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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′-