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BMP4 Regulates Vascular Progenitor Development in Human Embryonic Stem Cells Through a Smad-Dependent Pathway

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ABSTRACT

The signals that direct pluripotent stem cell differentiation into lineage-specific cells remain largely unknown. Here, we investigated the roles of BMP on vascular progenitor development from human embryonic stem cells (hESCs). In a serum-free condition, hESCs sequentially differentiated into CD34+CD31−, CD34+CD31+, and then CD34−CD31+ cells during vascular cell development. CD34+CD31+ cells contained vascular progenitor population that gives rise to endothelial cells and smooth muscle cells. BMP4 promoted hESC differentiation into CD34+CD31+ cells at an early stage. In contrast, TGFβ suppressed BMP4-induced CD34+CD31+ cell development, and promoted CD34+CD31− cells that failed to give rise to either endothelial or smooth muscle cells. The BMP-Smad inhibitor, dorsomorphin, inhibited phosphorylation of Smad1/5/8, and blocked hESC differentiation to CD34+CD31+ progenitor cells, suggesting that BMP Smad-dependent signaling is critical for CD34+CD31+ vascular progenitor development. Our findings provide new insight into how pluripotent hESCs differentiate into vascular cells. J. Cell. Biochem. 109: 363–374, 2010.

KEY WORDS: HUMAN EMBRYONIC STEM CELLS; ENDOTHELIAL CELLS; SMOOTH MUSCLE CELLS; BMP4; TGFβ

Human embryonic stem cells (hESCs) are pluripotent stem cells that not only have enormous potential as a source of therapeutic tissues, but also provide a unique system for studying human embryonic development, including cardiovascular lineage commitment [Levenberg et al., 2002; Goldberg-Cohen et al., 2006; Ferreira et al., 2007]. Recently, another breakthrough of the direct reprogramming of differentiated human somatic cells into "induced pluripotent stem" (iPS) cells was achieved by forced expression of pluripotency genes [Thomson et al., 1998; Takahashi et al., 2007; Yu et al., 2007]. Thereby, advancing patient-specific pluripotent stem cells and moving regenerative medicine closer to therapeutic applications. One remaining scientific challenge is how to direct pluripotent stem cells into desired cell types.

All regenerative strategies require revascularization of regenerated tissues. The generation of vascular cells from hESCs may improve tissue transplantation, reperfusion of ischemic tissues, and treatment of pathologies in which endothelial cell dysfunction exists. The hESC-derived endothelial cells (hESC-ECs) have been generated based on selections of CD31 or CD34 surface expression [Levenberg et al., 2002; Chen et al., 2007; Wang et al., 2007]. Our previously study demonstrated that hESC-ECs are capable of forming functional blood vessel in vivo [Wang et al., 2007]. We and others found that hESC-derived CD34+ cells have potential to give rise to hematopoietic, endothelial cells (ECs), and smooth muscle cells (SMCs) [Ferreira et al., 2007; Wang et al., 2007], suggesting that hESC-derived CD34+ cells are heterogeneous. It is unclear whether CD34+ cells contain a vascular progenitor population that gives rise to ECs and SMCs. In addition, signals to direct hESC differentiation to vascular lineage commitment are largely unknown.

Bone morphogenetic proteins (BMPs) belong to a transforming growth factor-beta (TGFβ) superfamily that plays critical roles in cell differentiation during embryonic development [Massague and Chen, 2000]. The TGFβ superfamily contains ~40 potential growth factors that are classified into two groups, based on receptors and downstream Smad molecules. BMPs activate Smad1, Smad5, and Smad8 (Smad1/5/8) signaling via the type I receptors ALK1, ALK2,

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ALK3, and ALK6 (ACVERL1, ACVER1, BMPR1A, and BMPR1B, respectively). The second group, including TGFβ, activin, and nodal activates Smad2/3 via ALK4, ALK5, and ALK7 (ACVR1B, TGFBR1, and ACVR1C, respectively) [Massague and Chen, 2000; Valdimarsdottir and Mummery, 2005]. TGFβ, activin, and nodal maintain hESC pluripotency, whereas blockage of BMP signaling is essential to maintain hESC pluripotency [Vallier et al., 2004; Beattie et al., 2005; James et al., 2005; Wang et al., 2005; Xu et al., 2005; Xiao et al., 2006]. Activation of BMP signaling promotes hESC differentiation into hematopoietic cells [Chadwick et al., 2003], trophoblast [Xu et al., 2002], extra-embryonic endoderm [Pera et al., 2004], and mesoderm [Zhang et al., 2008]. However, little is known about the roles of BMP and TGFβ in mediating hESC differentiation into vascular cells. Most of the studies were conducted in a medium containing serum or serum replacement that might contain factors to interfere with BMP and TGFβ signalings.

To define the effects of growth factors on vascular differentiation, we developed a serum-free culture system, and investigated vascular development from hESCs. We found that CD34+CD31+ cells have potential to give rise to vascular endothelial cells and SMCs. In the presence of VEGF and FGF2, the BMP proteins, including BMP2, BMP4, and BMP7, but not BMP9, promoted hESC differentiation into CD34+CD31+ cells. TGFβ and activin promoted hESC differentiation into CD34+CD31− cells that were unable to give rise to endothelial cells or SMCs. Furthermore, TGFβ and activin inhibited the development of BMP-induced CD34+CD31+ progenitor cells. Our study indicated that different members of TGFβ superfamily had distinct effects on hESC differentiation into vascular progenitor cells.

METHODS

CELL CULTURES AND MAINTENANCE OF hESCs

The hESC lines, H1 and H9, were obtained from WiCell Research Institute (Madison, WI). Human foreskin fibroblasts, Hs27 cells (ATCC, Manassas, VA) were used as feeder cells to maintain the hESCs. The hESCs (passages 29–60, ~500 colonies per 100-mm dish) were grown on mitotic-inactivated Hs27 cells in hESC growth medium containing DMEM/F-12 (Invitrogen, Carlsbad, CA), 20% knockout serum replacement (KSR, Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Mediatech, Inc, Herndon, VA), 0.1 mM beta-mercaptoethanol (Sigma, St. Louis, MO), and 4 ng/ml FGF2 (R&D Systems, Minneapolis, MN or PeproTech, Rocky Hill, NJ).

Hs27 cells were cultured in hESC growth medium without FGF2. Hs27 cells were used as feeder cells up to passage 15 to maintain hESCs. Hs27 cells (2×10^4 cells per cm^2) were inactivated by mitomycin C (10 μg/ml), and seeded on 0.1% gelatin-coated plates for hESC maintenance. The hESCs were split every 7–8 days by collagenase type IV treatment (1 mg/ml) (Invitrogen) and scraped mechanically. The hESC growth medium was changed daily, as we previously described [Chen et al., 2007; Wang et al., 2007].

DIFFERENTIATION OF hESCs

To induce hESC differentiation, undifferentiated hESCs on Hs27 feeder cells for 10 days were cultured in serum-free hESC differentiation medium (Supplementary Table I) with or without growth factors. The growth factors, including VEGF, FGF2, TGFβ (PeproTech), BMP-2, BMP4, BMP-7, BMP-9, and activin-AB (R&D Systems) are used as indicated in the Figures. Unless specifically stated, the concentrations of growth factors were 50 ng/ml. To block canonical BMP4 signaling pathway, 5 μM dorsomorphin (EMD Biosciences) was added in the differentiation medium. The differentiation media were changed every 3 days.

ISOLATION OF CD34+ PROGENITOR CELLS AND VASCULAR DIFFERENTIATION

Single cell suspensions of differentiated hESCs were obtained by treatment with collagenase B (2 mg/ml) at 37°C for 20 min, and passed through a 40-μm cell strainer (B&D Falcon), as previously described [Chen et al., 2007]. The CD34+ cells were positively selected using MACS immunomagnetic separation system (Miltenyi Biotec). After incubation with FcR Blocking Reagent, the cells were labeled with anti-CD34 MicroBeads for 30 min at 4°C, and processed through LS+ and then MS+ columns, or autoMACS (Miltenyi Biotec). To isolate subpopulations of CD34+ cells, the differentiated hESCs were labeled by anti-CD34-APC and anti-CD31-PE, and then sorted by FACS (FACSAria, B&D).

To generate hESC-ECs, the isolated cells were cultured on collagen I-coated 24-well plate (1×10^4 cells per well) in SFM (Invitrogen) or other basic medium with EC supplements (Supplementary Table I, EC medium). To generate hESC-SMCs, the isolated cells were cultured in MCDB131 (Invitrogen) or other basic medium containing SMC supplements (Supplementary Table I, SMC medium). The media were changed every 2–3 days.

Human umbilical vein endothelial cells (HUVECs) (Cambrex) were cultured in EGM-2MV medium containing 5% FBS, rhVEGF, FGF2, R^3-IGF-1, hydrocortisone, ascorbic acid, and heparin (Cambrex). Human aortic smooth muscle cells were growth in SmGM-2 medium containing 5% FBS, EGF, FGF2, GA-1000, and insulin (Cambrex).

WESTERN BLOTTING

To examine the phosphorylation of Smad stimulated by BMP4 or TGFβ, hESCs in differentiation medium containing VEGF and FGF2 were stimulated with and without BMP4 or TGFβ in the presence of BMP4 inhibitor (5 μM dorsomorphin) or TGFβ inhibitor (5 μM SB431542) for 30 min. The cells were lysed by RIPA buffer (1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 μM leupeptin, 150 mM NaCl, 50 mM Tris, pH 7.4) containing 1% protease inhibitor cocktail (Sigma). Western blots were performed with primary antibodies anti-Phospho-Smad1/5/8, anti-Phospho-Smad2/3 (all from Cell Signaling Technology), and secondary antibody anti-rabbit IgG-HRP (Sigma).

RT-PCR AND REAL-TIME PCR ANALYSES

Total RNAs from undifferentiated hESCs or differentiated hESCs at different time points were harvested by Trizol (Invitrogen). To eliminate DNA contamination, the RNA samples were treated with DNase (Invitrogen) and cleaned by RNeasy kit (QIAGEN) before the reverse transcription (RT) reaction. Total RNA (100 ng) was used for each RT reaction with SuperScript III (Invitrogen). All RNA samples

were adjusted to yield equal amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. Real-time PCR was performed on iQ5 thermal cycler (Bio-Rad). Oligonucleotide primers and PCR conditions are listed in the Supplementary Table II (RT-PCR) and Table III (real-time PCR).

IMMUNOHISTOCHEMISTRY

The cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min, and then incubated with 1% BSA for 30 min to block nonspecific binding. The cells were stained for 1 h with the primary antibodies: SSEA-1, SSEA-4, TRA-1-60, TRA-1-81 (all from Chemicon International Inc.), CD31, VE-cad (all from B&D Pharmingen), SMC α-actin (SMA), desmin (all from Abcam, Cambridge, MA), and calponin (Sigma). The cells were then incubated with the secondary antibodies, rabbit anti-mouse IgG-Alexa488 and Alexa594 (Invitrogen), respectively, for 1 h. DAPI (0.1 μg/ml) was used for cell nucleus staining. The results were checked by a fluorescence microscope.

FLOW CYTOMETRIC ANALYSIS

Single cell suspensions of differentiated hESCs were obtained as described above. The dissociated cells were washed and resuspended in PBS supplemented with 2% mouse serum to block nonspecific binding. Direct staining of fluorochrome-conjugated anti-human monoclonal antibodies included CD31-phycoerythrin (PE) and CD34-allophycocyanin (APC) (B&D Pharmingen). Isotype-matched controls (B&D Pharmingen) were used to determine the background staining. The samples were analyzed on a FACSCalibur (Becton Dickson) with CellQuest software. Data analysis was performed using CellQuest or FlowJo Software.

LDL-UPTAKE ASSAY

The hESC-ECs were cultured in serum-free endothelial cell medium containing dil-acetylated low-density lipoprotein (2 ng/ml) (Dil-Ac-LDL, Invitrogen) for 12 h. After washing twice with PBS, the cells were examined by a fluorescence microscope.

MATRIGEL VASCULAR NETWORK FORMATION

The assay was performed essentially as previously described [Chen et al., 2007; Wang et al., 2007]. Briefly, 24-well plates were coated with 200 μl/well Matrigel matrix (B&D Biosciences) at room temperature for more than 30 min. HUVECs or hESC-ECs (5×10^4 cells) were plated on Matrigel coated plates in 500 μl EGM-2 medium and incubated at 37°C in 5% CO_2. The structures were photographed by a phase-contrast microscopy (Nikon) after 16 h of incubation.

STATISTICAL ANALYSIS

The results were subjected to statistical analysis by the Student's *t*-test. For all analyses, values of $P < 0.05$ were considered statistically significant.

RESULTS

BMP4 PROMOTE hESC DIFFERENTIATION INTO CD34+ CELLS IN SERUM-FREE CULTURE

We previously established a monolayer hESC culture system to efficiently generate CD34+ progenitor cells without intermediate embryoid body (EB) formation, and demonstrated that CD34+ progenitor cells gave rise to hematopoietic and endothelial cells (hESC-ECs) [Chen et al., 2007; Wang et al., 2007]. To identify factors that direct hESC differentiation, we recently establish a serum-free hESC differentiation medium (Supplementary Table I). We and others demonstrated that BMP4, VEGF, and FGF2 promoted hematopoietic differentiation and facilitated CD34 generation from hESCs [Faloon et al., 2000; Li et al., 2001; Park et al., 2004; Kennedy et al., 2007; Wang et al., 2007; Pearson et al., 2008; Zhang et al., 2008]. To dissect the promoting effect of BMP4, VEGF, and FGF2 on CD34 generation from hESCs, various combinations of three factors were added into serum-free differentiation medium. Flow cytometric analyses demonstrated that neither BMP4 alone, nor the combination of VEGF and FGF2, was sufficient to promote hESC differentiation into CD34+ cells (Fig. 1A). The combination of BMP4 and VEGF or the combination of BMP4 and FGF2 increased CD34+ cells modestly (Fig. 1A). In the presence of VEGF and FGF2, BMP4 significantly facilitated hESC differentiation into CD34+ cells in the concentration dependent manner (Fig. 1B), suggesting that BMP signaling plays a crucial role in the development of CD34+ cells. Increasing BMP4 to 100 ng/ml modestly increased CD34+ cells, comparing with 50 ng/ml of BMP4 (data not shown). Our serum-free medium containing BMP4, VEGF, and FGF2 significantly increased the efficiency of hESC differentiation into CD34+ cells, compared to differentiation medium containing 15% FBS (Fig. 1A).

Ferreira et al. [2007] demonstrated that hESC-derived CD34+ cells have potential to give rise to ECs and SMCs. To further characterize the vascular differentiation potential of hESC-derived CD34+ cells, we formulated two serum-free media and tested several basic media for growth of ECs and SMCs from hESC-derived CD34+ cells (Supplementary Table I and Supplementary Figs. 1 and 2). The hESC-derived endothelial cells (hESC-ECs) were characterized after 7 days cultured in EC medium. Immunohistochemistry analyses indicated that CD31 and VE-Cad were expressed in most of hESC-ECs at the endothelial cell adherent junctions (Fig. 2A). The hESC-ECs incorporated Dil-Ac-LDL (Fig. 2B), and formed a vascular network in Matrigel (Fig. 2C), suggesting that the hESC-ECs from serum-free cultures phenotypically similar to those that we previously described in a serum-containing medium [Chen et al., 2007; Wang et al., 2007]. Approximately 1.3×10^4 hESC-ECs were obtained from 1×10^4 CD34+ cells that were originated from 1×10^5 hESCs (Supplementary Fig. 1A). Several endothelial markers, including CD31, VE-cad, vWF, VEGF, VEGFR2, EphB4, and ephrinB2, were expressed in hESC-EC as demonstrated by RT-PCR analysis (Fig. 2D). The pluripotent genes, Oct-4 and Nanog, were not detected in hESC-ECs, suggesting that no undifferentiated hESCs remained in the endothelial cell culture. After culturing CD34+ cells for 7 days in serum-free SMC medium (Supplementary Table I), approximately 50% cells expressed SMC markers, including smooth muscle α-actin (SMA), calponin, and desmin (Fig. 2E).

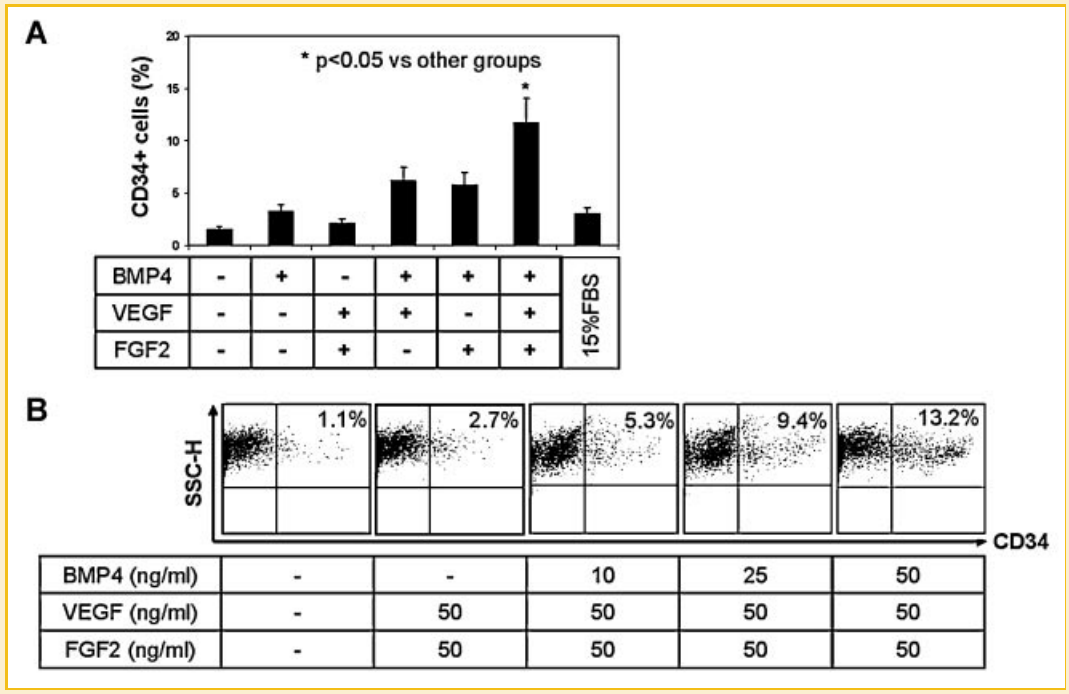


Fig. 1. BMP4, VEGF, and FGF2 promote development of CD34+ cells in serum-free culture. A: HESCs were maintained on Hs27 cells. The hESC differentiation was induced by switching hESC growth medium to serum-free differentiation medium with (+) or without (−) BMP4 (50 ng/ml), VEGF (50 ng/ml), FGF2 (50 ng/ml). After 12 days of differentiation, the cells were analyzed for CD34 expression by flow cytometry. Differentiation medium containing 15% defined-FBS was used as a control. Error bars represent standard deviation (* indicates $P < 0.05$ vs. others). B: The differentiation of hESCs was induced in serum-free media containing different concentrations of BMP4 in the presence of VEGF and FGF2, as indicated. The differentiated cells were analyzed after 12 days for CD34 expression by flow cytometry. Data are representative of three independent experiments.

Approximately 1.2×10^4 hESC-SMCs were obtained from 1×10^4 CD34+ cells (Supplementary Fig. 1B).

CD34+CD31+ SUBPOPULATION CONTAINS VASCULAR PROGENITOR CELLS

To identify potential vascular progenitor cells that have differentiation potential to become ECs and SMCs, we compared different subpopulations, including CD34−CD31− cells, CD34+CD31− cells, CD34+CD31+ cells, and CD34−CD31+ cells. The subpopulations were sorted by FACS, and analyzed for expression of EC- and SMC-specific genes. Real-time PCR analysis indicated that EC-specific genes, including VEGFR2, Tie2, VE-Cad, and vWF, were highly expressed in CD34+CD31+ cells (Fig. 3A). SMC-specific genes, including SMA and SM22α, calponin, and caldesmon, were highly expressed in CD34+CD31+ cells, and modestly expressed in other differentiated populations (Fig. 3B). As shown in Figure 3C and D, when FACS-sorted subpopulations were cultured in EC or SMC growth medium, CD34+CD31+ cells had high differentiation potential to become ECs and SMCs characterized by immunohistochemistry of CD31 and VE-cad, and SMA and calponin, respectively (Supplementary Fig. 2A and B). These results demonstrated that CD34+CD31+ cells derived from hESCs have EC and SMC differentiation capability, suggesting that CD34+CD31+ cells contain vascular progenitor cells.

To characterize the development of vascular progenitor cells, we assessed CD34 and CD31 expression at different time points during hESCs differentiation in serum-free medium containing BMP4, FGF2, and VEGF. As shown in Figure 3E, hESCs differentiated into CD34+ cells earlier than into CD31+ cells. By day 6, approximately 5% of cells were CD34+ cells, in which the majority were CD31− cells. CD34+CD31+ cells gradually emerged after day 6. By day 12, approximately 10% of cells were CD34+CD31+. CD31+/CD34− cells increased considerably after day 12. By day 18, ∼ 8% of differentiated hESCs were CD31+/CD34− cells, compared to ∼ 1% CD31+/CD34− cells at day 12. These data demonstrated a sequential development of CD34+CD31−, CD34+CD31+, and CD34−CD31+ cells during hESC differentiation. RT-PCR analysis indicated that the expressions of the pluripotent genes, Oct-4 and Nanog, were decreased gradually during hESC differentiation (Supplementary Fig. 3). The expressions of CD34, EC-specific genes (CD31 and VE-cad), and SMC-specific genes (Calponin and SMA) were increased during hESC differentiation. VEGFR2 (Flk1) and Tie2 were expressed in undifferentiated and differentiated hESCs, which is consistent with previous studies in hESC differentiation cultures [Levenberg et al., 2002, 2007; Gerecht-Nir et al., 2005; Zambidis et al., 2005].

SEQUENTIAL EFFECTS OF BMP4, VEGF, AND FGF2 ON THE GENERATION OF CD34+CD31+ PROGENITOR CELLS

Activation of BMP signaling blocks hESC self-renewal and promotes multilineage differentiation [Xu et al., 2002, 2005; Chadwick et al., 2003; Pera et al., 2004; Vallier et al., 2004; Beattie et al., 2005; James

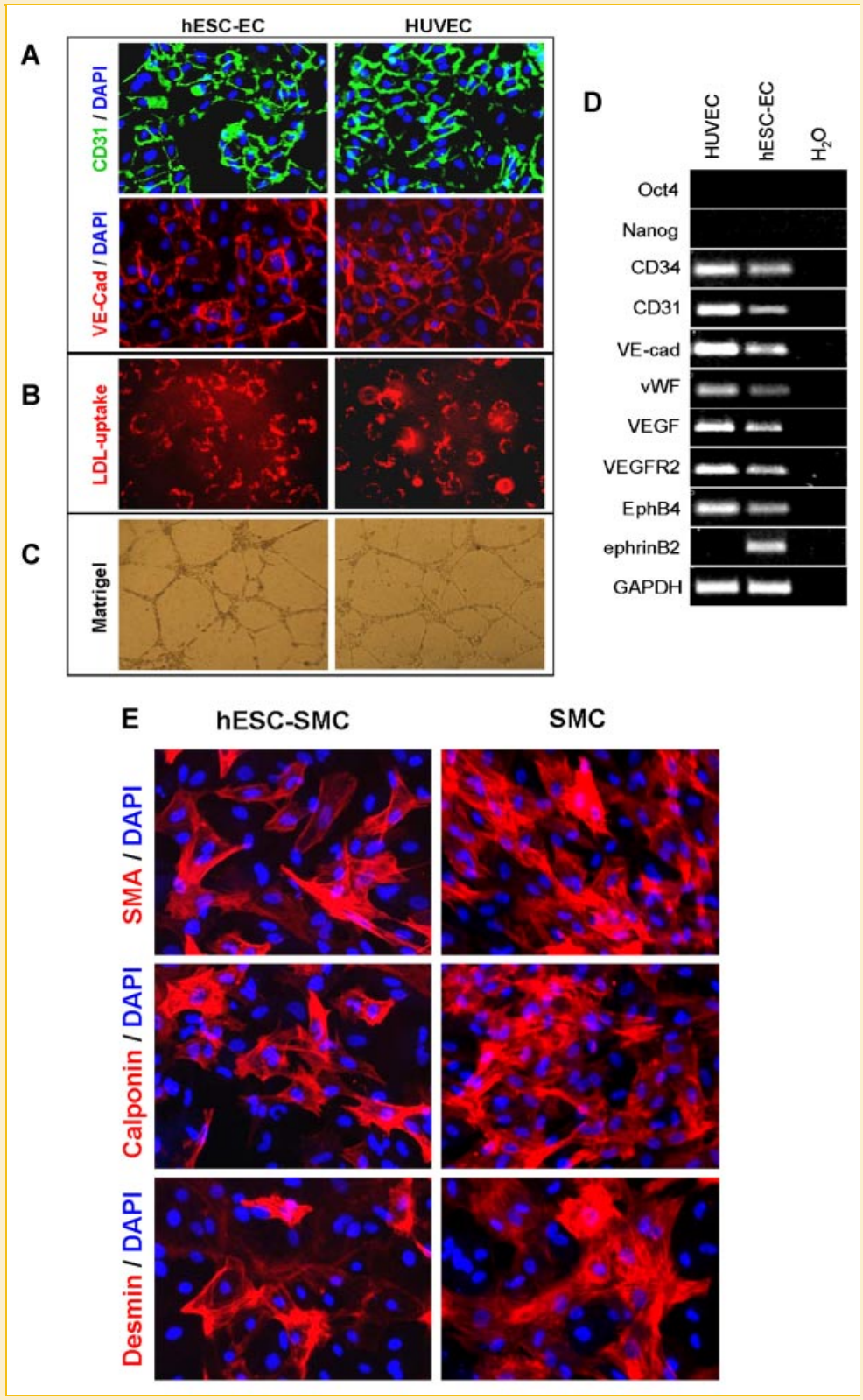


Fig. 2. Characterization of hESC-ECs and hESC-SMCs in serum-free media. The differentiation of hESCs was induced in serum-free medium containing BMP4, VEGF, and FGF2. After 12 days, the isolated CD34+ cells (1×10^4 cells/well) were cultured on collagen I-coated 24-well plates either in SFM medium containing EC growth supplements or in MCDB 131 medium containing SMC growth supplements for 7 days. A: The expression of EC markers, VE-cad and CD31, in hESC-ECs was analyzed by cell immunohistochemistry. DAPI was used for nuclear staining. B: The Dil-Ac-LDL incorporation assay. The hESC-ECs were incubated with 2 ng/ml of Dil-Ac-LDL for 12 h. The images were obtained with a fluorescent microscopy. C: Matrigel assay for vascular network formation. The hESC-ECs were loaded on a 24-well plate containing 200 μl matrigel. The images were taken with a phase-contrast microscopy after 16 h. D: Gene expression analysis of hESC-ECs by RT-PCR. HUVECs were used as a control in A–D. E: The expression of SMC-specific markers, including SMA, calponin, and desmin, was examined in hESC-SMCs by cell immunohistochemistry. Human primary aortic SMCs were used as a control. DAPI was used for nuclear staining.

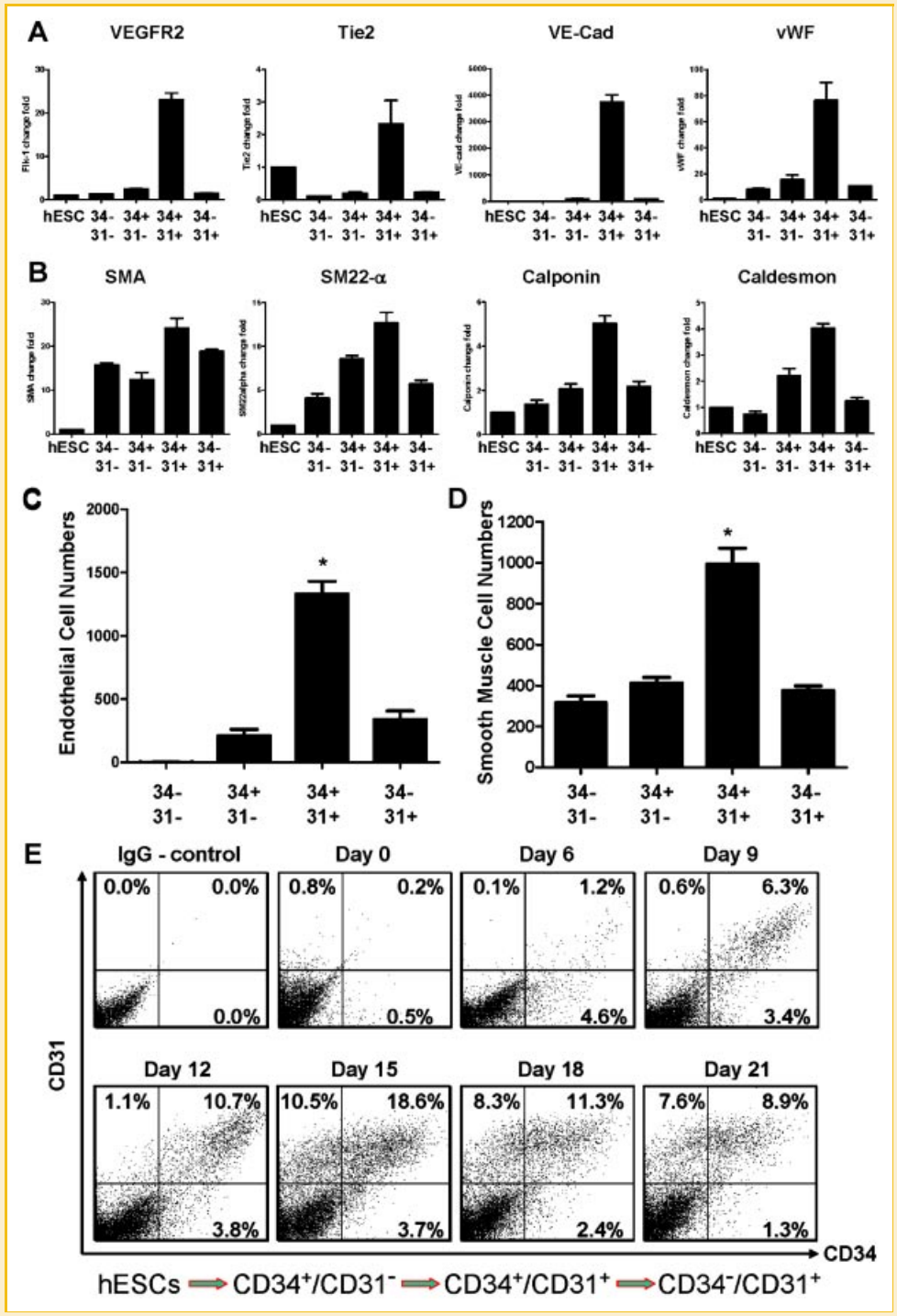
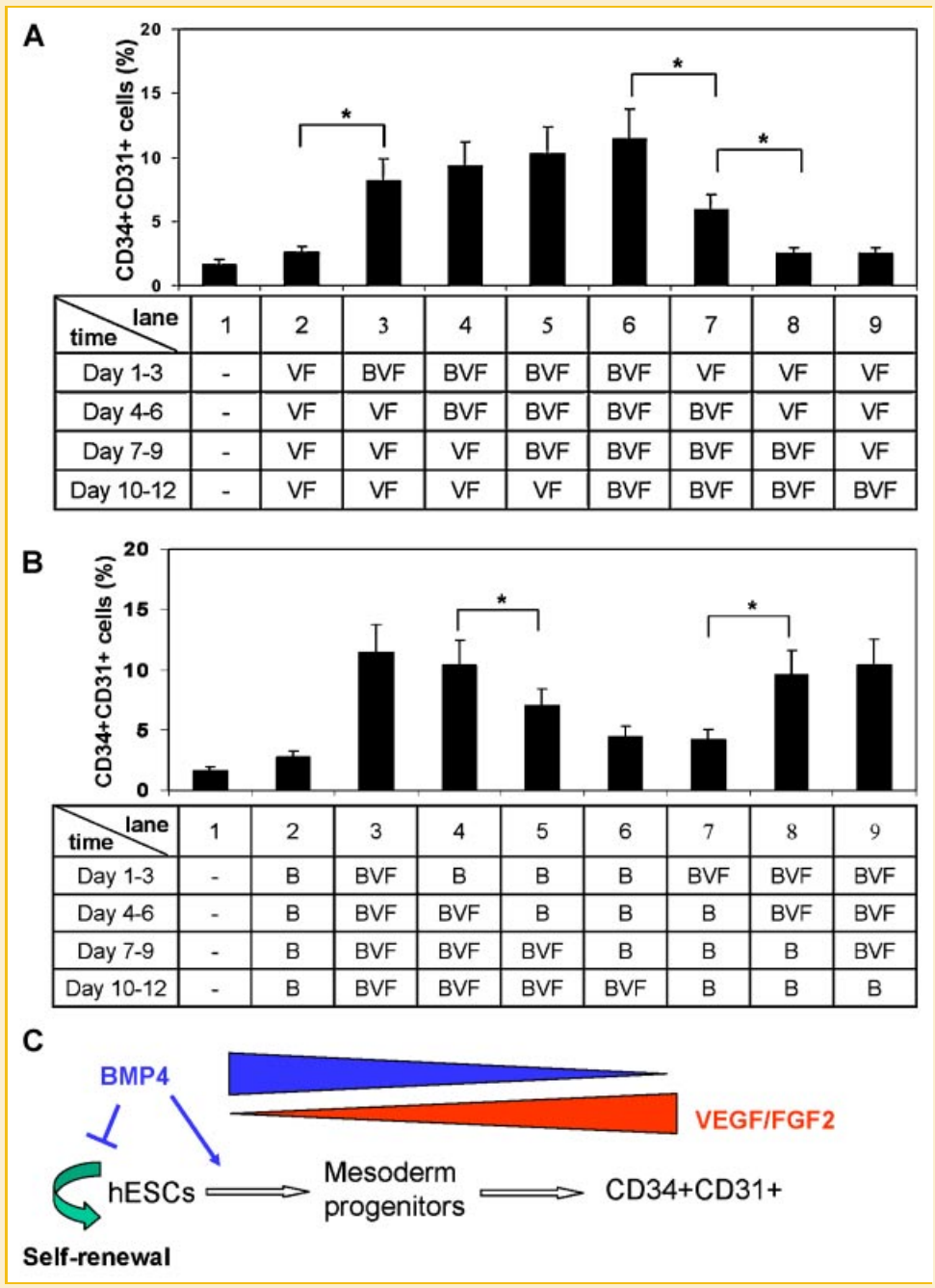


Fig. 3. Vascular progenitor cells in CD34+CD31+ population. After 12 days of hESC differentiation, CD34−CD31− cells, CD34+CD31− cells, CD34+CD31+ cells, and CD34−CD31+ cells were sorted by FACS. Real-time PCR were used to analyze EC-specific genes: VEGFR2, Tie2, VE-cad, and vWF (A), and SMC-specific genes: SMA, SM22alpha, calponin, and caldesmon (B). Undifferentiated hESCs were used as a control (hESC). GAPDH was used to normalize the gene expression. The isolated subpopulation cells (1×10^4 cells/well) were cultured on collagen I-coated 24-well plates either in EC growth medium or in SMC growth medium. The CD31 and VE-cad positive cells were measured as hESC-ECs by immunohistochemistry (C). The SMA and calponin positive cells were measured as hESC-SMCs by immunohistochemistry (D). DAPI was used for nuclear staining in C. D: Error bars represent standard deviation ($^*P < 0.05$ vs. other groups). E: Flow cytometric analyses CD34 and CD31 expression during hESC differentiation at different time points. Data are representative of three independent experiments.

et al., 2005; Wang et al., 2005; Xiao et al., 2006]. To test whether BMP4 initiates hESC differentiation into CD34+CD31+ progenitor cells, we added BMP4 to the differentiation medium at various times in the presence of VEGF and FGF2 (Fig. 4A). Without BMP4, VEGF and FGF2 were insufficient to induce hESC differentiation into CD34+CD31+ cells (Fig. 4A, lane 2). The addition of BMP4 from day 1 to 3 significantly increased CD34+CD31+ cells (Fig. 4A, lane 3). The continuous addition of BMP4 from day 4 and later moderately increased CD34+CD31+ cells (Fig. 4A, lanes 4–6). In addition, the absence of BMP4 from day 1 to 3 significantly decreased CD34+CD31+ cells (Fig. 4A, lane 7), whereas the absence of BMP4 from day 4 to 6 further decreased CD34+CD31+ cells



time \ lane	1	2	3	4	5	6	7	8	9
Day 1-3	-	VF	BVF	BVF	BVF	BVF	VF	VF	VF
Day 4-6	-	VF	VF	BVF	BVF	BVF	BVF	VF	VF
Day 7-9	-	VF	VF	VF	BVF	BVF	BVF	BVF	VF
Day 10-12	-	VF	VF	VF	VF	BVF	BVF	BVF	BVF

time \ lane	1	2	3	4	5	6	7	8	9
Day 1-3	-	B	BVF	B	B	B	BVF	BVF	BVF
Day 4-6	-	B	BVF	BVF	B	B	B	BVF	BVF
Day 7-9	-	B	BVF	BVF	BVF	B	B	B	BVF
Day 10-12	-	B	BVF	BVF	BVF	BVF	B	B	B

Fig. 4. Sequential effects of BMP4, VEGF, and FGF2 on CD34+CD31+ progenitor cells. The hESCs were induced to differentiation in serum-free differentiation medium containing various combinations of BMP4, VEGF, and FGF2. BMP4 (B), VEGF and FGF2 (VF), and BMP4, VEGF and FGF2 (BVF) were presented for different periods during hESC differentiation. The differentiation was set for 4 stages: day 1–3, day 4–6, day 7–9, and day 10–12. The CD34+CD31+ cells were analyzed after 12 days by flow cytometry. Error bars represent standard deviation ($^*P < 0.05$ vs. compared group). C: Schematic diagram of sequential effects of BMP4, VEGF, and FGF2 on hESC differentiation into CD34+CD31+ cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Fig. 4A, lane 8). To examine the effect of VEGF and FGF2 on development of CD34+CD31+ progenitor cells, we added VEGF and FGF2 to the differentiation medium at different time points in the presence of BMP4 (Fig. 4B). We found VEGF and FGF2 had no significant effect on CD34+CD31+ progenitor cells from hESCs differentiation before day 4 (Fig. 4B, lanes 4 and 7). The promoting effect of VEGF and FGF2 on the development of CD34+CD31+ progenitor cells gradually increased after day 4. Taken together, our data suggest that BMP4 is important to promote hESC differentiation into CD34+CD31+ progenitor cells in the early stage, mainly before day 6, whereas VEGF and FGF2 facilitates CD34+CD31+ cell development after day 4 (Fig. 4C).

DISTINCT ROLES OF TGFβ FAMILY MEMBERS IN CD34+CD31+ CELL DEVELOPMENT

BMPs are members of the TGFβ superfamily. To determine whether other members of the TGFβ family regulate vascular differentiation, we examined the effect of BMP2, BMP7, and BMP9, as well as TGFβ1 or activin in serum-free hESC differentiation medium containing VEGF and FGF2. Figure 5A shows that BMP2 and BMP7 promoted hESC differentiation into CD34+CD31+ cells effectively; whereas BMP9 had no effect on hESC differentiation into CD34+CD31+ cells. The addition of TGFβ1 or activin increased CD34+ cells; however, most of the CD34+ cells induced by TGFβ1 and activin were CD31 negative (Fig. 5B). The TGFβ1-induced or activin-induced CD34+CD31− cells were unable to give rise to either ECs or SMCs (Supplementary Fig. 4). To determine if TGFβ1 or activin blocks BMP4 effect on CD34+CD31+ progenitor cells, TGFβ1 or activin was added to differentiation medium in the presence of BMP4. As Figure 5B shown, TGFβ1 or activin increased CD34+ cells, but abolished BMP4 effect on induction of CD34+CD31+ cells, thereby suggesting that TGFβ signaling negatively regulates the development of CD34+CD31+ cells during hESC differentiation. To determine whether TGFβ1 alters the kinetics of CD31 and delays CD31 expression, we examined the effect of TGFβ1 and activin on the development of CD34+ cells, CD31+ cells, and CD34+CD31+ cells for a longer period up to 21 days. Flow cytometric analysis showed that the CD34 was expressed with similar kinetics during hESC differentiation in the presence of BMP4, TGFβ1, or activin (Fig. 5C); whereas TGFβ1 or activin was unable to promote hESCs to differentiate into CD31+ cells and CD34+CD31+ cells, even with longer culture periods (Fig. 5D,E).

Smad-DEPENDENT PATHWAY MEDIATE BMP EFFECTS ON CD34+CD31+ CELL GENERATION

Canonical BMP4 signaling is through the Smad1/5/8-dependent pathway. We examined whether the effect of BMP4 signaling on the generation of CD34+CD31+ vascular progenitor cells is dependent on Smad pathway during hESC differentiation. The activation of BMP receptors can be specifically blocked by a small molecule BMP inhibitor, dorsomorphin [Yu et al., 2008]. In hESCs, BMP4 induced the phosphorylation of Smad1/5/8 that was markedly inhibited by dorsomorphin (Fig. 6A), whereas the TGFβ inhibitor, SB431542, blocked the phosphorylation of Smad2/3 induced by TGFβ1 (Fig. 6B). The hESC differentiation into CD34+CD31+ progenitor cells was abolished by dorsomorphin (Fig. 6C), whereas SB431542 abolished TGFβ-induced CD34+CD31− cells (data not shown). These data suggest that BMP4 induces CD34+CD31+ cells in hESCs through the Smad-dependent pathway.

DISCUSSION

Due to the low proliferation potential in terminal differentiated cells, we attempted to characterize vascular progenitor differentiation in hESCs. We formulated three serum-free media to generate vascular progenitor cells from hESCs, hESC-ECs, and hESC-SMCs, respectively. Our new formulated serum-free media allowed us to investigate factors that direct hESC differentiation into vascular cells without interference from factors derived from sera.

We and other previously demonstrated that the hESC-derived CD34+ cells have differentiation potential to give rise to hematopoietic cells, endothelial cells (ECs), and smooth muscle cells (SMCs) [Kaufman et al., 2001; Vodyanik et al., 2005; Chen et al., 2007; Ferreira et al., 2007; Wang et al., 2007], suggesting that CD34+ cell are heterogenous. To characterize vascular progenitor population in CD34+ cells, we compared CD34+CD31− cells, CD34+CD31+ cells, CD34−CD31− cells, and CD34−CD31+ cells for their EC and SMC potentials by gene expression analysis, and by their ability to grow EC and SMC medium. Our data indicated that CD34+CD31+ cells derived from hESCs have both endothelial and SMC differentiation capability, although it is not clear whether this occurs at a clonal level. Approximately 1.3×10^4 hESC-ECs and 1.2×10^4 hESC-SMCs were obtained from 1×10^4 CD34+CD31+ cells. Many cells died in EC and SMC cultures. It could be due to cell damage caused by enzyme treatment and FACS during cell isolation. Our previous study found that CD34+ cells isolated from autoMACS sorting survived better than FACS sorting [Wang et al., 2007]. However, it is technically challenging to isolate CD34+CD31+ cells by autoMACS. Another possibility is that only a subset of CD34+CD31+ population has vascular progenitor potential.

A study of mouse ES cells by Yamashita et al. suggested that VEGFR2+ (Flk1+) vascular progenitor cells differentiated into either ECs or SMCs that were dependent on VEGF or PDGF-BB, respectively [Yamashita et al., 2000]. A subpopulation of VEGFR2+ cells from mouse ES cells contain cardiovascular progenitor cells that give rise to cardiomyocyte, endothelial cells, and vascular SMCs [Kattman et al., 2006; Moretti et al., 2006]. Recent study of hESCs by Yang et al. [2008] demonstrated that VEGFR2+ cells from hESCs contain cardiovascular population that displays cardiac, endothelial, and vascular smooth muscle potential. Our study demonstrated that CD34+CD31+ cells have endothelial and SMC potential. In the future, we will examine whether CD34+CD31+ cells and VEGFR2+ cells contain an overlap population that has high vascular progenitor potential.

Srivastava et al. [2007] recently demonstrated that CD34+CD31− cells that are induced by hematopoietic factors, including SCF, IL-3, TPO, and VEGF, contain hematopoietic progenitors. We found that TGFβ-induced CD34+CD31− cells did not express GATA-2 and were unable to form hematopoietic colonies in methylcellulose cultures (data not shown). Instead, TGFβ-induced CD34+CD31−

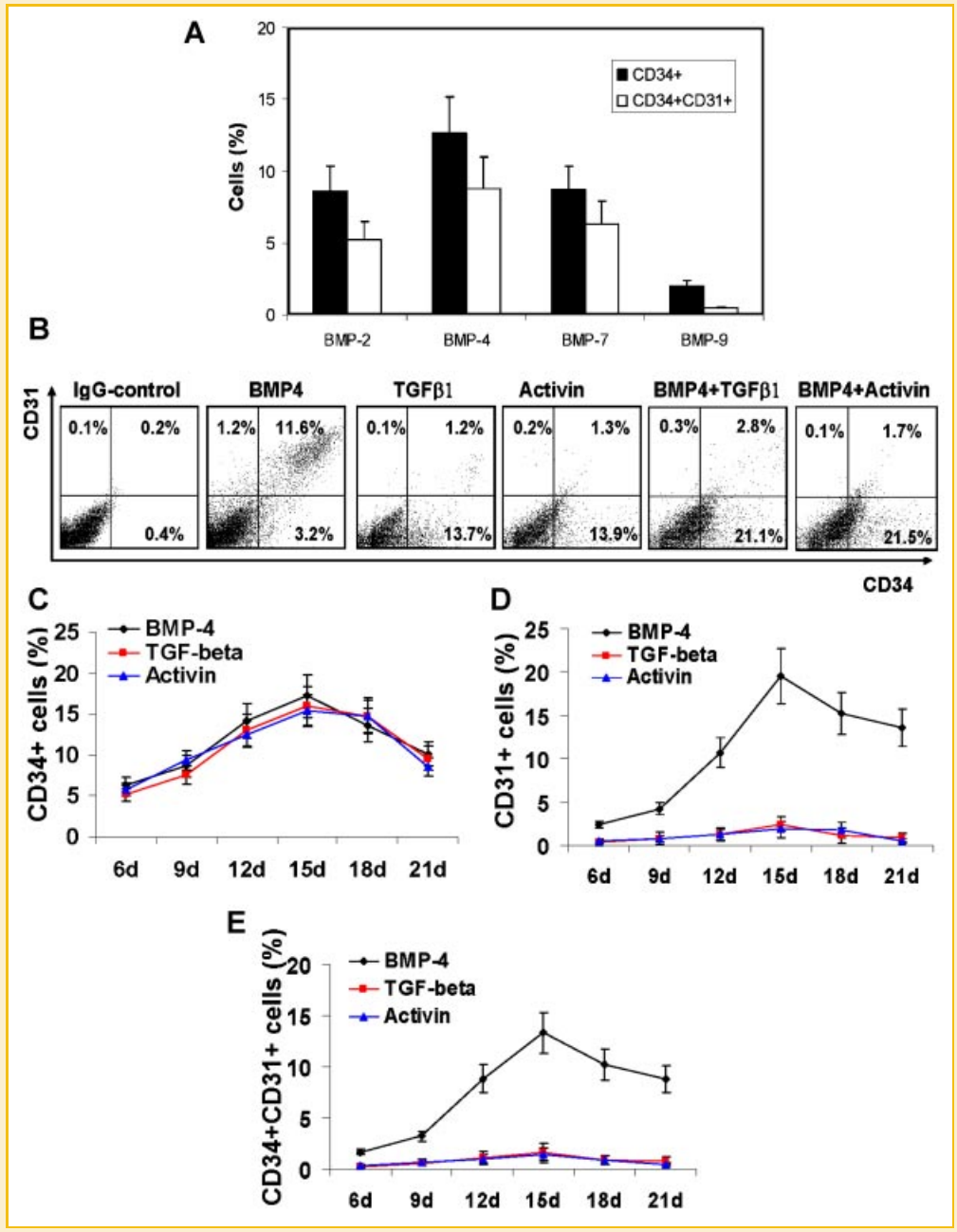


Fig. 5. Distinct roles of TGFβ family members in the development of CD34+CD31+ cells. A: The hESCs were differentiated in serum-free differentiation medium containing VEGF and FGF2, and different TGFβ family members. (A) The differentiation medium contained BMP2, BMP4, BMP7, or BMP9, as indicated. After 12 days, the expression of CD34 and CD31 were analyzed by flow cytometry. Error bars represent standard deviation from 3 experiments. (B) The differentiation medium contained BMP4, TGFβ1, or activin, as indicated. After 12 days, the expression of CD34 and CD31 were analyzed by flow cytometry. Data were a representative of 3 independent experiments. (C), (D), and (E) Kinetic analyses of CD34+ cells, CD31+ cells, CD34+CD31+ cells. The differentiation medium contained BMP4, TGFβ1, or activin. The CD34+ cells and CD31+ cells were analyzed by flow cytometry at different time points.

cells expressed some ectodermal markers (data not shown). Our data contradicted a previous study that activin and TGFβ primarily induced mesodermal markers, whereas BMP4 and FGF2 activated ectodermal and mesodermal markers in hESCs [Schuldiner et al., 2000]. The contradictory results could be due to different culture systems, the presence of serum, and the time that the factor is added. CD34 is highly expressed in the skin stem cells niche [Tumbar et al., 2004] and could be a marker of mouse skin epithelial stem cells [Trempus et al., 2003; Sur et al., 2006]. It is unclear whether TGFβ-induced CD34+CD31− cells contain skin epithelial progenitors.

The activation of BMP2, BMP4, and BMP7 signaling is mediated by ALK2, ALK3, and ALK6, while BMP9 activation is predominantly mediated by ALK1 [Massague and Chen, 2000; Scharpfenecker et al., 2007]. Our study demonstrated that BMP2, BMP4, and BMP7, but

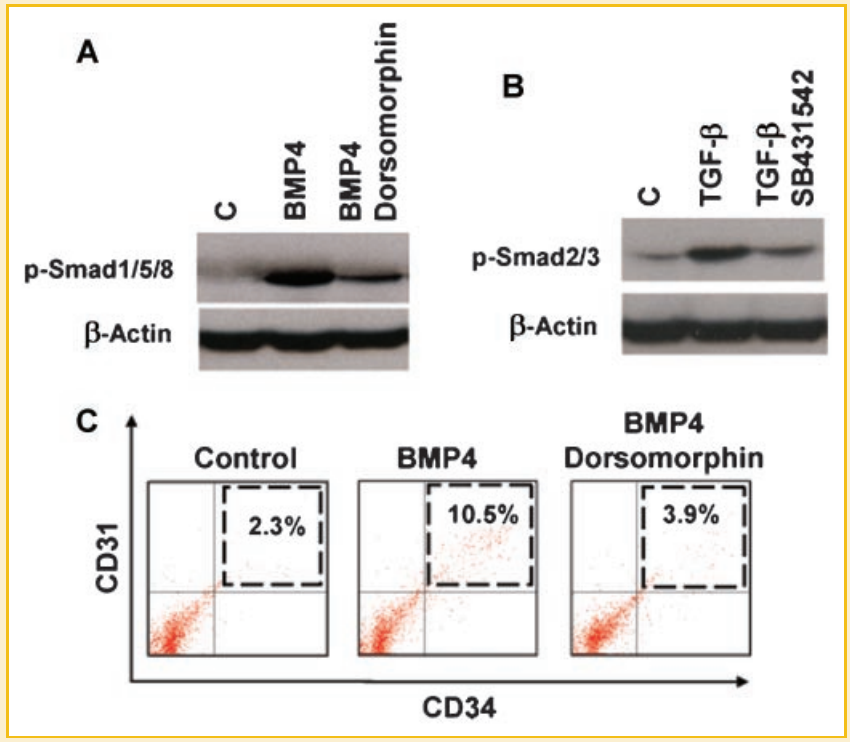


Fig. 6. Smad-dependent pathway mediates BMP4 effect on CD34+CD31+ cell generation. A: The hESCs were stimulated by BMP4 (50 ng/ml) with or without dorsomorphin (5 μM) for 30 min. The phosphorylation of Smad1/5/8 was examined by Western blot analysis. B: The hESCs were stimulated by TGFβ1 (50 ng/ml) with or without SB431542 (5 μM) for 30 min. The phosphorylation of Smad2/3 was examined by Western blot analysis. C: The hESCs were induced to differentiation in serum-free medium containing BMP4, VEGF, and FGF2 in the presence or absence of dorsomorphin (5 μM). The expression of CD34+ and CD31+ cells was analyzed after 12 days by flow cytometry. The serum-free differentiation medium without BMP4 and inhibitors was used as a control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

not BMP9, facilitated CD34+CD31+ cell development. Dorsomorphin, which specifically inhibits the BMP type I receptors ALK2, ALK3, and ALK6 [Yu et al., 2008], blocked BMP4-induced Smad1/5/8 phosphorylation and CD34+CD31+ cell development in hESCs. These data suggested that ALK2, ALK3, and ALK6, but not ALK1, might be involved in hESC differentiation in a ligand type-specific manner. Future study will investigate which BMP receptor is involved in vascular progenitor development in hESCs. In addition to canonical BMP4 signaling, BMPs can signal through Smad-independent pathways, notably via MAP kinases, including the ERK, JNK, and p38 MAP kinase pathways that are involved in VEGF and FGF2 signaling [Derynck et al., 2001; Miyazono et al., 2005]. In the future, we will investigate whether VEGF and FGF2 signaling interact with BMP4-Smad dependent or SMAD-independent pathway to facilitate vascular development.

Retinoic acid (RA) induces the differentiation of mouse ES cells and hESCs into SMCs [Drab et al., 1997; Huang et al., 2006]. We tested whether RA promotes SMC growth from CD34+CD31+ cells. We found that RA had no significant effect on CD34+CD31+ cell differentiation into SMCs, whereas PDGF-BB promotes CD34+CD31+ cells to SMCs in serum-free medium (data not shown), suggesting that the promoting effect of PDGF-BB and RA on SMCs occurs at a different time window.

Taken together, our study demonstrated that BMPs promotes hESC differentiation into CD34+CD31+ vascular progenitor cells.

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