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# Cooperative Effect of Erythropoietin and TGF-β Inhibition on Erythroid Development in Human Pluripotent Stem Cells

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## ABSTRACT

Patient-specific human induced-pluripotent stem cells (hiPSCs) represent important cell sources to treat patients with acquired blood disorders. To realize the therapeutic potential of hiPSCs, it is crucial to understand signals that direct hiPSC differentiation to a hematopoietic lineage fate. Our previous study demonstrated that $CD34^+CD31^+$ cells derived from human pluripotent stem cells (hPSCs) contain hemato-endothelial progenitors (HEPs) that give rise to hematopoietic cells and endothelial cells. Here, we established a serum-free and feeder-free system to induce the differentiation of hPSC-derived $CD34^+CD31^+$ progenitor cells to erythroid cells. We show that extracellular matrix (ECM) proteins promote the differentiation of $CD34^+CD31^+$ progenitor cells into $CD235a^+$ erythroid cells through $CD41^+CD235a^+$ megakaryocyte-erythroid progenitors (MEP). Erythropoietin (EPO) is a predominant factor for $CD34^+CD31^+$ progenitor differentiation to erythroid cells, whereas transforming growth factor beta (TGF-β) inhibits the development of $CD34^+CD31^+$ progenitor cells. Apoptosis of progenitor cells is induced by TGF-β in early erythroid differentiation. Suppression of TGF-β signaling by SB431542 at early stage of $CD34^+CD31^+$ progenitor differentiation induces the erythroid cell generation. Together, these findings suggest that TGF-β suppression and EPO stimulation promote erythropoiesis of $CD34^+CD31^+$ progenitor cells derived from hPSCs. J. Cell. Biochem. 116: 2735–2743, 2015. 

**KEY WORDS:** ERYTHROID PROGENITORS; HUMAN PLURIPOTENT STEM CELLS; HEMATOPOIETIC STEM AND PROGENITOR CELLS; TGF-BETA

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are capable of expansion indefinitely and of differentiation into various types of tissue cells. The properties of self-renewal and differentiation ensure them not only have the potential as cell sources for regenerative medicine, but also provide a unique model system for studying lineage commitment and early human development, including ontogeny of the hematopoietic system [Murry and Keller, 2008; Passier et al., 2008]. Recent studies of hESCs and hiPSCs have advanced our understanding of hPSC differentiation into hematopoietic stem and progenitor cells (HSPCs) through the formation of embryoid bodies (EBs), and/or co-culture with hematopoietic-supporting stromal cells [Kaufman et al., 2001; Ng et al., 2005; Vodyanik et al., 2005]. Erythroid cells have been generated from hPSCs in several laboratories [Lu et al., 2008, 2010; Ma et al., 2008; Dias et al., 2011). However, factors and signals that direct hPSC commitment to erythroid lineage remain largely unknown.

TGF-β and related factors exert autocrine and/or paracrine effects on hematopoiesis by regulating cell proliferation, differentiation, and survival [Blank and Karlsson, 2015]. Administration of TGF-β into mice results in suppressing erythropoietin (EPO)-dependent erythropoiesis in vivo [Chuncharunee et al., 1993]. During hPSC differentiation, suppression of TGF-β signaling by SB431541, a specific TGF-β signaling inhibitor, promotes definitive hematopoiesis and the generation of hematopoietic progenitors from hemogenic endothelial cells [Kennedy et al., 2012; Wang et al., 2012; Sturgeon et al., 2014]. However, it has been shown that TGF-β1 and TGF-β3 are positive regulators mediating hematopoietic differentiation from hESC in coculture with the AGM and fetal liver

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derived stromal cells [Ledran et al., 2008]. Knockout TGF-β receptor I impairs erythroid development [Dickson et al., 1995; Larsson et al., 2001]. A possible explanation of contradicted findings of TGF-β function in hematopoiesis is that TGF-β effects depend on the developmental stage and other niche components. We recently demonstrated that positive and negative effects of TGF-β on the development of $CD34^+CD31^+$ hemato-endothelial progenitors (HEPs) dependent differentiation time window of hPSCs [Bai et al., 2013].

We established a serum-free and feeder-free system to differentiate $CD34^+CD31^+$ progenitors into erythroid cells. Here, we show that extracellular matrix (ECM) proteins play a critical role in the differentiation of $CD34^+CD31^+$ progenitor cells into erythroid cells through megakaryocyte-erythroid progenitor (MEP) intermediate, a process predominantly regulated by EPO. TGF-β signaling negatively regulates erythroid progenitor development at early stage of erythroid differentiation, while TGF-β inhibition at late stage had no or less effect on erythroid cell generation. These findings suggested that TGF-β suppression coordinates EPO signaling to promote erythroid progenitor development.

## MATERIALS AND METHODS

### CELL LINES

H1 and H9 hESC lines were obtained from WiCell research Institute (Madison, WI). The HDFa-YK26 and TZ1 hiPSCs were kind gifts from Dr. Ren-He Xu at University of Connecticut Health Center [Wang et al., 2009]. BC1 hiPSCs were obtained from Dr. Linzhao Cheng at Johns Hopkins University [Chou, 2011]. The hPSCs were cultured on mitotic-inactivated MEF feeder cells in medium containing DMEM/F-12 (Life Technologies, Carlsbad, CA), 20% knockout serum replacement (KSR, Life Technologies), 0.1 mM nonessential amino acids (Life Technologies), 2 mM L-glutamine (Mediatech, Inc, Herndon, VA), 0.1 mM β-mercaptoethanol (Sigma Aldrich, St, Louis, MO), and 5 ng/ml FGF2 (PeproTech, Rocky Hill, NJ). The hPSCs were subcultured every 5–6 days by Accutase treatment followed by mechanical scrapping and the medium was changed daily. In some experiments, hPSCs were cultured on Vitronectin-coated plates in E8 medium (Life Technologies), and growth media was changed every day. The hPSCs were sub-cultured every 4 to 5 days with a treatment of 0.5 mM EDTA for passaging.

### FORMATION OF EMBRYOID BODIES (EBS) AND ERYTHROID DIFFERENTIATION

To induce hPSC differentiation, hESCs and hiPSCs were treated with Accutase at 37°C for 3–5 min to remove feeder cells and generate single cell suspension by passing dissociated cells through a 40 μm cell strainer as previously described [Bai et al., 2012, 2013]. EBs were formed by hanging-drop method and cultured in hPSC medium without FGF2. After 2 days, EBs were cultured in serum-free differentiation medium containing IMDM/F12 (1:1), 0.5% BSA (Sigma Aldrich), 1% ITS-X (Life Technologies), 1% chemical defined lipid (Life Technologies), 2 mM Glutamax (Life Technologies), 50 μg/ml L-ascorbic acid (Sigma Aldrich), and 450 μM 1-Thioglycerol (Sigma Aldrich). The differentiation media were changed every 2 days with growth factors as indicated in Fig. 1A. EBs were harvested between day 8 to 10, and treated with TrypLE (Life Technologies) to dissociate EBs [Bai et al., 2013]. $CD34^+CD31^+$ cells were isolated by fluorescence-activated cell sorting (FACS) with FACSAria II (BD Biosciences, Franklin Lakes, NJ). In some experiments, the $CD34^+CD31^+$ cells were positively selected by using the MultiSort immunomagnetic separation system (Miltenyi Biotec, San Diego, CA) following the manufacturer's instruction.

For erythroid differentiation, isolated $CD34^+CD31^+$ cells were then cultured at $2 \times 10^4$ cells/ml in differentiation medium containing stem cell factor (SCF, 50 ng/ml), Flt3-ligand (F3L, 50 ng/mL), erythropoietin (EPO, 3U/mL), thrombopoietin (TPO, 50 ng/ml), in a 12-well plate coated with collagen I (BD Biosciences) or other ECM proteins for 15 days. Hematopoietic clusters containing round cells were emerged from adherent monolayer around day 2–3.

### FLOW CYTOMETRIC ANALYSIS

Hematopoietic cells in suspension were harvested at different days, and analyzed by flow cytometry in PBS with 1% fetal bovine serum (FBS) and 1 mM EDTA. Samples were incubated with isotypic antibodies or indicated monoclonal antibodies for 30 min at 4°C. After the incubation with antibodies, cells were washed three times with PBS prior to analysis. 7-Aminoactinomycin Via-Probe staining solution (BD) was added to samples just before analysis to exclude dead cells. The data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR). The monoclonal antibodies used for identification and characterization of cell subsets included CD41, CD11b, CD14, CD33, CD45, CD71, and CD235a (eBiosciences).

### CFC ASSAY (HEMATOPOIETIC COLONY-FORMING CELL ASSAY)

Hematopoietic colony-forming cell (CFC) assay was performed as described in our previous studies [Chen et al., 2007; Wang et al., 2007]. Briefly, cells ($1 \times 10^4$) were mixed with MethoCult® methylcellulose-based media containing EPO (H4434, StemCell Technologies, Vancouver, Canada), following manufacturer's instruction. The hematopoietic colonies were photographed and counted after 2 weeks of culture. Each type of colony was classified according to morphology. Each assay was performed in triplicate.

### QUANTITATIVE REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

Total RNAs from undifferentiated hPSCs and EBs at different time points were isolated by using Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). To eliminate DNA contamination, RNA samples were treated with DNase I (RNase free), following manufacturer's instruction. Total RNA (1 μg) was used for each reverse transcription reaction with SuperScript III (Invitrogen). Real-time qPCR was performed on an iQ5 thermal cycler (Bio-Rad Laboratories, Hercules, CA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard for normalization. Specific primers for each globin were as followings: ε-globin (embryonic) forward (GCCTGTGGA GCAAGATGAAT) and reverse (GCGGGCT TGAGGTTGT); γ-globin (fetal) forward (CTTCAAGCTCCTGGGAAATGT) and reverse (GCAGAATAAAGCCTACCTTGAAAG); β-globin (adult) forward (GGCACCTTTGCCACACTG) and reverse

(CACTGGTGGGGTGAATTCTT). Expression levels were estimated by minimal cycle threshold values (Ct) normalized to the reference expression of GAPDH in each sample.

### STATISTICAL ANALYSIS

Results were expressed as mean ± SD and analyzed by software GraphPad prism 5. Statistical significance was determined using an unpaired Student t-test. Results were considered statistically significant when values of $P$ less than 0.05.

## RESULTS

### ESTABLISHMENT OF A SERUM-FREE AND FEEDER-FREE SYSTEM TO GENERATE CD34+CD31+ PROGENITOR CELLS FROM HPSCS

Considering undefined factors in EB differentiation and co-culture with hematopoiesis-supporting stromal feeder cells, we established a two-step process to induce erythropoiesis in hPSCs in serum-free and feeder-free condition (Fig. 1A). The first step was to differentiate hPSCs to generate $CD34^+CD31^+$ progenitor cells. The second step was to isolate $CD34^+CD31^+$ progenitor cells, and induce their differentiation into erythroid cells.

We previously used BMP4, VEGF, and FGF2 to induce hPSC differentiation into $CD34^+CD31^+$ progenitor cells [Bai et al., 2010, 2013]. To investigate whether hematopoietic factors promote the development of $CD34^+CD31^+$ progenitor cells from mesodermal progenitors, we added various factors, including VEGF (V), FGF2 (F), SCF (S), F3L (L), TPO (T) from day 6 to day 10 during EB differentiation. VEGF and FGF-2 enhanced the development of $CD34^+CD31^+$ progenitor cells in a serum-free differentiation condition, whereas addition of hematopoietic factors, SCF, F3L, and TPO, had no effect on the development of $CD34^+CD31^+$ progenitor cells (Fig. 1B), suggesting that exogenous hematopoietic factors are not required for the development of $CD34^+CD31^+$ progenitor cells from mesoderm precursors during EB differentiation. To confirm endothelial and hematopoietic differentiation potential of $CD34^+CD31^+$ progenitor cells [Bai et al., 2013], $CD34^+CD31^+$ progenitor cells were isolated from EB day 10, and were induced for hematopoietic differentiation. Both adherent cells and round-shape cells were observed after 4 days. Adherent cells expressed endothelial cell marker, VE-cadherin (VE-Cad), whereas some of round hematopoietic cells expressed erythroid marker, CD235a (Glycophorin A) (Fig. 1C).

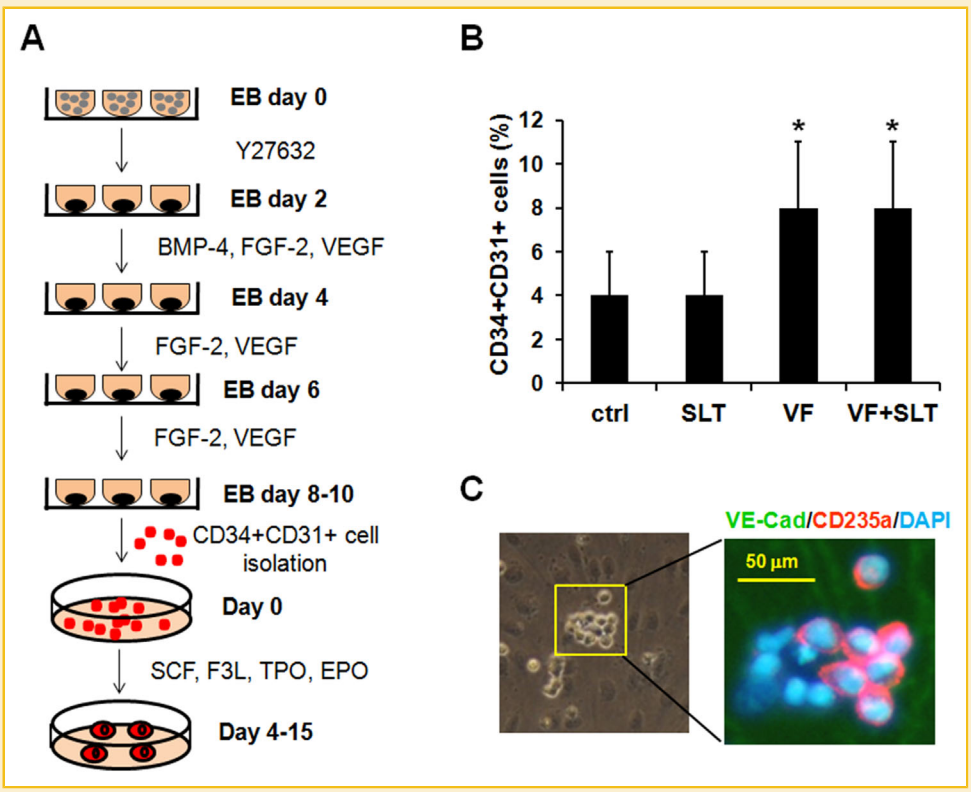


Fig. 1. Development of $CD34^+CD31^+$ progenitor cells from hPSCs. A: Schematic outline of experimental design. EB differentiation was performed by a hanging-drop method. After 2 days, EBs were collected into ultralow attachment dishes with growth factors as indicated. B: $CD34^+CD31^+$ cells were analyzed after 10 days of EB differentiation by flow cytometry. The cytokines were added after EB day 6, as following groups: i) Control (ctrl), ii) SCF, F3L, and TPO (SLT), iii) VEGF and FGF2 (VF), and iv) combination of group 2 and 3 (VF+SLT). Data were represented as Mean ± SD from three independent experiments. $^*P < 0.05$ between experimental group and control group (ctrl). C: Representative images of endothelial and hematopoietic cells derived from CD34+CD31+ progenitor cells. Hematopoietic clusters were analyzed by staining with anti-human CD235a-PE. Endothelial cells were identified by anti-human CD144-FITC. Nuclei were detected by DAPI.

### PROMOTING EFFECT OF EXTRACELLULAR MATRIX PROTEINS ON HEMATOPOIETIC DIFFERENTIATION FROM CD34+CD31+ CELLS

Collagen I is a major extracellular matrix (ECM) protein in bone marrow, which provide niche to anchor hematopoietic stem and progenitor cells (HSPCs) [Scadden, 2007]. To mimic hematopoietic-supporting niche in a feeder-free condition, we explored the effect of ECM on hematopoietic differentiation from $CD34^+CD31^+$ progenitor cells. $CD34^+CD31^+$ cells isolated from EB day 8–10 were cultured on regular tissue culture plates or plates coated with collagen I. We observed few cells grown on regular tissue culture plates, whereas the numbers of cells on collage I-coated plates were significantly increased. Similar results were obtained from hESCs or hiPSCs (Fig. 2A). To assess the effect of different ECM proteins on cell proliferation, we cultured $CD34^+CD31^+$ progenitor cells on plates coated with various ECM, including gelatin, collagen I, and fibronectin. CCK-8 assay was used to determine cell growth index at different time points (Fig. 2B). Compared with regular tissue culture plates, cells cultured on plates coated with collagen I, fibronectin, or collagen/fibronectin had a significant increased proliferative index (Fig. 2B). To monitor endothelial-to-hematopoietic transition (EHT) from $CD34^+CD31^+$ progenitor cells, we assess hematopoietic clusters that emerged from adherent cells and then formed semi-attached round cells within 2–4 days (Fig. 2C). The numbers of hematopoietic clusters were 10 to 15-fold higher in ECM-coated plates than in regular tissue culture plates (Fig. 2D), suggesting that ECM proteins foster hematopoietic development from $CD34^+CD31^+$ progenitor cells.

### ERYTHROID DIFFERENTIATION OF $CD34^+CD31^+$ PROGENITOR CELLS PROMOTED BY EPO

To investigate factors that direct erythroid differentiation, we cultured $CD34^+CD31^+$ cells on collagen I-coated plates in a medium supplemented with various hematopoietic growth factors to induce erythroid differentiation. As shown in Fig. 3A, the numbers of cells cultured in the presence of SCF (S), F3L (L), and EPO (E), or in the presence of SCF, F3L, TPO (T), and EPO were significantly higher than those cultured without EPO (SL or SLT). The majority cells cultured with SCF, F3L, TPO, and EPO for 6 days expressed erythroid marker, CD235a, but not CD45, a pan-hematopoietic marker (Fig. 3B). The erythroid progenitor cells continued to proliferate and reached to a four-fold increase between day 6–10 (Fig. 3C). The number of erythroid cells was not or modestly increased by day 15, compared to that by day 10, indicating that the proliferation of erythroid progenitor cells reaches to a peak by day 10.

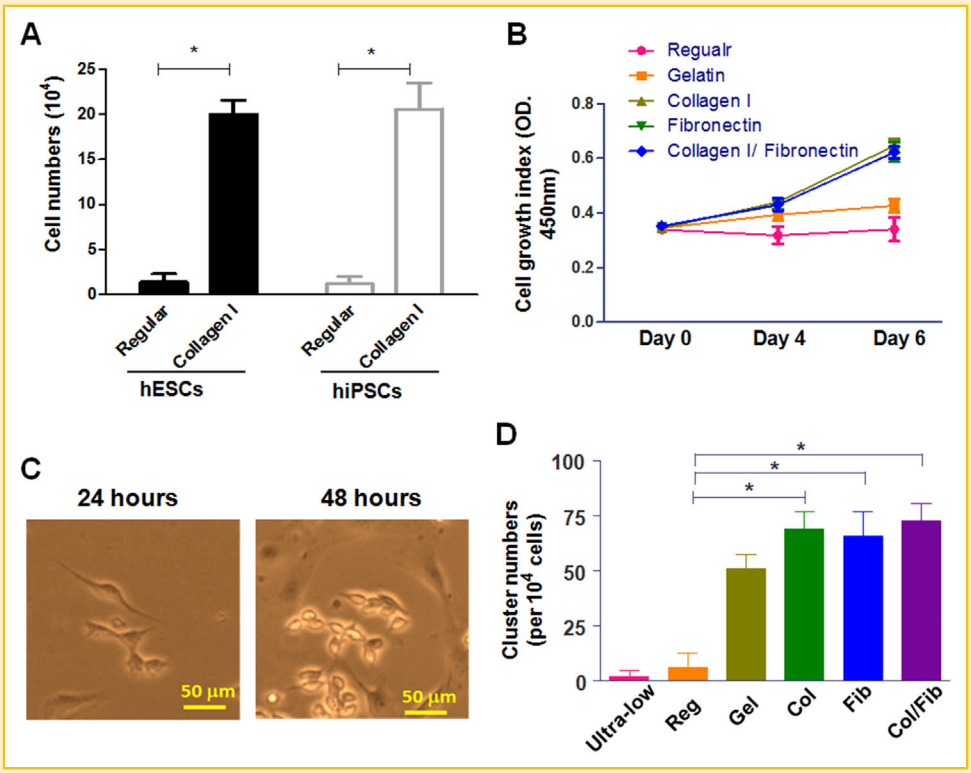


**Fig. 2.** Effect of extracellular matrix (ECM) proteins on hematopoietic differentiation. A: Isolated $CD34^+CD31^+$ cells from hESCs or hiPSCs were cultured on plates coated without (Regular) or with collagen-I. The cell numbers were counted after 6 days. B: Growth of hPSC-derived cells on regular tissue culture plates (Regular), or plates coated with gelatin, collagen-I, fibronectin, and mixture of collagen-I and fibronectin (1:1). WST-8 cell proliferation kit was used to determine the growth index by measuring absorbance at 450 nm wavelength using a microplate reader. C: Representative images of adherent cells at 24 hours and hematopoietic clusters at 48 h. D: Development of hematopoietic clusters. $CD34^+CD31^+$ cells from EB day 10 were cultured in ultra-low attachment plates (Ultra-low), regular tissue culture plates (Reg), or plates coated with gelatin (Gel), collagen-I (Col), fibronectin (Fib), mixture of collagen-I and fibronectin (Col/Fib). The numbers of hematopoietic clusters were counted on day 4 of erythroid differentiation. Data were represented as Mean ± SD from three independent experiments.

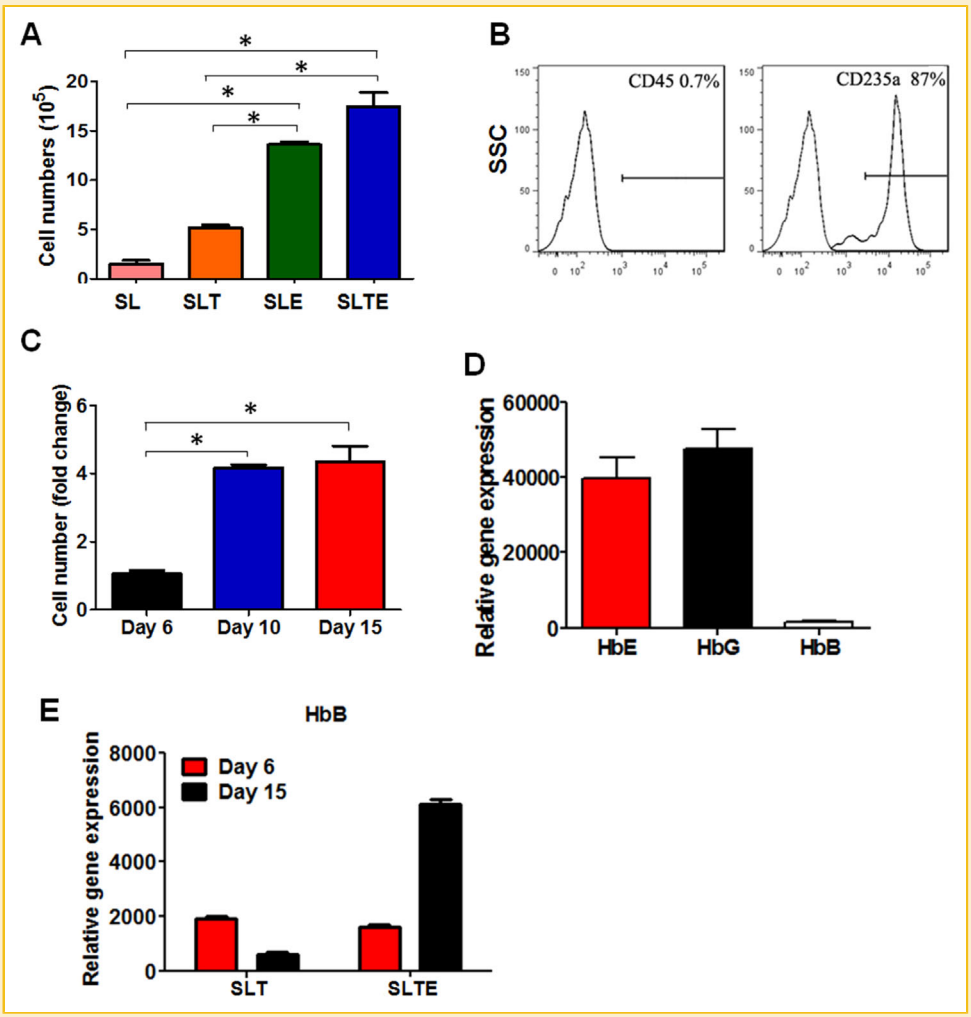


**Fig. 3.** EPO effect on erythroid differentiation of CD34+CD31+ progenitor cells. A: $CD34^+CD31^+$ progenitor cells isolated from EB day 10 were cultured on collagen I coated plated in differentiation medium containing i) SCF and F3L (SL), ii) SCF, F3L, and TPO (SLT), iii) SCF, F3L, and EPO (SLE), or iv) SCF, F3L, TPO, and EPO (SLTE). After 6 days, the numbers of cells in suspension were counted. B: A representative flow cytometric analysis of cells harvested from suspension after 6 days of erythroid differentiation $CD34^+CD31^+$ progenitor cells in the presence of SCF, F3L, TPO, and EPO. C: $CD34^+CD31^+$ progenitor cells from EB day 10 were cultured on collagen I in the presence of SCF, F3L, TPO, and EPO. The cell numbers of erythroid cells at different time points were counted during erythroid differentiation. D: Hemoglobin expression. $CD34^+CD31^+$ progenitor cells were induced to erythroid differentiation in the presence of SCF, F3L, TPO and EPO. RNA samples were harvested after 6 days of erythroid differentiation. Real-time RT-PCR was performed using specific primers for embryonic hemoglobin (HbE), fetal hemoglobin (HbG), and adult hemoglobin (HbB). GAPDH was used as an internal standard. E: HbB expression. $CD34^+CD31^+$ progenitor cells were induced to erythroid differentiation in the presence of SCF, F3L, and TPO (SLT) or SCF, F3L, TPO and EPO (SLTE). RNA samples were harvested on day 6 and day 15, and subjected for real-time RT-PCR analysis. GAPDH was used as an internal standard. Data were represented as Mean ± SD from three independent experiments.

Gene expression pattern of hemoglobin variants represents the progress of erythropoiesis during the development. The majority of cells primitive hematopoiesis in yolk sac are primitive erythroid cells that remain nucleated and synthesize mainly embryonic hemoglobin (ε, or HbE), whereas fetal hemoglobin (γ, or HbG) and adult hemoglobin (β, or HbB) are mostly synthesized in fetal liver and adult bone marrow during definitive erythropoiesis from HSCs [Palis, 2014]. Using hemoglobin as an indicator, we tested whether $CD34^+CD31^+$ progenitor cells differentiate to primitive and/or definitive erythrocytes. As shown in Fig. 3D, revealed by real-time RT-PCR, both embryonic globin (HbE) and fetal-globin (HbG) were highly expressed in erythroid cells after 10 days of differentiation, whereas the level of adult globin (HbB) expression was very low. Adult globin (HbB) expression significantly increased from day 6 to day 15 (Fig. 3E) in the presence of EPO. Our data suggested that both primitive- and definitive-erythropoiesis occur during the differentiation of $CD34^+CD31^+$ progenitor cells.

We next determined whether EPO promotes erythroid differentiation from megakaryocyte-erythroid progenitor (MEP). The MEP population that expresses both erythroid marker CD235a and megakaryocytic marker CD41 was analyzed by flow cytometry during erythroid differentiation. By day 6, approximately 50% cells

were $CD41^+CD235a^+$ MEP without EPO, whereas $CD41^+CD235a^+$ cells decreased to 8% in the presence of EPO (Fig. 4A). $CD235a^+CD41^-$ erythroid cells were 9% without EPO, and 78% with EPO (Fig. 4A), suggesting that EPO is a major factor to direct erythroid differentiation from MEP. Kinetic analysis showed that $CD41^+CD235a^+$ MEP were gradually decreased during erythroid differentiation in the presence of EPO (Fig. 4B). By day 15, the majority cells were $CD235a^+CD41^-$ erythroid cells, whereas $CD41^+CD235a^+$ MEP, $CD41^+CD235a^-$ megakaryocytes, and $CD14^+$ and $CD33^+$ myeloid cells were less than 1% (Fig. 4C).

## NEGATIVE EFFECT OF TGF-β SIGNALING ON THE DEVELOPMENT OF ERYTHROID PROGENITORS

We previously demonstrated that TGF-β signaling play a role in regulating hematopoietic and vascular progenitors from hPSCs [Bai et al., 2010, 2013]. To determine a role TGF-β signaling in the development of $CD34^+CD31^+$ progenitor cells, we added TGF-β1 and SB421543, a specific inhibitor of TGF-β signaling, to EB culture between day 6 and day 10. As shown in Fig. 5A, additional TGF-β1 had no significant effect on the generation of $CD34^+CD31^+$ progenitor cells, whereas inhibition of TGF-β signaling by SB421543 enhanced the generation of $CD34^+CD31^+$ progenitor cells, consistent with our previous study [Bai et al., 2013].

To further investigate the role of TGF-β signaling in the development of erythroid progenitors, we added TGF-β1 or SB431542 at different time points during erythroid differentiation from $CD34^+CD31^+$ progenitor cells. When TGF-β1 was added at early stage of erythroid differentiation (day 0 to 2), the numbers of erythroid cells were significantly decreased (Fig. 5B). Further addition of TGF-β1 had no or moderate effect on erythroid cell generation. The negative effect of TGF-β on erythroid development was diminished when TGF-β1 was added into culture after 2 days of erythroid differentiation (Fig. 5B). Conversely, the numbers of erythroid cells were significantly increased in the presence of SB431542 during early stage of erythroid differentiation (day 0–4), whereas further inhibition of TGF-β signaling had no significant effect (Fig. 5C). These data suggested that the negative effect of TGF-β signaling is restricted in the early stage of erythroid differentiation from $CD34^+CD31^+$ progenitor cells.

To determine whether TGF-β signaling inhibits erythroid progenitor generation, we performed CFC assay to quantify the numbers of erythroid progenitor cells in the presence of TGF-β1 for 6 days. Consistent with cell numbers, the number of colonies for BFU-E and CFU-E were significantly decreased in the presence of TGF-β1 (Fig. 5D), suggesting that the potential of erytthroid progenitor cells from $CD34^+CD31^+$ progenitor cells is inhibited by TGF-β signaling. TUNEL assay was used to detect DNA fragmentation that results from apoptotic cells during the early stage differentiation of $CD34^+CD31^+$ progenitor cells. In the presence of TGF-β1, the number of apoptotic cells were significantly increased (Fig. 5E), suggesting that suppression of TGF-β signaling enhances progenitor cell survival during erythroid differentiation.

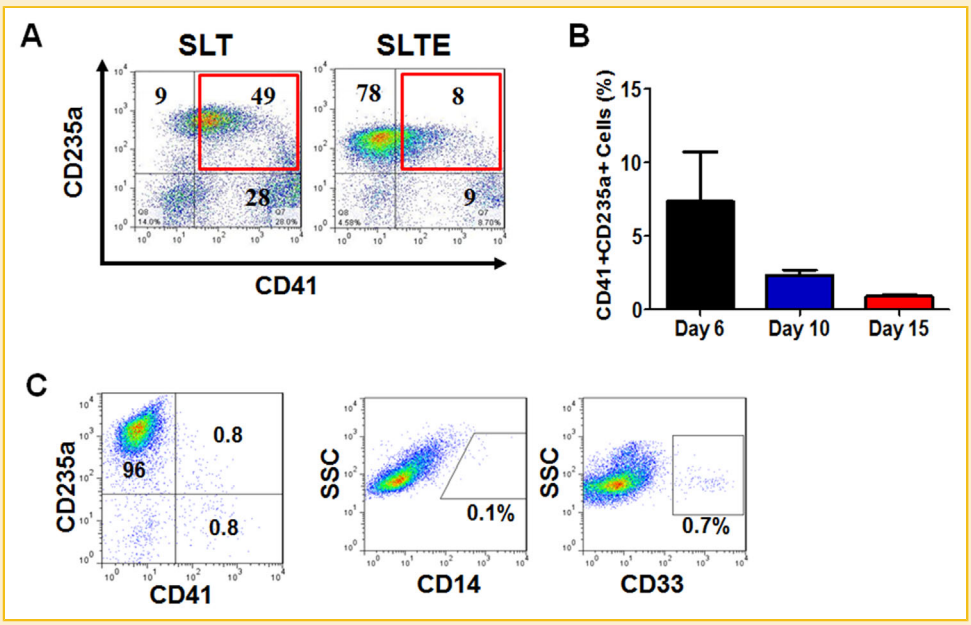


Fig. 4. EPO effect on megakaryocyte-erythroid progenitor (MEP) differentiation. A: Representative flow cytometry of $CD41^+CD235^+$ MEP during erythroid differentiation. $CD34^+CD31^+$ progenitor cells were cultured on collagen I in the presence of SCF, F3L, and TPO (SLT) or SCF, F3L, TPO, and EPO (SLTE). Cells in suspension were harvested after 6 days of erythroid differentiation. $CD41^+CD235^+$ MEP were analyzed by flow cytometry. B: MEP at different days during erythroid differentiation. $CD34^+CD31^+$ progenitor cells were cultured on collagen I in the presence of SCF, F3L, TPO, and EPO (SLTE). Cells in suspension were harvested on day 6, 10, and 15 during erythroid differentiation. $CD41^+CD235^+$ MEP were analyzed by flow cytometry. Data were represented as Mean ± SD from three independent experiments. C: Flow cytometry of CD41, CD235a, CD14, and CD33 after 15 days of erythroid differentiation in the presence of SCF, F3L, TPO, and EPO (SLTE).

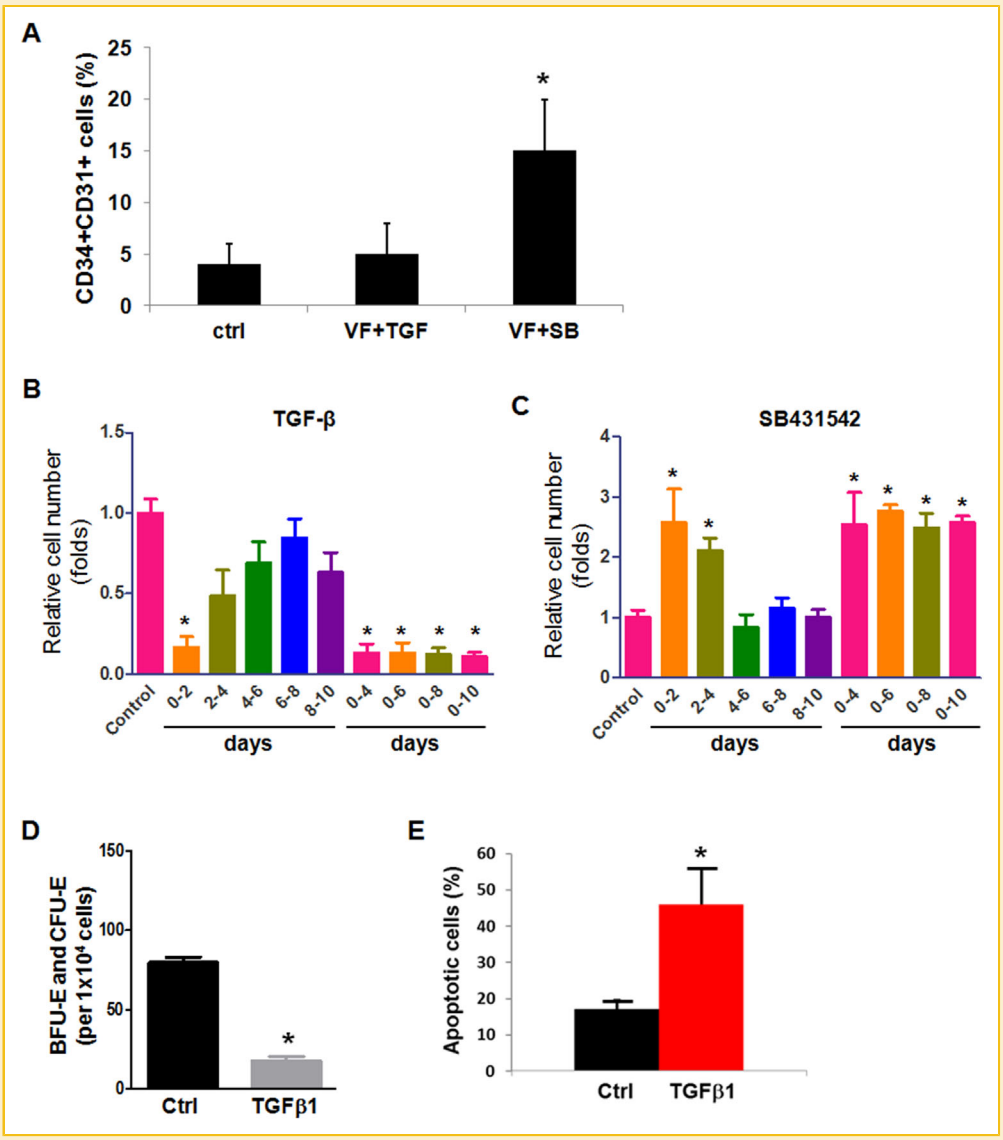


**Fig. 5.** Effect of TGF-β signaling on erythroid differentiation. A: Effect of TGF-β signaling on the development of $CD34^+CD31^+$ progenitor cells. $CD34^+CD31^+$ progenitor cells were analyzed after 10 days of EB differentiation by flow cytometry. The cytokines were added after EB day 6, as following groups: i) Control (ctrl), ii) VEGF, FGF2, and TGF-β1 (VF+TGF), and iv) VEGF, FGF2, and SB431542 (VF+SB). The number of erythroid cells on day 10. B,C: TGF-β effect on erythroid cell generation. $CD34^+CD31^+$ progenitor cells were induced to erythroid differentiation in the presence of TGF-β1 (B) or SB431542 (C). TGF-β1 or SB431542 was added into differentiation medium for different days. The number of erythroid cells were counted on day 10. D: CFC assay. Erythroid cells on day 6 of erythroid differentiation with or without TGF-β1 were subjected for CFC assay. Hematopoietic colonies were counted on day 14. E: TUNEL assay. $CD34^+CD31^+$ progenitor cells were induced to erythroid differentiation with or without TGF-β1. Apoptotic cells were analyzed after 48 h by TUNEL assay. Data were represented as Mean ± SD from three independent experiments. $^*P < 0.05$ between experimental group and control group (ctrl).

## DISCUSSION

Stem cells reside in specialized niches and their behavior are regulated by coordination of environmental signals and intrinsic programs [Fuchs et al., 2004; Sugiura et al., 2008]. The primary function of the niche is to retain stem cells through anchoring stem cells to the extracellular matrix (ECM), and provides growth factors and/or other signals for stem cell self-renewal and differentiation. Adhesion molecules play an important role in the interaction between microenvironment and stem cells [Simmons et al., 1997]. Therefore, mimicking niche components in vitro in a defined condition provides a potential strategy to generate functional HSPCs from hPSCs. Our results showed that ECM proteins significantly enhance hematopoietic differentiation from $CD34^+CD31^+$ progenitor cells (Fig. 2). Interestingly, gelatin-coated plates had less effect on the generation of hematopoietic cells than other extracellular matrix-coated plates (collagen-, fibronectin-, collagen/fibronectin-coated plates). Gelatin is denatured collagen protein. It is possible

that the natural structures of EMC proteins provide a better anchor for $CD34^+CD31^+$ progenitor cells, whereas denatured collagen is less efficient to interact with integrins on $CD34^+CD31^+$ progenitor cells. A recent study shows that Tenascin C, an ECM protein, promotes definitive hematopoiesis from hPSCs [Uenishi et al., 2014]. It is unclear whether Tenascin C, collagen I, and fibronectin bind to same integrin targets to promote the generation of different lineage hematopoietic cells. Nevertheless, our data indicated that ECM proteins provide the appropriate niche cues for promoting hematopoietic generation from $CD34^+CD31^+$ progenitor cells derived from hPSCs.

Megakaryocyte-erythroid progenitors (MEP) are common bipotent progenitors that give rise to erythrocytes and megakaryocytes [Klimchenko et al., 2009]. To characterize kinetics of MEP development during erythroid differentiation of $CD34^+CD31^+$ progenitor cells, we assessed the cells that express both erythroid marker, CD235a, and megakaryocytic marker, CD41, by flow cytometry. In the early stage of the process of erythroid differentiation, $CD41^+CD235a^+$ cells emerged transiently and then gradually decreased, especially in the presence of EPO (Fig. 4). In the presence of TPO, a primary factor of megakaryocytic development [Bacon et al., 1995; Sabath et al., 1995], approximately half of hematopoietic cells in suspension were $CD41^+CD235a^+$ cells. It has been demonstrated that TPO also promotes the early stage hematopoiesis and erythroid progenitor development [Papayannopoulou et al., 1996; Ratajczak et al., 1997; Tanimukai et al., 1997; Liu et al., 1999]. Interestingly, in the presence of both TPO and EPO, $CD41^+CD235a^+$ MEP were decreased and $CD235a^+CD41^-$ erythroid cells were increased significantly, indicating that EPO signaling is more effect to direct hematopoietic lineage development than TPO signaling. Our results are consistent with hPSC differentiation in OP9 coculture system [Klimchenko et al., 2009].

It has been demonstrated that TGF-β signaling promotes primitive hematopoietic development in hPSCs [Kennedy et al., 2012; Sturgeon et al., 2014]. To investigate the effect of TGF-β signaling on $CD34^+CD31^+$ progenitor development, we added TGF-β1 or SB431542 after mesoderm induction during EB differentiation. While TGF-β1 had no significant effect on the $CD34^+CD31^+$ progenitor development, addition of SB431542 significantly increased the development of $CD34^+CD31^+$ progenitor cells (Fig. 5A). It is possible that endogenous expression of TGF-β1 or related molecules in EBs is sufficient to suppress $CD34^+CD31^+$ progenitor development. Considering that TGF-β1 or related factors induce apoptosis in a variety of tissues [Jang et al., 2002], we examined whether the negative effect of TGF-β signaling on erythroid development is due to apoptosis. When isolated $CD34^+CD31^+$ progenitor cells were induced for erythroid differentiation on collagen I, TGF-β1 suppressed erythroid cell generation by inducing apoptosis (Fig. 5D), consistent with finding that TGF-β inhibitory effect only occurred in early stage of erythroid development from $CD34^+CD31^+$ progenitor cells.

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## AUTHOR CONTRIBUTIONS

Y.X and H.B: Designed research and performed research. Y.L and D. L.H: Performed research. T.C: Designed research and wrote the paper. Z.Z.W: Designed research, analyzed data, and wrote the paper. There are no conflict interests.

## DISCLOSURE

No competing financial interests exist.

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