in the presence of 100ng/mL KL, FL, and Tpo, and polybrene (4μg/mL). GFP+ postnatal hematopoietic stem/progenitor cells were isolated using MoFlo (Dako, Denmark), after 72 hours of lentivirus transduction.

3. Immunoblot

FUEG- and FUEG-Bmi-1-transduced 293T cells and CB-derived CD34+ cells (30,000 cells per sample) were boiled for 5 min in Laemmli sample buffer before separation on 12% SDS-acrylamide gels. Proteins were transferred to nitrocellulose filters (Millipore) in Tris-glycine

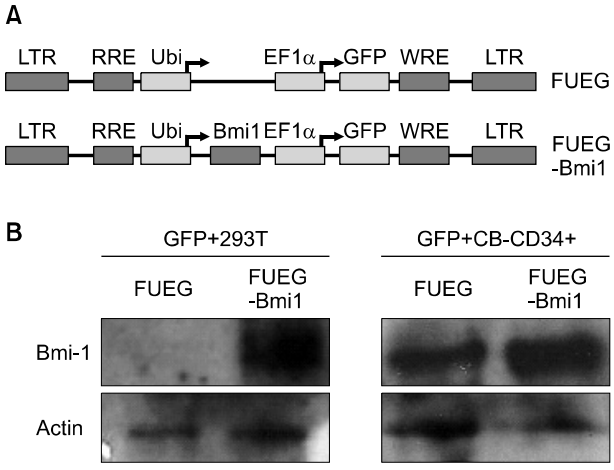

Fig. 1. Construction of FUEG and FUEG-Bmi-1 lentivirus and detection of elevated Bmi-1 expression in transduced 293T and human cord blood (CB) CD34+ cells. (A) A schematic image of FUEG and FUEG-Bmi-1 lentivirus used in this study. (B) Immunoblot of whole-cell protein extracts from transduced 293T and GFP+FACS-sorted human CB-derived CD34+ cells with anti-Bmi-1 and anti-Actin antibodies.

buffer 9V for 1.5 hr using a semidry Electroblotter (Bio-Rad, Hercules, CA). Membranes were blocked and incubated with antibodies. An ECL kit was used for detection according to manufacturer's instruction (Roche, Basel, Switzerland).

4. Hematopoietic colony forming cell (CFC) assay

GFP+ transduced cells were plated at 300 or 1×10^3 cells/mL in 35-mm Petri dishes containing IMDM, 1.2% methylcellulose (Dow Chemical, Midland, MI), 30% FBS, 57.2 μm β-ME, 2mm glutamine, 0.5mm hemin (Sigma-Aldrich, MA), 20ng/mL KL, 20ng/mL granulocyte-macrophage colony stimulating factor (GM-CSF; 20ng/mL, R&D Systems), interleukine-3 (IL-3; 20ng/mL, R&D Systems), granulocyte colony stimulating factor (G-CSF; 20ng/mL, Amgen, Thousand Oaks, CA), and erythropoietin (Epo; 6units/mL, Amgen). Colonies were scored after 14 days. Secondary CFC assay was performed with 1×10^4 cells/mL that were harvested from primary CFC dish. To define the Epo dependence, erythroid colony formation was determined in the presence of KL (10ng/mL) alone or KL plus two concentrations of Epo (1unit/mL, or with 6units/mL).

5. Co-culture with stromal cells

Cobblestone area-forming cell (CAFC) assay was performed by plating 1×10^4 CB CD34+ cells on to MS-5 monolayers in T12.5 tissue-culture flasks (Becton Dickinson, Franklin Lanes, NJ). Cobblestone areas (CA) were scored each week after 5 weeks of culture. CA was defined as a group of at least 10 phase-contrast dark cells tightly associated beneath the MS-5 monolayer. Mitotically inactivated OP-9 and MS-5 were used to find the expanding ability of transduced hematopoietic cells by plating 1×10^3 CB-CD34 +cells in T12.5 tissue-culture flasks (Becton Dickinson, Franklin Lake, NJ). Weekly demi-depopulations were performed and non-adherent cells were counted. CFC assays were performed after 5 weeks of co-culture.

6. Cytokine-stimulated suspension cultures

Suspension cultures were established by plating 2.5×10^4 GFP+ CB cells in each well of 24-well plates in QBSF medium supplemented with KL (20ng/mL), FL (20ng/mL), Tpo (20ng/mL) and Epo (6units/mL). After 10 days of culture, phenotypes of cultured cells were analyzed by flow cytometry. Harvested cell pellets were suspended in PBS containing 0.3% BSA. After antibody binding for 30 minutes at 4°C, the cells were washed twice and re-suspended in PBS containing 3% BSA, and finally stained with 1 μg/mL 7-aminoactinomycin D (7-AAD, BD Pharmingen, San Diego, CA) viability dye at room temperature. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) through Cell Quest acquisition software (Becton Dickinson). Results of flow cytometry were analyzed by FlowJo software (Tree Star, Inc., Ashland, OR). Following mouse anti-human antibodies were used; anti-CD34-APC and anti-Glycophorin A-PE (BD Pharmingen). Control staining with appropriate isotype-matched control antibodies (BD Pharmingen) was included.

RESULTS

1. Lentiviral transduction of Bmi-1 into human CB CD34+ cell

Purified human CB CD34+ cells were pre-stimulated (4 hours) and transduced with FUEG or FUEG-Bmi-1 lentivectors (Fig. 1A). Transduction efficiencies were 30±5% for FUEG and 15±5% for FUEG-Bmi-1 as determined with flow cytometric analysis of GFP+ cells at 72 hours after transduction (data not shown). Increased expression of Bmi-1 in transduced 293T cell (more than 90% transduction efficiency) and GFP+ CB CD34+ cells were confirmed by Western blot (Fig. 1B).

2. Forced expression of Bmi-1 promotes the formation of erythroid colonies in an erythropoietin dependent manner

GFP+ CB CD34+ cells were isolated by flow cytometry and cultured on methylcellulose plate in the presence of KL, GM-CSF, IL-3, G-CSF and Epo. In primary CFU assay, the total number of colonies was increased in FUEG-Bmi-1 group as compared to control group and this was attributed exclusively to an increase in BFU-E (Fig. 2A). Most of the colonies expressed

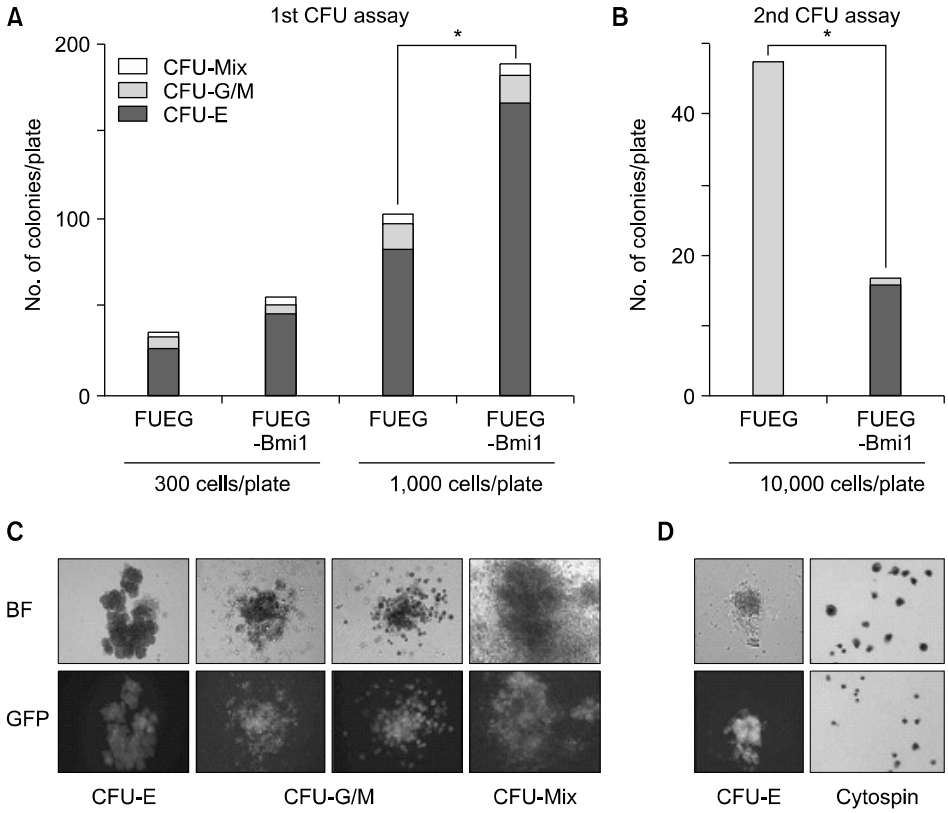

Fig. 2. Ectopic Bmi-1 expression in CB CD34+ cells enhances the formation of both primary and secondary erythroid colonies. Numbers of erythroid colonies in primary (A) and secondary (B) The number of colony-forming cells (CFCs) was significantly increased in FUEG-Bmi-1 group (*$P<0.05$, Error bar is not shown). (C) Representative images for bright field (BF) and GFP expression (GFP) of each type of colonies from primary CFC assay in FUEG-Bmi-1 group. (D) Representative images for GFP expression of CFU-E and cytospin images of the cells obtained from secondary colonies in FUEG-Bmi-1 group.

GFP, indicating that the transgenes are in good function (Fig. 2C). Secondary CFU assay in the presence of KL, GM-CSF, IL-3, G-CSF and Epo was performed with the cells harvested from primary colonies. The total number of secondary CFU decreased significantly in the FUEG-Bmi-1 group, as compared to the control; all the colonies in FUEG-Bmi-1 group were erythroid lineage (Fig. 2B). Secondary colonies also expressed GFP (Fig. 2D). Cytospin preparations showed that the secondary erythroid colonies contained differentiated enucleated erythroid cells (Fig. 2D).

To determine the effects of Epo on BFU-E formation, isolated GFP+ CB CD34+ cells were subjected to CFU assay in the presence of KL and different concentrations of Epo (Fig. 3). Without Epo or with a low concentration of Epo (1IU/mL), there was no increase in numbers of BFU-E in the FUEG-Bmi-1 group, compared to that of the control group (Fig. 3A, B). However, a higher concentration of Epo (6IU/mL) significantly enhanced BFU-E formation in FUEG-Bmi-1 group (Fig. 3C). Our data show that the overexpression of Bmi-1 favored erythroid differentiation in an Epo-dependent manner; however, we could not find an effect of Bmi-1 overexpression on the self-renewal or expansion of hematopoietic stem/progenitor cells.

3. Overexpression of Bmi-1 does not enhance self-renewal or proliferation of human postnatal CD34+ stem/progenitor cells

After transduction of FUEG or FUEG-Bmi-1 lentivirus, GFP+ CB CD34+ cells were isolated by FACS cell sorter and plated on MS-5 for CA forming assay. Except for the first week, the CA number in sequential weeks did not show a significant difference among non-trans-

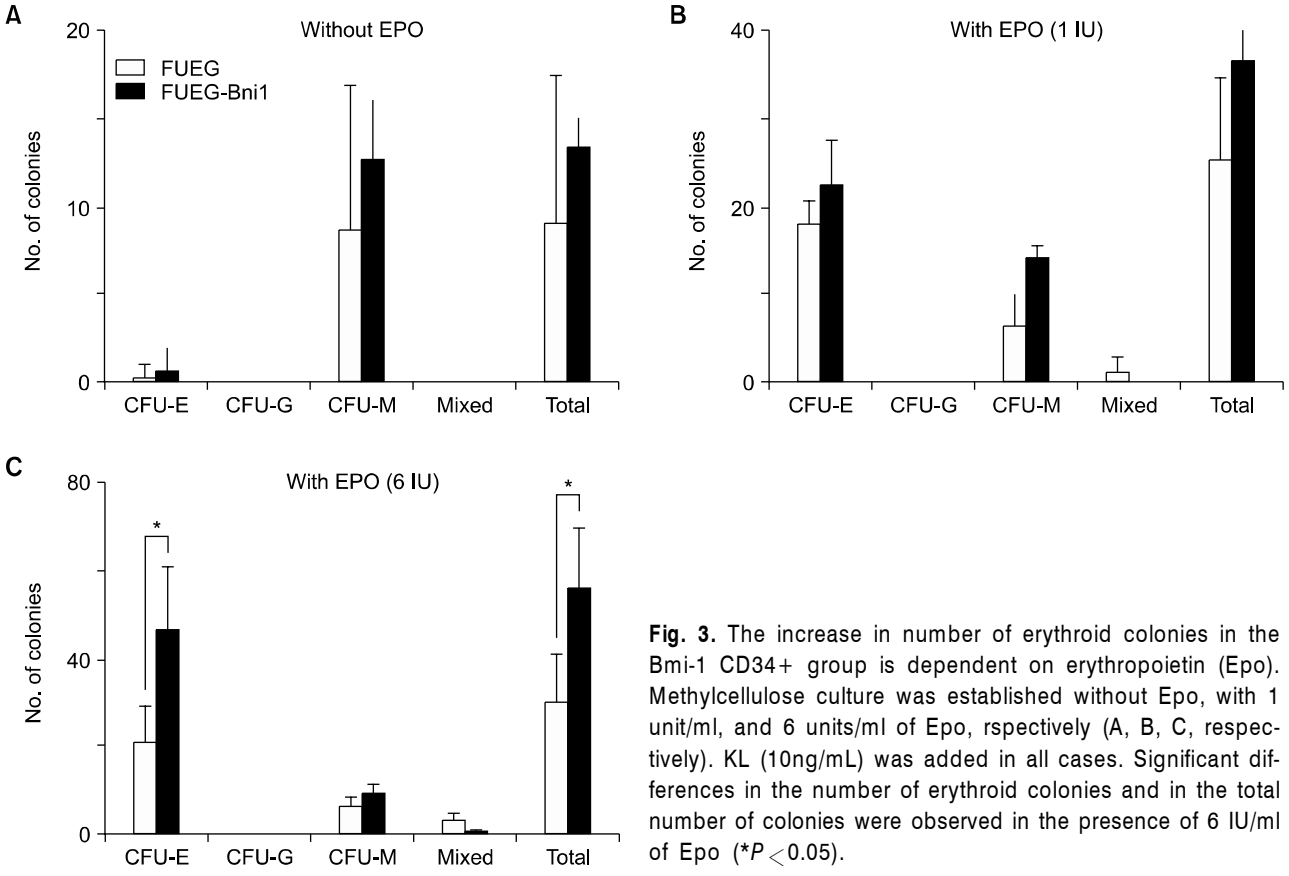

Fig. 3. The increase in number of erythroid colonies in the Bmi-1 CD34+ group is dependent on erythropoietin (Epo). Methylcellulose culture was established without Epo, with 1 unit/ml, and 6 units/ml of Epo, rspectively (A, B, C, respectively). KL (10ng/mL) was added in all cases. Significant differences in the number of erythroid colonies and in the total number of colonies were observed in the presence of 6 IU/ml of Epo ($*P < 0.05$).

duced, FUEG, and FUEG-Bmi-1 group (Fig. 4A). When transduced CD34+ cells were cultured on irradiated OP-9 for 5 weeks, the number of non-adherent cells from each group was not significantly different (Fig. 4B). After 5 weeks of culture, CFC assays from non-adherent cells of each group showed significantly lower numbers of CFC in the FUEG-Bmi-1 group compared with the FUEG group (Fig. 4C). When Bmi-1-expressing CB-CD34+ cells were plated on irradiated MS-5, the number of non-adherent cells did not change during 5-week co-culture (Fig. 4D). CFC assays of non-adherent cells from 5-week culture on MS-5 culture showed a significantly lower number of CFC in the FUEG-Bmi-1 group compared to the FUEG group (Fig. 4E). These data indicates that enforced expression of Bmi-1 does not enhance self-renewal or expansion of HSCs, as determined by the CA forming ability, proliferating ability, and generation of progenitors by week 5.

4. Cytokine-stimulated serum-free culture of transduced human CD34+ cells reveals that ectopic expression of Bmi-1 enhances erythroid differentiation

GFP+ cells were isolated by FACS and cultured in stroma-free condition with serum-free medium supplemented with KL, FL, Tpo, and Epo. As shown in Fig. 5A and B, at day 7 of stroma-free culture, CD34+ cells were not maintained and Glycophorin A+cells were increased in FUEG-Bmi-1 group compared to FUEG group, although total cell number from each group did not show significant difference. These data suggest that Bmi-1 expression enhances erythroid differentiation.

DISCUSSION

We have demonstrated that overexpression of Bmi-1 in human CB CD34+ cells resulted in the increased their ability to form erythroid colonies in primary and secon-

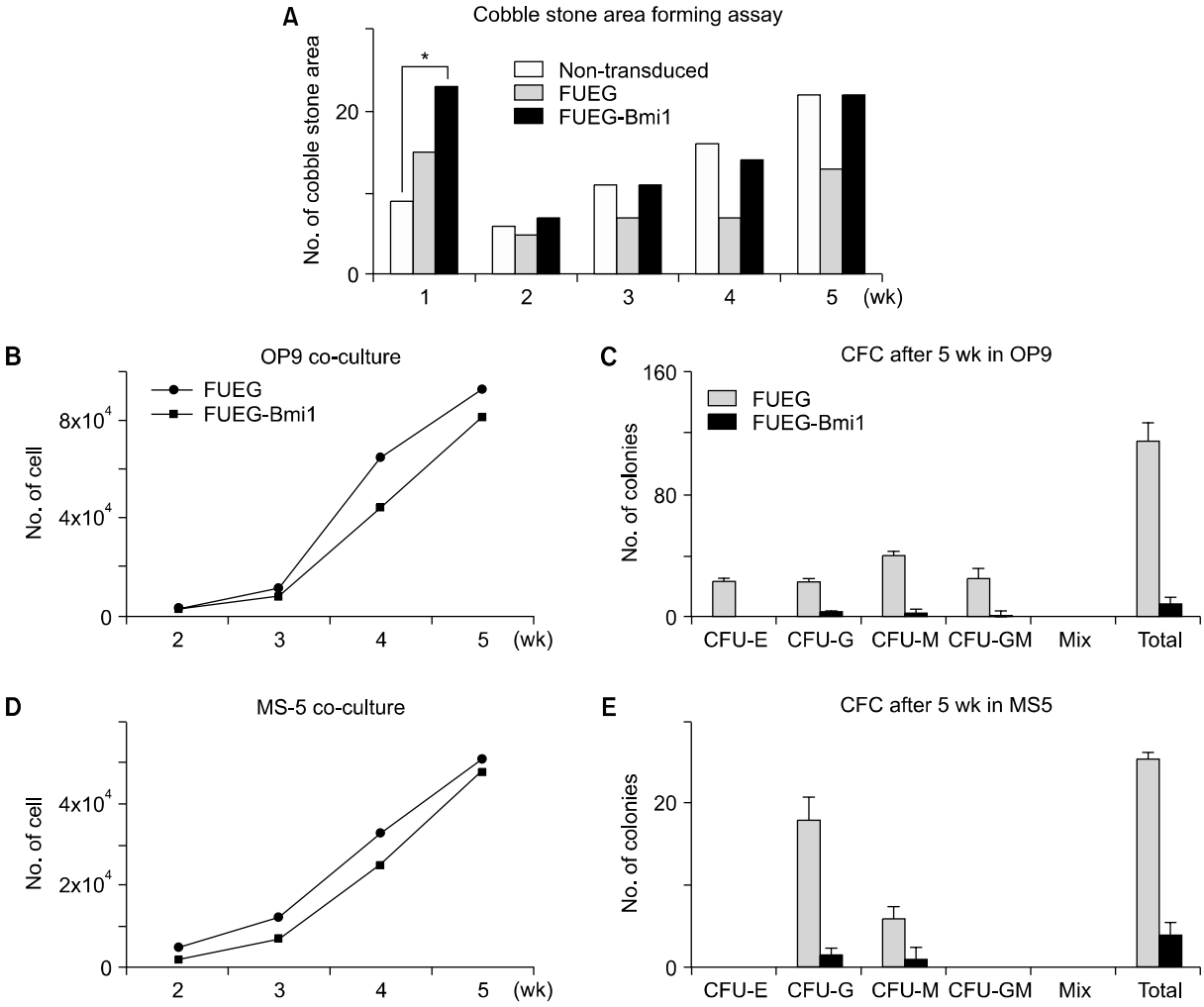

Fig. 4. Forced expression of Bmi-1 does not increase self-renewal of CB HSC. (A) Results of cobblestone area forming assay showed that there is no significant difference between non-transduced, FUEG and FUEG-Bmi-1-transduced groups (Error bar is not shown). (B, D) Number of non-adherent cells in co-cultures of CD34+ cells on OP-9 and MS-5 stromal cells was not different between the two groups (Error bar is not shown). (C, E) Methylcellulose cultures were established with non-adherent cells obtained from 5-week co-cultures on OP-9 and MS-5 stromal cells. A significant decrease in total number of 5 week colonies in FUEG-Bmi-1 group was observed.

dary colony forming assay in an Epo-dependent manner and did not promote self-renewal, as judged by week 5 CAFC assay and LCT-IC assay. Suspension cultures of Bmi-1-expressing CD34+ cells resulted in a decrease in CD34 expression and an increase in Glycophorin A expression. Our data suggest that ectopic expression of Bmi-1 enhances differentiation to the erythroid lineage without an increase in self-renewal or proliferation of human postnatal HSCs.

A number of murine studies have showed Bmi-1 to be an indispensable regulator of mouse HSCs as well as of mouse NSCs and neoplastic cells, via p16 INK4a/ARF or p21-Rb cell cycle regulatory pathway.[3,4,6-9,11,13,14] Ectopic expression of Bmi-1 in mouse HSCs enhanced the self-renewals measured by competitive repopulation and increased number of high proliferation multilineage CFCs. This results is not consistent with out data, although this present data was based on human postnatal CD34+ cells.[7] It is possible that the difference is due to the species used. It is also possible that the results could be due to differences in level of transcription or translation among methodologies of gene delivery, because we used the lentiviral introduction of Bmi-1.

Iwama group showed that increased number of the

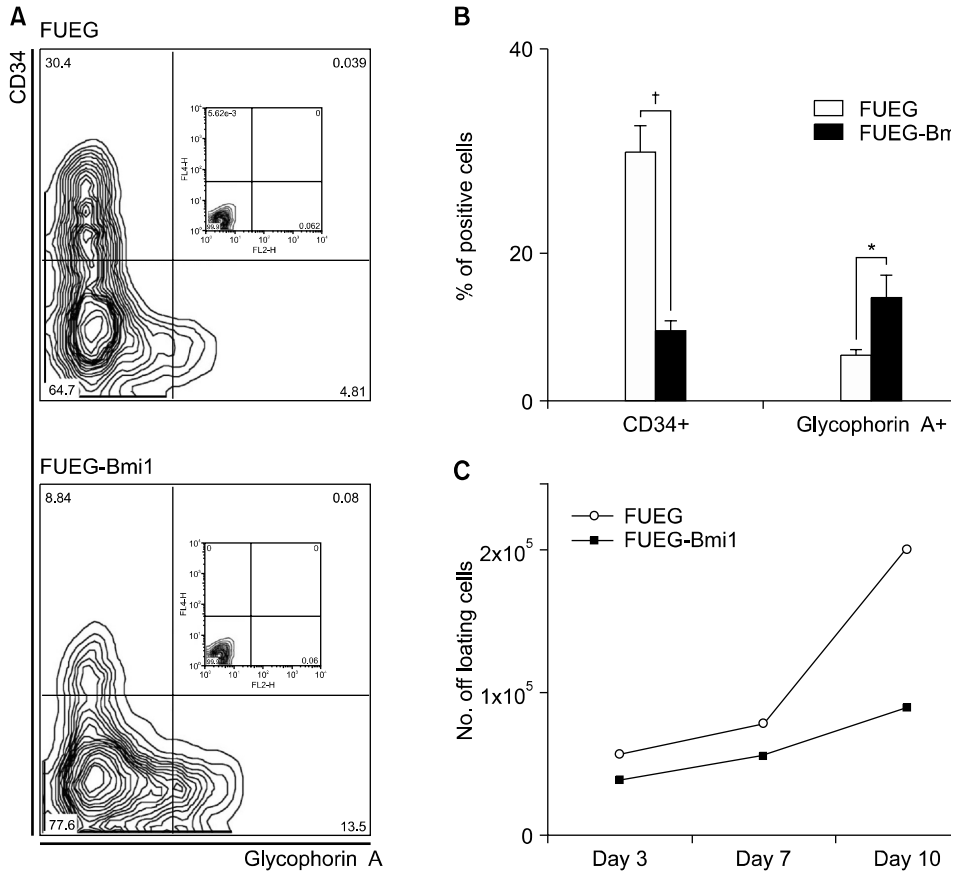

Fig. 5. Overexpression of Bmi-1 promotes erythroid differentiation of CB CD34+ cells. GFP+ CB CD34+ cells were cultured in serum-free medium supplemented with KL, Flt3 ligand, Tpo, and Epo. (A) Representative images of flow cytometric analysis using anti-CD34 and anti-Glycophorin A antibodies after 7 days of culture in serum-free condition. (B) Quantification of CD34+ and Glycophorin A+ cells in stroma free culture for 7 days showed a significant decrease of CD34+ cells and an increase of Glycophorin A+ cells. (C) Total number of the cells in stromal-free culture did not show significant difference (Error bar is not shown).

multilineage CFC unit in Bmi-1 transduced group,[7] which is partially similar to our data. However, our study showed the increased formation of erythroid colonies but not multilineage CFU-Mix. This inconsistency can be related that initial hematopoietic samples (mouse adult bone marrow CD34-KSL cells versus human postnatal CD34+ cells) in each study are different. The mouse study used more enriched population of HSC than on human CB CD34+ cells. So we postulate that Bmi-1 is necessary for maintaining the stemness in mouse adult bone marrow CD34-KSL cells and/or Bmi-1 has other function in expanding human postnatal CD34+ cells.

Perinatal CB is believed to contain embryonic hematopoietic cells, which decrease abruptly in number during aging due to the expansion of cells lacking fetal hemoglobin mRNA.[16] Previously, it is reported that Bmi-$1^{-/-}$ mice had a similar number of HSC and an identical *in vivo* repopulation frequency of HSCs in fetal liver cells, compared with wild type mice.[6] These mouse fetal liver HSCs from Bmi-$1^{-/-}$ showed normal migrations towards the chemokine SDF-1α, which may imply that Bmi-1 can be dispensible in embryonic or fetal HSCs as well as, possibly, perinatal CD34+ cells. These previous studies suggest that the function of Bmi-1 in human perinatal HSCs is not necessarily identical to that of mouse adult bone marrow HSCs.[7] These observations raise the possibility that Bmi-1 has new roles in fetal hematopoiesis or in the regulation of perinatal HSCs.

Proposed signaling pathway of Bmi-1 in HSCs and NSCs is the Bmi-1-mediated suppression of p16 INK4a/ARF-Rb or p21-Rb cell cycle regulatory pathway, sug-

gesting the important role of Rb (Retinoblastoma) in Bmi-1 function.[4,6,14] The phosphorylation of Rb is related with the regulation of cell cycle, especially G1-S phase, which points the role of Bmi-1 in HSC and NSC.[6,17] Recently, it has been reported that the role of Rb in HSC is not intrinsic, but rather extrinsic as demonstrated in the interaction between hematopoietic cells and the niche in $Rb^{-/-}$ mice.[18] This study suggests that there could be other downstream target or mechanism (other than Rb) for the function of Bmi-1 in, at least, HSCs. The functional failure of Bmi-1-/- HSCs in previous studies could be resulted from the abnormal environment in bone marrow, not from their cell intrinsic way.

In summary, we found that Bmi-1 overexpression did not promote self-renewal or expansion of human CB CD34+ cells; however, it induced differentiation of the cells toward erythroid lineage, which is not in line with previous studies using murine HSCs. Our data raises possibilities that Bmi-1 has other signaling pathways in human CB CD34+ cells.

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요 약

배경: Bmi-1은 Polycomb-group에 속하는 유전자로서 생쥐의 일반 줄기세포 및 백혈병 유발 줄기세포의 자기갱신를 조절하는 인자이며 세포의 성장, 분화 및 생존에 관여한다. 본 연구에서는 Bmi-1의 과발현이 인간 제혈대 유래 CD34양성세포의 성장 및 분화에 미치는 영향을 평가하였다.

방법: Bmi-1을 CD34양성세포에 렌티바이러스를 이용하여 과발현 시킨 후 집락형성분석, 기질세포와의 공조배양 및 사이토카인 첨가하의 액상배양을 시행하였다.

결과: Bmi-1의 과발현은 CD34양성세포의 일차 및 이차 적혈구계 집락 형성을 erythropoietin 의존적으로 증가시켰다. Bmi-1을 과발현시킨 CD34양성세포를 기질세포와의 5주간 공조배양을 시행하고 cobblestone area-forming cell 분석과 배양 후 수확한 세포의 집락형성분석을 시행한 결과 조혈줄기세포의 자기갱신이 감소하는 현상을 확인하였다. 사이토카인 첨가하의 액상배양에서는 Bmi-1이 발현되는 조혈세포가 적혈구계통으로 분화되는 현상을 관찰하였다.

결론: 인간 제대혈 유래 조혈세포에서의 Bmi-1과발현은 적혈구 계통으로의 분화를 촉진하는 반면에 자기갱신은 감소시킨다.

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