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Inhibition of erythropoiesis by Smad6 in human cord blood hematopoietic stem cells

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ABSTRACT

Bone morphogenetic proteins (BMPs) that belong to the transforming growth factor-β (TGF-β) superfamily cytokines, play crucial roles in hematopoiesis. However, roles of Smad6 in hematopoiesis remained unknown in contrast to the other inhibitory Smad (I-Smad), Smad7. Here we show that Smad6 inhibits erythropoiesis in human CD34$^+$ cord blood hematopoietic stem cells (HSCs). Smad6 was specifically expressed in CD34$^+$ cord blood HSCs, which was correlated with the expression of BMP2/4/6/7 and BMP type I receptor (BMPRI). BMP-specific receptor-regulated Smads (R-Smads), Smad1 and Smad5 in cooperation with Smad4 induced transcription of the *Smad6* gene. Instead of affecting cell cycle, apoptosis, self-renewal, and stemness of CD34$^+$ cells, Smad6 knockdown enhanced, whereas Smad6 overexpression suppressed erythropoiesis in stem cell culture and colony formation assay. Consistently, Smad6 suppressed the expression of the genes essential for erythropoiesis, such as Kruppel-like factor 1 (erythroid) (KLF1/EKLF) and GATA binding protein 2 (GATA-2). Promoter analyses showed that Smad6 repressed Smad5/4-induced transcription of the *Klf1* gene. Thus, our data suggest that Smad6 indirectly maintains stemness by preventing spontaneous erythropoiesis in HSCs.

1. Introduction

Hematopoietic stem cells (HSCs) possess the ability of quiescence, self-renewal and multi-potency to raise all functional blood cells [1]. Transforming growth factor-β (TGF-β) superfamily cytokines play the pivotal roles in maintenance of stemness of HSCs as well as in complex processes of differentiation into various hematopoietic cells from HSCs [1–4]. Bone morphogenetic proteins (BMPs), members of TGF-β superfamily were originally identified as factors that induce the formation of bone and cartilage [5]. BMPs bind with type I and type II BMP receptors. The type II receptor kinase transphosphorylates the type I receptor, which transmits the specific signals through Smads. BMP receptor-regulated Smads (R-Smads), Smad1 and Smad5 form complexes with common Smad (co-Smad), Smad4 and translocate to the nucleus to regulate target gene transcription [5].

Studies in hematopoietic development in vertebrate embryos and human cord blood cells have shown the importance of Smad-mediated BMP signaling in erythropoiesis. BMP4 is the most crucial BMPs in embryonic erythropoiesis [6–9], and Smad5 is the R-Smad to induce the transcription factors crucial for erythropoiesis [10–16].

Inhibitory Smads (I-Smads) function as antagonists of R-Smad/co-Smad signaling. I-Smads, Smad6 and Smad7 prevent the activation of R-Smads by occupying the activated type I receptors. Smad6 preferentially inhibits BMP signaling [5,17]. By contrast, Smad7,

Abbreviations: BMPs,bone morphogenetic proteins; TGF-β, transforming growth factor-β; I-Smad, inhibitory Smad ; HSCs, hematopoietic stem cells; BMPRI, BMP type I receptor; R-Smads, receptor-regulated Smads; KLF1/EKLF, Kruppel-like factor 1 (erythroid); GATA, GATA binding protein; co-Smad, common Smad; ALK, activin receptor-like kinase; TβRI, TGF-β type I receptor; SCF, stem cell factor; IL, interleukin; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; Epo, erythropoietin; 7-AAD, 7-amino-actinomycin D; PI, propidium iodide; GFP, green fluorescent protein; EYFP, yellow fluorescent protein; GPA, glycophorin A; CFU-E, colony-forming unit-erythroid; BFU-E, burst-forming unit-erythroid; CFU-GM, colony-forming unit-granulocyte macrophage.

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initially identified as a TGF-β-inducible antagonist of TGF-β signaling [18], interacts with BMP type I receptor (BMPRI) and activin receptor-like kinase (ALK)-1 in addition to TGF-β type I receptor (TβRI) [19], thereby inhibiting signaling pathways of both BMP and TGF-β. Studies with overexpression systems have shown that Smad7 promotes myeloid differentiation and self-renewal of HSCs [20,21]. Smad6 was used as a tool to block BMP signaling [11]. However, physiological role of BMP-specific I-Smad, Smad6 in the regulation of hematopoiesis remained largely unknown. Here, we report that $CD34^+$ cell-specific expression of Smad6 prevents Smad5/Smad4-induced erythropoiesis in human cord blood HSCs.

2. Materials and methods

2.1. Human cord blood cell culture and viral transduction

Human cord blood was obtained from healthy full-term pregnancies at the Department of Obstetrics and Gynecology, Gil Hospital, Gachon University, Incheon, Korea. Written informed consent was obtained from all volunteers in accordance with the Declaration of Helsinki. The Institutional Review Board at Gil Hospital approved this study. $CD34^+$ cells were sorted by AriaII (BD Bioscience) after enrichment of lineage marker-negative cells using a Lineage Cell Depletion kit (Miltenyi Biotec) according to the manufacturer's instructions. Isolated $CD34^+$ cells were cultured for 48 h in X-VIVO15 serum-free medium (Lonza) containing 100 ng/mL each of stem cell factor (SCF) and Flt3 ligand, 20 ng/mL each of interleukin (IL) -6, IL-3, and granulocyte colony-stimulating factor (G-CSF) subsequently transduced for 3 days with the lentiviral particles using a shSmad6-pLL3.7 vector [22], shControl-pLL3.7 vector, Smad6-pLT-CMV-X-EYFP vector, and empty pLT-CMV-X-EYFP vectors in three consecutive rounds. Cytokines were purchased from PeproTech.

2.2. Colony formation assay

A total of 500–10^3 cord blood cells in MethoCult H4435 (StemCell Technologies) supplemented with 20 ng/mL of SCF, IL-3, IL-6, G-CSF, granulocyte macrophage colony-stimulating factor (GM-CSF), and 1 U/mL of erythropoietin (Epo) were plated in duplicate in the presence or absence of BMP4 (25 ng/mL) or TGF-β (1 ng/mL). Plates were scored on day 14 using an inverted microscope (Nikon Eclipse TS100) at 40× magnification.

2.3. Immunocytochemistry

$CD34^+$ and $CD34^-$ cells were fixed with 4% formaldehyde. For proximity ligation assay (PLA), fixed cells were permeabilized by 0.1% Triton X-100 (Sigma) for the staining with Rabbit MINUS Duolink II in situ PLA kits (OLINK) and rabbit anti-Smad6 antibody (Cell Signaling Technology). Slides were observed using a confocal microscope, LSM700 (Carl Zeiss) at 400× magnification. PLA signals were quantified using BlobFinder software (Centre for Image Analysis, Uppsala University).

2.4. Flowcytometry

CD34-PE-Cy7, CD38-APC, CD36-APC, CD11b-APC, CD14-PE-Cy7, and glycophorin A (GPA)-PE were obtained from BD Biosciences. Isotype controls were stained in parallel. For cell cycle analysis, DNA contents and Ki-67, a nuclear cell proliferation-associated antigen expressed in all active stages of the cell cycle were determined by FITC Mouse Anti-Human Ki-67 Set (BD Pharmingen). Briefly, cells were fixed by cold 80% ethanol overnight at −20 °C, then washed twice in phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA) and stained with Ki-67-FITC for 30 min at room temperature (10^6 cells/100 μL). Finally, cells were washed and resuspended in 7-amino-actinomycin D (7-AAD) solution. Phosphatidylserine exposure was measured using Annexin V Apoptosis Detection Kit APC (eBioscience). Cells were resuspended in 1× binding buffer (10^5 cells/100 μL) and incubated with Annexin V-APC for 15 min at room temperature in the dark. Cells were washed, resuspended in 1× binding buffer (200 μL). Propidium iodide (PI) staining solution was added into each sample and analyzed by flowcytometry immediately. For cell cycle and apoptosis assays, the cells in different quadrants in green fluorescent protein $(GFP)^+$ gate for shSmad6-pLL3.7 vector-transduced $CD34^+$ cells or enhanced yellow fluorescent protein $(EYFP)^+$ gate for Smad6-pLT-CMV-X-EYFP vector-transduced $CD34^+$ cells were analyzed. Stained samples were acquired by LSRII (BD Bioscience) and analyzed by FlowJo (Tree Star).

2.5. RNA isolation and quantitative RT-PCR

Total RNA was extracted and amplified from cord blood $CD34^+$ and $CD34^-$ cells using a CellAmp whole transcriptome amplification kit according to the manufacturer's instructions (TaKaRa). Real-time quantitative PCR was performed using an ABI 7900 Analyzer with SYBR Green Master Mix (Applied Biosystems). The following primers were used: GAPDH 5′-ACCACAGTCCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCTGTA-3′, Smad1 5′-AAATTGCCTACATGTTTCAATACC-3′, 5′-AAAGCCTATTTCTGTTACTGTAAATCC-3′, Smad2 and Smad3 were quantitated by presynthesized TaqMan Gene Expression Assays (Applied Biosystems): Smad2 (Hs00183425_m1), Smad3 (Hs00969210_m1), and GAPDH (Hs99999905_m1), Smad4 5′-TTGCTTCCACTTGAATGCTG-3′, 5′-CTTCAAAGGGGACACAAAA-3′, Smad5 5′-TCGAAGAGGATTGTAATCATGG-3′, 5′-CCTACAGTGCAGCCACTAGC-3′, Smad6 5′-TACCACTTCAGCCGGCTCTG-3′, 5′-AGTACGCCA CGCTGCACCAGT-3′, Smad7 5′-TACCGTGCAGATCAGCTTTG-3′, 5′-TTTGCATGAAAAGCAAGCAC-3′, BMP2 5′-TCAAGCCAAACACAAACAGC-3′, 5′- AGCCACAATCCAGTCATTCC-3′, BMP4 5′-ACGGTGGG AAACTTTTGATG-3′, 5′-CGA TCGGCTAATCCTGAA-3′, BMP6 5′-AAGA AGGCTGGCTGGAATTT-3′, 5′-GAAGGGCTGCTTGTCGTAAG-3′, BMP7 5′-TTTTCTGGATCCTCCATTGC-3′, 5′-CAAAAGCCATATGCTGCT CA-3′, ALK1 5′-GAAGAAGGTGGTGTGTGTGG-3′, 5′-TCTGAGCTAGGC CTGAGAGG-3′, ActRI 5′-CCATTACCCACGTGACACC-3′, 5′-CAGAGTTT AAAT GCACGTAATGG-3′, ActRII 5′-GCATCTTGATTGAACATCATTTAC C-3′, 5′-GGGATATGGGTTGAGACTGC-3′, BMPRI 5′-AAGCCTTGAACATCGT CCTG-3′, 5′-TCCTCTGGGAGCTTCTCTG-3′, BMPRII 5′-TAAG CTGTC TGAAGCCTTGC-3′, 5′-TCAGCTTTCATAGTGGCATCC-3′, CDKN1 A (p21) 5′-ATGAAATTCACCCCCTTTCC-3′, 5′-CCCTAGGCTGTGCTCACT TC-3′, BMI1 5′-ATGCAGCTCATCCTTCTGCT-3′, 5′-GCATCACAGTCATTGCTGCT-3′, Flt3 ligand 5′-GATGCAGAAGAAGCGATGTA TCA-3′, 5′-AGGTGTGAGGACATTCCGAAAC-3′, Oct4 5′-TCCCATGCATT CAAACTGAGG-3′, 5′-CCAAAACCCTGGCACAAACT-3′, Hoxa9 5′-CGG TGATTTAGGTAGTTTCCTGTTG-3′, 5′-GTAATGAAGGCAGTTCGTGCT G-3′, GPA 5′-CAAACGGGACACATATGCAG-3′, 5′-TCCAATAACACCAG CCATCA-3′, KLF1 5′-CCCCTCCTTCCTGAGTTGTT-3′, 5′-GTGGGAGCT CTTGGTGTAGC-3′, GATA-1 5′-CCAAGCTTCGTGGAACTCTC-3′, 5′-CC TGCCCGTTTACTGACAAT-3′, and GATA-2 5′-GTCACTGACGGAGAGCATGA-3′, 5′-GCCTTCTGAACAGGAACGAG-3′. The relative mRNA levels to GAPDH were calculated by the comparative C_t method.

2.6. Luciferase assay

The proximal promoter regions (−2.0-kilobase) of Smad6, KLF1, and GATA-1 were generated by PCR from human blood genomic DNA using PrimeSTAR™ HS DNA Polymerase (TaKaRa) and the primers: Smad6 5′-AAACTCGAGATAGTAACGACTCAATACGCAC-3′, 5′-AAAAGATCTCTCCCCCGCTCGGCTCTCTCTC-3′, KLF1 5′-AAACA CA CAATTATATGTGCACG-3′, 5′-AAACCTCAAGCCTCCTCTTCCTC-3′, GA TA1 5′-AAAGGTACCAG GTACTCAATA AATAAATAGG-3′, 5′-

AAAAAGCTTCTG GC TGGCCTTGG CCTTTGAG-3′. Products were verified by sequencing and subcloned into the pGL4 luciferase vector (Promega) using KpnI and HindIII sites. 293T cells were transfected with resulting reporter constructs or an empty pGL4 plasmid in various combinations with Flag-tagged Smad6, Smad1, Smad5, Smad4, Runx1 using polyethylenimine. Total DNA amount was adjusted by empty pcDNA3 plasmid. Cells were lysed at 48 h after transfection. Firefly luciferase and β-galactosidase activities were measured consecutively using luminometer.

2.7. Statistics

Statistical analyses were performed using an unpaired Student's *t*-test. A P-value < 0.05 was considered to indicate statistical significance.

3. Results and discussion

3.1. Specific expression of Smad6 in CD34⁺ human cord blood HSCs

Screening of the mRNA expression levels of Smads in CD34⁺ and CD34⁻ human cord blood cells showed that Smad6 mRNA was expressed in CD34⁺ cells, but not in CD34⁻ cells (Fig. 1A, the *y*-axis is presented on a logarithmic scale). The basal expression level of Smad6 mRNA was relatively lower than other Smads, as previously reported using murine HSCs and the Lhx2-hematopoietic progenitor cell line [23]. PLA confirmed the specific expression of Smad6 protein in CD34⁺ cells (Fig. 1B). Smad6 protein was localized in the cytoplasm of CD34⁺ cells (Fig. 1B). Because Smad6 is a BMP-inducible antagonist of BMP signaling [5,17], we examined the mRNA expression of BMP ligands and BMP receptor components in CD34⁺ and CD34⁻ human cord blood cells. BMP2, BMP4, BMP6, BMP7, and BMPRI were expressed significantly higher in CD34⁺ cells compared with CD34⁻ cells, although their expression levels were very low even in CD34⁺ cells (Fig. 1C, the *y*-axis is presented on a logarithmic scale). These data suggest that the miniscule autocrine BMP signaling induces Smad6 in human cord blood HSCs. To confirm the responsible R-Smads for BMP signaling to induce transcription of the *Smad6* gene, 2.0-kilobase pair of the 5′-untranslated region of the human *Smad6* gene was inserted into the pGL4 luciferase vector to perform promoter assay. Both Smad1 and Smad5 induced *Smad6* luciferase activity in combination with Smad4, and Smad1 was more potent than Smad5 (Fig. 1D). These data are consistent with the previous report that characterizes the mouse *Smad6* promoter [26]. In contrast to our results, Smad5 induces the mouse *Smad6* promoter (9.5-kilobase) activity more efficiently than Smad1, which might be due to the involvement of the distal region of the *Smad6* promoter [26].

Inhibition of Runx1 activity by Smad6 and upregulation of Smad6 by Runx1 play crucial roles in embryonic hematopoiesis [24,25]. However, Runx1 had no significant effect on Smad1/5-induced *Smad6* proximal promoter activity (Fig. 1D). This result is consistent with the previous report showing that Runx1 regulates

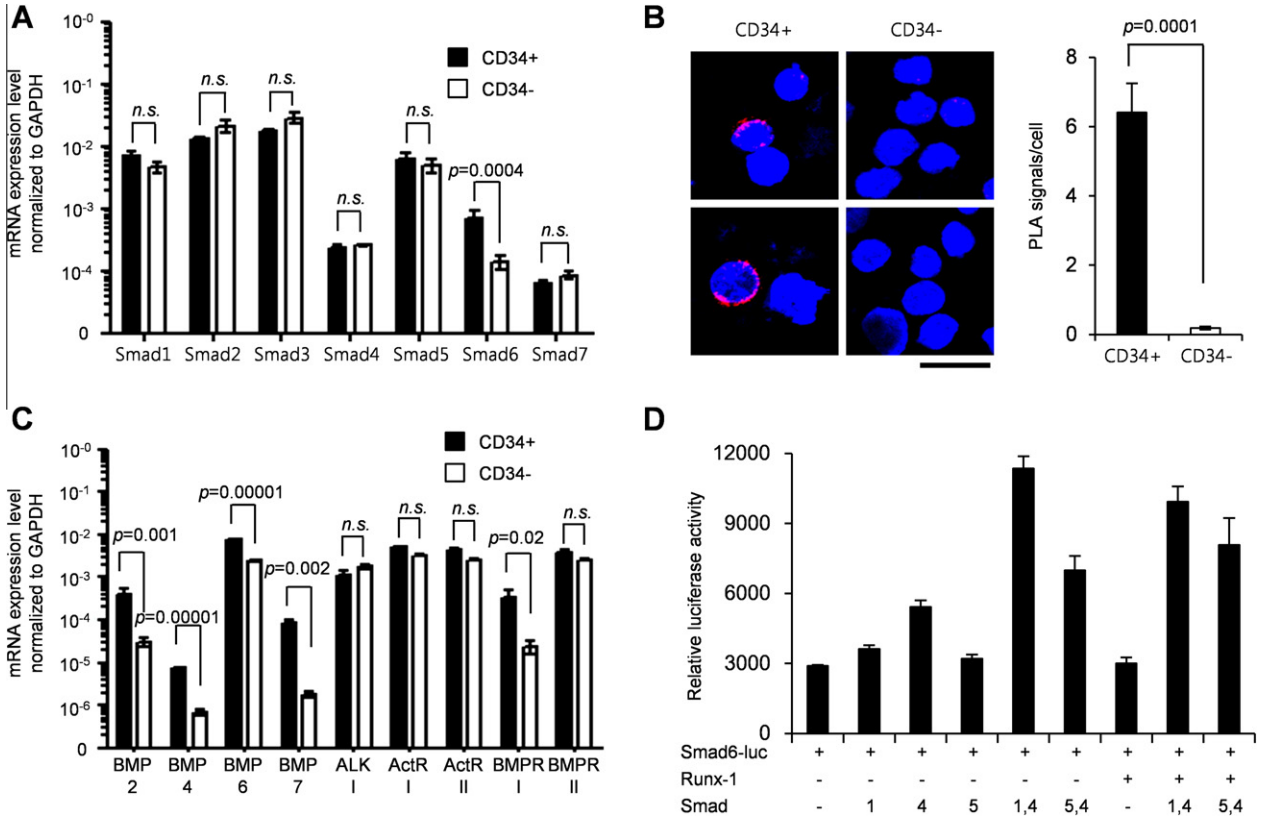

Fig. 1. Specific expression of Smad6, BMP4, and BMPRI in CD34⁺ human cord blood HSCs. (A) Total RNA was extracted from the freshly isolated CD34⁺ and CD34⁻ cord blood cells. The mRNA levels of Smads relative to GAPDH were determined by quantitative RT-PCR. (B) Expression of Smad6 protein in the freshly isolated CD34⁺ and CD34⁻ human cord blood cells was determined by proximity ligation assay. Images were acquired using a confocal microscope, LSM700. Scale bars represent 10 μm. PLA signals were quantified using BlobFinder software. (C) The mRNA levels of BMPs and BMP receptors relative to GAPDH were determined by quantitative RT-PCR. (D) 293T cells were transfected with the *Smad6* reporter construct with the indicated combinations of Smad1, Smad5, Smad4, Smad6, and Runx1. Total DNA amount was adjusted by empty pcDNA3 plasmid. Firefly luciferase activity was measured with a luminometer and normalized to β-galactosidase activity. Data are shown as mean ± SEM (n = 3–5 experiments). The y-axes of the graphs in (A) and (C) are presented on a logarithmic scale. P values were calculated by unpaired Student's t test.

the *Smad6* promoter activity via an upstream enhancer, but not proximal promoter in mouse HPC-7 hematopoietic stem/progenitor cells and human K562 erythroleukemic cells [25].

3.2. Smad6 is not required for the stemness of HSCs

To examine the role of Smad6 in $CD34^+$ cells, we applied knockdown and overexpression systems using lentiviral vectors labeled with GFP or EYFP. $CD34^+$ cells transduced with shSmad6-pLL3.7 vector or shControl-pLL3.7 vector for knockdown, and $CD34^+$ cells transduced with Smad6-pLT-CMV-X-EYFP vector or empty pLT-CMV-X-EYFP vector for overexpression were cultured in HSC condition with high concentration (100 ng/mL) of SCF and Flt3 ligand. Knockdown of Smad6 mRNA and overexpression of Smad6 mRNA by lentiviral vectors were confirmed by quantitative RT-PCR (Fig. 2A). Nearly 60% of the cells maintain $CD34^+$ phenotype in this culture condition (Fig. 2B). Unlike Smad7, which promotes self-renewal of HSCs [21], neither knockdown nor overexpression of Smad6 affected the $CD34^+$ cell numbers, cell cycle, and apoptosis of $CD34^+$ cells (Fig. 2B–D). Consistently, neither knockdown nor overexpression of Smad6 affected mRNA expression of the stem cell markers: Oct4, Flt3 ligand, and Hoxa9, cell cycle regulator: CDKN1A (p21), and self-renewal regulator: BMI1 [21,27] (Fig. 2E and F). These data suggest that the specific expression of Smad6 in HSCs is not required for the stem cell features.

3.3. Smad6 inhibits erythropoiesis in HSCs

CD36 and glycophorin A (GPA) are used as erythroid markers, because they are specifically expressed on normal human erythrocytes and erythroid progenitors [28]. GPA^+CD36^+ cells in HSC culture transduced with shSmad6-pLL3.7 vector were increased by 1.5-fold, whereas GPA^+CD36^+ cells in HSC culture transduced with Smad6-pLT-CMV-X-EYFP vector were decreased by almost 40% compared with the vector controls (Fig. 3A). By contrast, transduction of neither shSmad6-pLL3.7 vector nor Smad6-pLT-CMV-X-EYFP vector affected $CD11b^+/CD14^+$ cells (Fig. 3A). Namely, knockdown of Smad6 increased, whereas overexpression of Smad6 decreased erythroid cells without affecting myeloid cells. These data suggest that the specific expression of Smad6 in HSCs prevents erythropoiesis, but not myelopoiesis in human HSC culture with high concentration of SCF and Flt3 ligand. Therefore, the role of Smad6 in hematopoiesis is also distinct from Smad7, which augments myeloid differentiation at the expense of lymphoid commitment [20]. The early phase of erythroid differentiation is highly dependent on Epo [29]. However, knockdown of Smad6 was sufficient to enhance early erythropoiesis even in the absence of Epo (Fig. 3A). These data suggest that Smad6 prevents spontaneous erythropoiesis in HSC.

Next, we examined the role of Smad6 in Epo-induced erythropoiesis. We performed colony formation assay with methylcellulose media containing Epo, IL-3, IL-6, G-CSF, and GM-CSF in addition to low concentration of SCF (20 ng/mL). Cells transduced

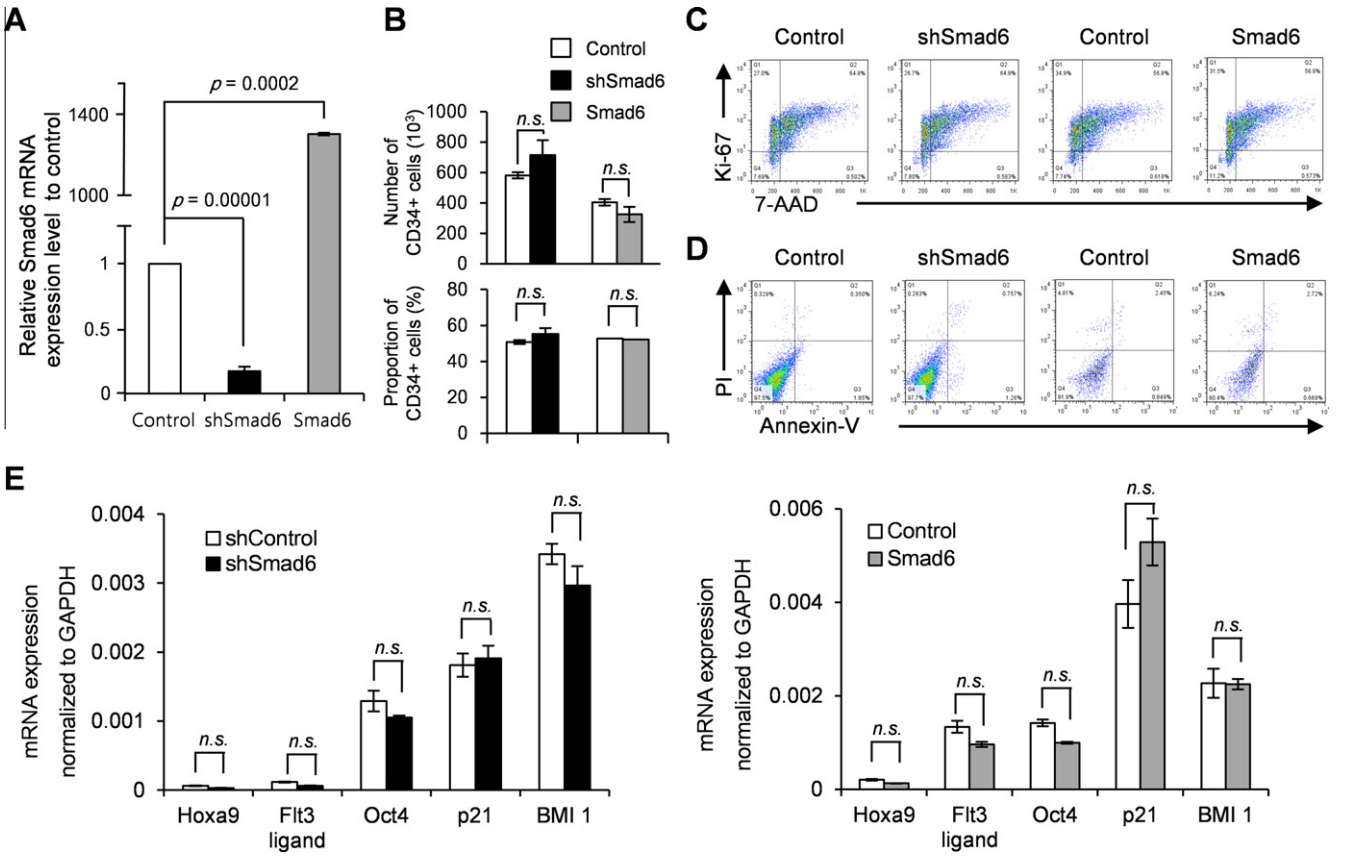

Fig. 2. Smad6 is not required for the stemness of HSCs. Human $CD34^+$ cord blood cells were cultured in X-VIVO15 serum-free medium (Lonza) containing 100 ng/mL each of SCF and Flt3 ligand, 20 ng/mL each of IL-6, IL-3, and G-CSF for 5 days. (A) The relative mRNA levels of Smad6 in the cells transduced with the indicated lentiviral vectors (black) to the cells transduced with the control vectors (white) were determined by quantitative RT-PCR. (B) Cell number and percentage of $CD34^+$ cells, (C) cell cycle, and (D) apoptosis of the cells transduced with the indicated lentiviral vectors (black) and the cells transduced with the control vectors (white) are shown. Representative dot plots are shown in (C) and (D). (E) The mRNA expression levels of Hoxa9, Flt3 ligand, Oct4, p21, and BMI1 relative to GAPDH in the cells transduced with shSmad6-pLL3.7 vector or control vector (left graph), and in the cells transduced with Smad6-pLT-CMV-X-EYFP vector or control vector (right graph) were determined by quantitative RT-PCR. Data are shown as mean ± SEM (n = 3–5 experiments). P values were calculated by unpaired Student's t test.

with shSmad6-pLL3.7 vector showed the significant increase in colony-forming unit-erythroid (CFU-E) and burst-forming unit-erythroid (BFU-E) compared with the control cells transduced with the control vector (Fig. 3B). BMP4 is known as an inducer of early and stress erythropoiesis [6–9]. Knockdown of Smad6 by shSmad6-pLL3.7 vector was more potent than the treatment with high concentration of BMP4 (25 ng/mL) to increase erythroid colony formation. Knockdown of Smad6 was capable of increasing erythroid colony formation even in the presence of high concentration of BMP4 (Fig. 3B). Oppositely, overexpression of Smad6 by Smad6-pLT-CMV-X-EYFP vector significantly suppressed the formation of CFU-E and BFU-E (Fig. 3C). These data suggest that Smad6 has an inhibitory effect on erythropoiesis in HSC culture with autocrine miniscule concentration of BMPs as well as in Epo-induced differentiation culture with exogenous high concentration of BMP4.

Neither knockdown nor overexpression of Smad6 showed significant effects on colony-forming unit-granulocyte macrophage (CFU-GM) and the inhibition of colony formation by TGF-β (Fig. 3B and C). Thus, Smad6 does not affect G-CSF/GM-CSF-induced myeloid differentiation and Smad6 does not function as I-Smad for TGF-β.

Taken together, we found that Smad6 prevented both spontaneous erythropoiesis in human cord blood HSCs and Epo-induced early erythropoiesis.

3.4. Smad6 represses Smad5/4-induced transcription of KLF1

Erythropoiesis is orchestrated by the complex networks of gene regulation [29]. Kruppel-like factor 1 (erythroid) (KLF1/EKLF), GATA binding protein 2 (GATA-2), and GATA binding protein 1 (GATA-1) are the essential erythroid lineage-restricted transcriptional regulators. We examined their mRNA expression levels in $CD34^+$ cells in HSC culture with high concentration (100 ng/mL) of SCF and Flt3 ligand. Smad6 knockdown with shSmad6-pLL3.7 vector increased KLF1 and GATA-2 significantly, which was suppressed by Smad6 overexpression with Smad6-pLT-CMV-X-EYFP vector (Fig. 4A). These data suggest that Smad6 suppresses spontaneous erythropoiesis of HSCs by repressing the expression of KLF1 and GATA-2.

BMP4 signaling through Smad1 has been implicated to play a role in induction of KLF1 and GATA-1 during embryoid body differentiation in serum-free media [11]. More recently, deletion of Smad1 in embryoid body has been reported to expand the pool of hematopoietic progenitors with enhanced expression of Gata1, Runx1 and EKLF [30]. By contrast, studies using gene deletion systems in vivo have demonstrated that BMP4 signaling via Smad5 rather than Smad1 plays crucial role in erythropoiesis [13–16]. Thus, we examined the role of Smad6 in BMP-R Smad-mediated induction of the *Klf1* gene and the *Gata1* gene. Fragments (2.0-kilobase pair) of the 5′-untranslated regions of the human *Klf1* gene and the *Gata1* gene were inserted into the pGL4 luciferase vector to generate promoter reporter constructs. Smad5, but not Smad1 induced the *Klf1* promoter activity in the presence of Smad4, which was significantly inhibited by Smad6 (Fig. 4B). Thus, Smad6 represses Smad5/4-induced transcription of the *Klf1* gene. The disparity between the reports on the involvement of Smad1 [11,30] and our results might be due to the difference in the stages of erythropoiesis.

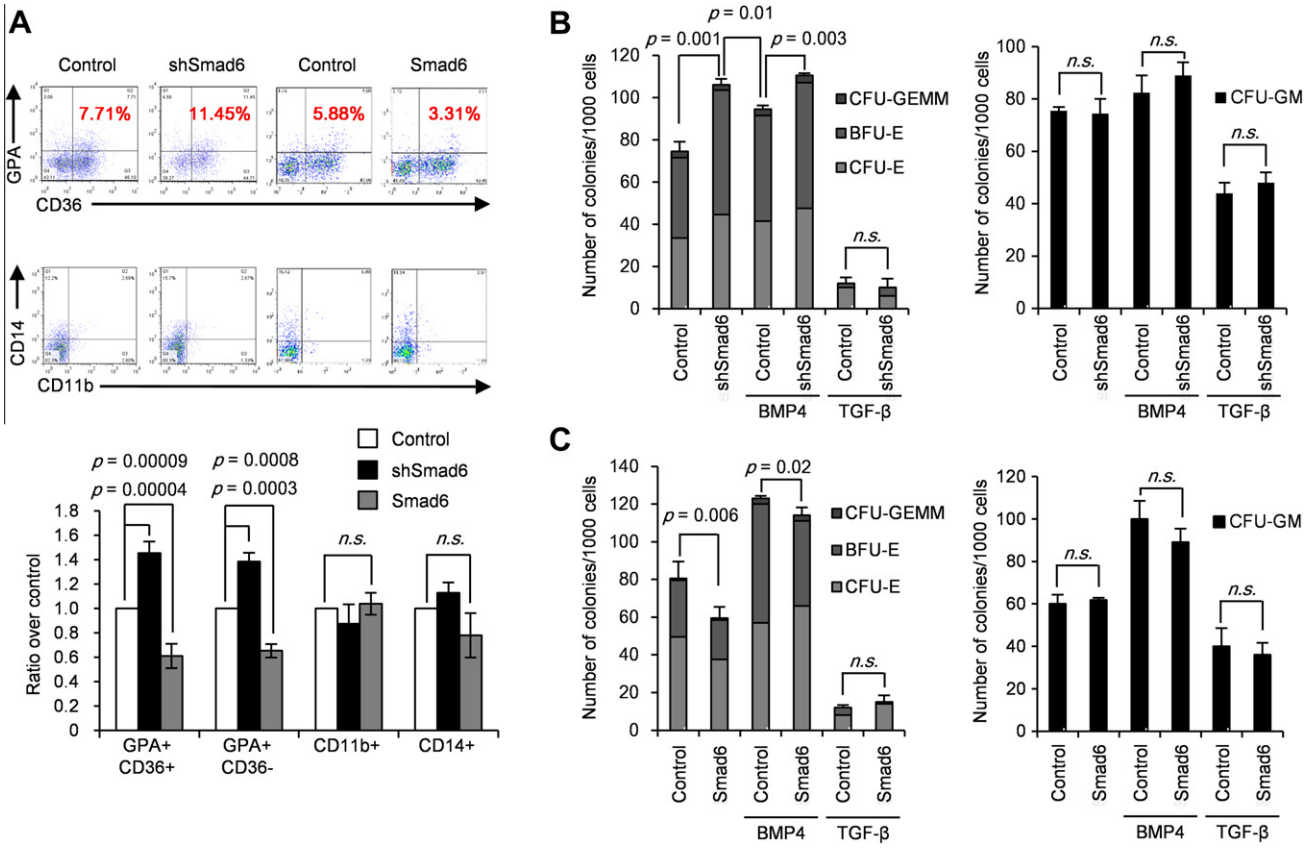

Fig. 3. Smad6 knockdown facilitates, whereas Smad6 overexpression inhibits erythropoiesis. (A) Expression of GPA, CD36, CD14, and CD11b on cord blood cells cultured in X-VIVO15 serum-free medium containing 100 ng/mL each of SCF and Flt3 ligand, 20 ng/mL each of IL-6, IL-3, and G-CSF for 5 days was determined by flowcytometry. The graph shows the ratios of GPA^+CD36^+ cells and the ratios of $CD14^+CD11b^+$ cells in $CD34^+$ cord blood cells transduced with shSmad6-pLL3.7 vector or Smad6-pLT-CMV-X-EYFP vector to those in the cells transduced with the control vectors. Colony formation of the cells transduced with (B) shSmad6-pLL3.7 vector, (C) Smad6-pLT-CMV-X-EYFP vector, and the corresponding control vector in the presence or absence of BMP4 (25 ng/mL) or TGF-β (1 ng/mL) was determined on day 14. Data are shown as mean ± SEM ($n = 3$ experiments). *P* values were calculated by unpaired Student's *t* test.

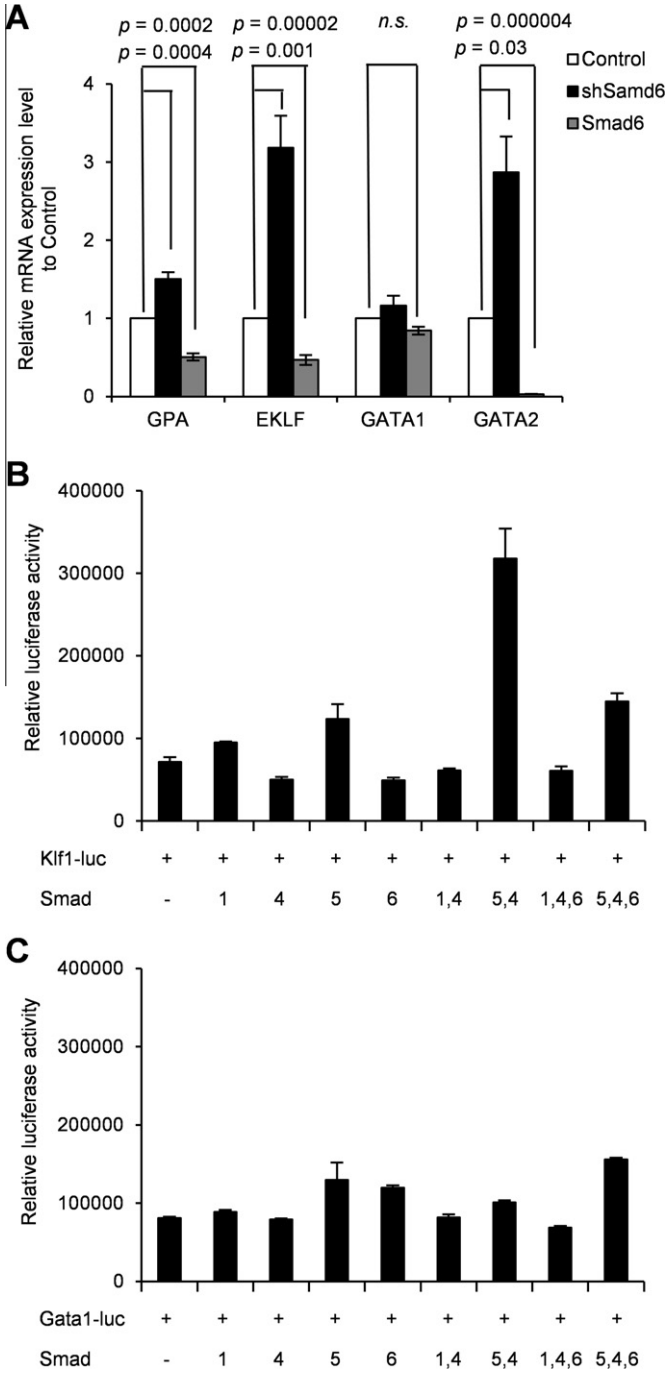

Fig. 4. Smad6 represses transcription of the erythroid lineage-restricted transcriptional regulators. (A) Total RNA was extracted from $CD34^+$ cord blood cells transduced with shSmad6-pLL3.7 vector or Smad6-pLT-CMV-X-EYFP vector, and the corresponding control vectors cultured with 100 ng/mL each of SCF and Flt3 ligand, 20 ng/mL each of IL-6, IL-3, and G-CSF for 5 days. The mRNA expression levels of GPA, KLF1, GATA-1, and GATA-2 relative to GAPDH were determined by quantitative RT-PCR. The graph shows the ratios of mRNA levels in $CD34^+$ cells transduced with shSmad6-pLL3.7 vector or Smad6-pLT-CMV-X-EYFP vector to those in $CD34^+$ cells transduced with the control vectors. 293T cells were transfected with (B) *Klf1* reporter, (C) *Gata1* reporter constructs with the indicated scombinations of Smad1, Smad5, Smad4, and Smad6. Total DNA amount was adjusted by empty pcDNA3 plasmid. Firefly luciferase activity was measured with a luminometer and normalized to β-galactosidase activity. Data are shown as mean ± SEM ($n = 3$ experiments). *P* values were calculated by unpaired Student's *t* test.

Despite of the existence of GC-rich Smad binding element [31] in the promoter region of the *Gata1* gene, the *Gata1* promoter activity was unaffected by Smad1/5/6 (Fig. 4C), which was consistent with the mRNA expression pattern (Fig. 4A). Further studies are required to determine the cooperative effects between BMP-Smads and other transcription factors to induce transcription of the *Gata1* gene, such as hypoxia-inducible factor 1 and p73 [32,33].

In conclusion, this study shows a novel function of Smad6 in HSC: HSC-specific expression of Smad6 suppresses spontaneous erythropoiesis, thereby indirectly maintains stem cell features.

Acknowledgments

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