BMP9 Induces Cord Blood–Derived Endothelial Progenitor Cell Differentiation and Ischemic Neovascularization via ALK1

Jihye Kim, Minhyung Kim, Yoonjeong Jeong, Wook-bin Lee, Hyojin Park, Ja-Young Kwon, Young-Myeong Kim, Daehee Hwang, Young-Guen Kwon

Objective—Modulating endothelial progenitor cells (EPCs) is essential for therapeutic angiogenesis, and thus various clinical trials involving EPCs are ongoing. However, the identification of environmental conditions and development of optimal methods are required to accelerate EPC-driven vasculogenesis.

Approach and Results—We evaluated gene expression profiles of cord blood–derived EPCs and endothelial cells to identify the key factors in EPC→endothelial cell differentiation and to show that transforming growth factor-β family members contribute to EPC differentiation. The expression levels of activin receptor-like kinase 1 (ALK1) and its high-affinity ligand, bone morphogenetic protein 9 (BMP9) were markedly changed in EPC→endothelial cell differentiation. Interestingly, BMP9 induced EPC→endothelial cell differentiation and EPC incorporation into vessel-like structures by acting on ALK1 expressed on EPCs in vitro. BMP9 also induced neovascularization in mice with hindlimb ischemia by increasing vessel formation and the incorporation of EPCs into vessels. Conversely, neovascularization was impaired when ALK1 signaling was blocked. Furthermore, EPCs exposed to either short- or long-term BMP9 stimulation demonstrated these functions in EPC-mediated neovascularization.

Conclusions—Collectively, our results indicated that BMP9/ALK1 augmented vasculogenesis and angiogenesis, and thereby enhanced neovascularization. Thus, we suggest that BMP9/ALK1 may improve the efficacy of EPC-based therapies for treating ischemic diseases. **(*Arterioscler Thromb Vasc Biol*. 2015;35:2020-2031. DOI: 10.1161/ATVBAHA.115.306142.)**

Key Words: activin receptors ■ endothelial cells ■ endothelial progenitor cells ■ growth differentiation factor 2 ■ ischemia ■ neovascularization, pathologic

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Circulating endothelial progenitor cells (EPCs) participate in vasculogenesis after incorporation into regions of neovascularization and differentiation into endothelial cells (ECs) and promote angiogenesis by the production of angiogenic growth factors.[1–3] Thus, EPC-induced vasculogenesis provides a novel therapeutic approach for patients with heart and limb ischemia.[4,5] To achieve efficient and therapeutically useful EPC grafting, various trials have tested local EPC delivery, promotion of EPC mobilization, enhancement of EPC function, and in vitro EPC expansion.[6–9] Select cytokines trigger rapid EPC expansion in culture systems and improve EPC angiogenic potency, which are essential for advancing the grafting efficacy of therapeutic EPCs.[4,7,10] For example, local administration of stromal-derived factor-1 induces neovascularization after EPC transplantation by recruiting EPCs to pre-existing vessels and increasing their incorporation into the developing vasculature.[8,11] As well, ex vivo EPC priming with stromal-derived factor-1 significantly enhances subsequent EPC transplantation efficacy.[12] EPC maturation is also enhanced in the presence of cytokines and growth factors, such as vascular endothelial growth factor (VEGF)-A, fibroblast growth factor-2, and insulin-like growth factor.[13] $CD34^+$ EPC→EC differentiation is induced by VEGF, which increases bone marrow–derived EPC engraftment in the developing vasculature of neonatal mice.[7] However, EPC biology is complex, and our understanding of the precise mechanisms that regulate EPC differentiation is limited. To improve the focus and safety of clinical trials using EPCs, we need to identify the angiogenic factors that enhance EPC differentiation and functional incorporation into the neovasculature of ischemic tissues.

The transforming growth factor-β (TGF-β) superfamily exerts multiple effects on most cell types, depending on the cellular and environmental contexts. Members of the TGF-β

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Nonstandard Abbreviations and Acronyms

ALK1	activin receptor-like kinase 1
BMP9	bone morphogenetic protein 9
CA-ALK1	constitutively active ALK1
EC	endothelial cell
EPC	endothelial progenitor cell
ES	embryonic stem cells
hEPCs	human EPCs
HUVEC	human umbilical vein cell
KDR	kinase insert domain receptor
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor

family have been linked to vascular formation and the modulation of vascular inflammatory responses and remodeling.[14–16] The TGF-β family also plays important roles in the self-renewal, maintenance of pluripotency, and differentiation of embryonic stem cells (ES).[17,18] Specific ligands induce TGF-β signaling by forming a heterotetrameric complex of type I and II receptors, which subsequently initiate Smad phosphorylation in a type I receptor– and cell type–dependent fashion.[15] Among TGF-β type I receptors, activin receptor-like kinase-1 (ALK1) is specifically expressed in blood vessels during embryogenesis[14] and adult stages.[19] In addition, ALK1 mutations have been linked to type II hereditary hemorrhagic telangiectasia, which is characterized by hemorrhages and vascular malformations.[16] Recent studies have revealed that the ubiquitously expressed type I receptor ALK5 inhibits EC migration and proliferation, whereas EC-restricted ALK1 promotes both processes after stimulation with TGF-β1.[20,21] Interestingly, recent studies have also shown that bone morphogenetic protein 9 (BMP9) binds with high affinity to ALK1 in ECs.[22,23] BMP9 circulates in human plasma at high concentrations and helps to maintain the maturation status of blood vessels.[24,25] However, the precise function of BMP9 in vascular physiology remains unclear.

In this study, we evaluated the ALK1 expression in EPCs and then investigated the role of its potential ligand BMP9 in regulating EPC differentiation and function. We likewise analyzed the ALK1 and Smad1/5 pathways to explore the underlying mechanisms. To investigate the possible implication of BMP9 in EPC-mediated neovascularization further, we evaluated the therapeutic efficiency of the administration of BMP9 with EPCs in a mouse hindlimb ischemia model. Our resulting observations revealed a novel role for BMP9/ALK1 in EPC-mediated neovascularization, and suggested that BMP9 may serve as a viable new target for limb ischemia.

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Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Identification of Angiogenic Regulators During EPC Differentiation

EPCs were isolated from human cord blood by density gradient and cultured on fibronectin-coated plates (Figure IA in the online-only Data Supplement). We confirmed EPC identity by the uptake of DiI-labled acetylated low-density lipoprotein (Figure IB in the online-only Data Supplement). After 7 days of cell culture, the EPCs formed distinct colonies on fibronectin-coated dishes (Figure IC in the online-only Data Supplement). The EC-like colony-forming EPCs differentiated into outgrowing EPCs[26,27] (Figure ID in the online-only Data Supplement) that expressed the EC marker VE-cadherin (Figure IE in the online-only Data Supplement) and exhibited endothelial phenotypes including tube formation (Figure IF in the online-only Data Supplement). Furthermore, we evaluated the expression of multiple previously described markers for various cell types,[28] including CXCR4 (EPCs), CD45 (hematopoietic cells), and CD31 and kinase insert domain receptor (KDR; ECs; Figure IG in the online-only Data Supplement).

To understand the molecular signatures associated with the differentiation of EPCs, we generated mRNA expression profiles at days 5, 7, 9, 13, and 17 of EPC differentiation using the HumanHT-12 v3 Expression BeadChip Kit, which uses 48 000 genome-wide probes. First, we identified 8284 differentially expressed genes with >1.5-fold differences when compared with undifferentiated, early EPCs (day 5). These 8284 differentially expressed genes were categorized into 76 clusters based on gene function (Table I in the online-only Data Supplement). Among these, 14 clusters were defined as major patterns during EPC differentiation, given the large cluster size (>50 genes) and their consistent expression patterns (Figure 1A). We then performed enrichment analysis of the gene ontology biological processes and Kyoto Encyclopedia of Genes and Genomes pathway database searches for the 14 gene clusters (Figure 1B) using DAVID (The Database for Annotation, Visualization and Integrated Discovery) Functional Annotation Bioinformatics Microarray Analysis software.[29] Most of the immune response-related genes were expressed at early differentiation stages and continuously decreased in expression as endothelial differentiation progressed, whereas expression of angiogenic functional genes related to wound healing and actin cytoskeleton increased concurrently with EPC→EC maturation (Figure 1B). Furthermore, EPC→EC differentiation also activated signaling pathways involved in angiogenesis,[30–33] including the TGF-β, BMP, Notch, and integrin signaling pathways.

Among the 14 clusters, we focused on the 7 upregulated clusters that apparently contributed to promoting EPC differentiation. We then identified 493 potential angiogenic regulators (Table II in the online-only Data Supplement) that could contribute to EPC differentiation by selecting hub-like molecules with a significant (P<0.01) number of interactors in the 7 upregulated clusters based on the interactome data.[34–38] We then performed enrichment analysis of the gene ontology biological processes and Kyoto Encyclopedia of Genes and Genomes pathways for the 493 potential angiogenic regulators (Table). Interestingly, these regulators included 24 components involved in TGF-β signaling and 47 components involved in the regulation of the actin cytoskeleton that most closely interacted with the TGF-β signaling pathway (Figure II in the online-only Data Supplement). After reconstruction of the network model delineating the functional roles of TGF-β signaling using the 24 and 47 components, we found that the expression of ALK1, a TGF-β type 1 receptor, was most

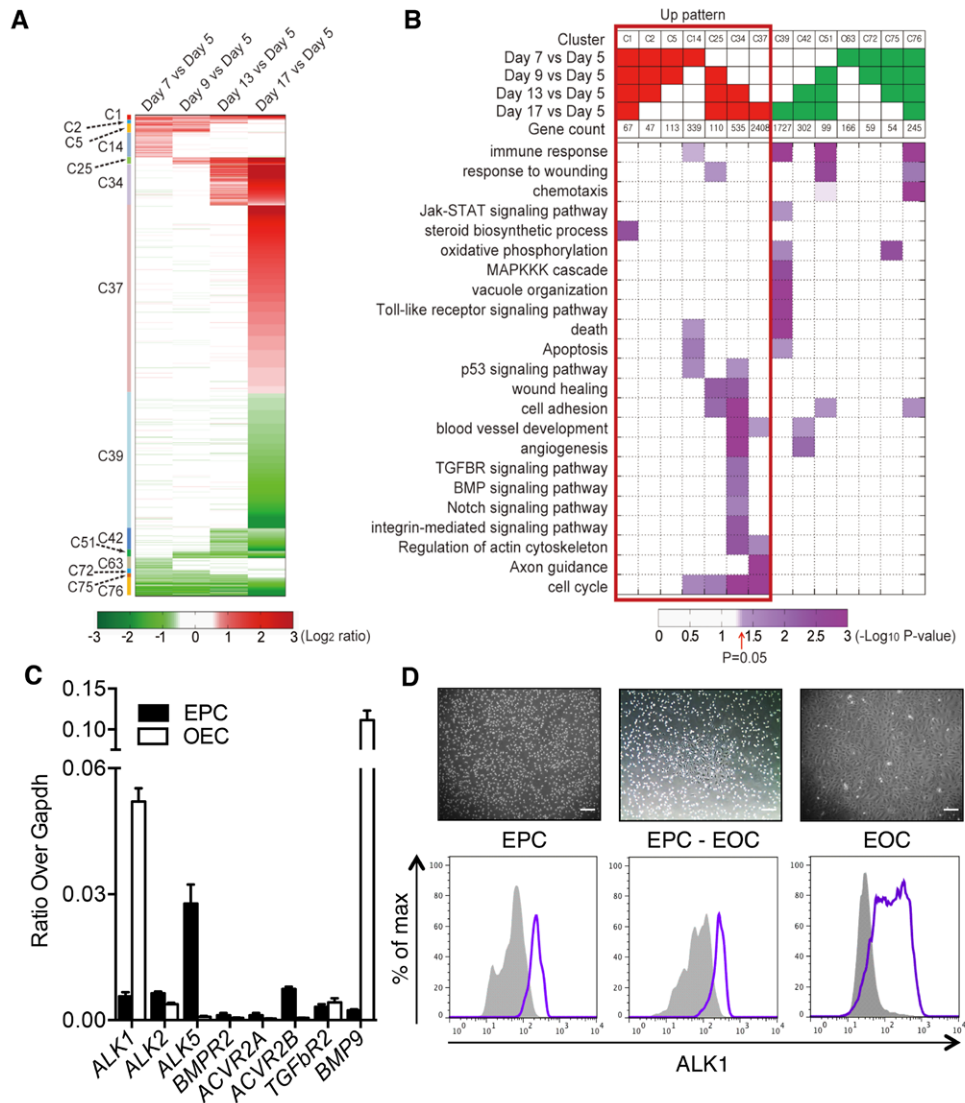

Figure 1. Identification of activin receptor-like kinase 1 (ALK1) during endothelial progenitor cell (EPC)→endothelial cell (EC) differentiation. Major clusters of gene expression change and associated cellular processes during EPC→EC differentiation. **A**, Fourteen major clusters (C1–2, C5, C14, C25, C34, C37, C39, C42, C51, C63, C72, and C75–76) of the genes showing differential expression during EPC differentiation. Gene expression change during EPC differentiation (day 7 to day 17) is shown as $\log_2$-fold-changes using day 5 as a control. Red and green colors indicate up- and downregulation, respectively. (positive and negative $\log_2$-fold-changes; see the color bar). **B**, Gene ontology biological process and Kyoto Encyclopedia of Genes and Genomes pathways represented by the genes in individual clusters. Color gradient indicates $-\log_{10}(P)$, where P is the enrichment P value obtained from the DAVID (The Database for Annotation, Visualization and Integrated Discovery) software (see the color bar). **C**, Expression of ALK1 and other transforming growth factor (TGF)-β family genes measured by a quantitative reverse transcriptase-polymerase chain reaction analysis. The transcript levels were normalized to GAPDH mRNA levels. **D**, Phase-contrast figures showing EPC morphology at each stage. Scale bars, 150 μm. ALK1 expression was determined by fluorescence cytometry. EPC: day 5 to 7; EPC–endothelial outgrowth cells (EOC): EPC→EC transitioning, day 25.

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notably changed during EPC differentiation (Figure II in the online-only Data Supplement).

Expression of ALK1 in EPCs and Identification of BMP9

First, we determined the expression patterns of key ligands and receptors in the TGF-β signaling pathway to evaluate their correlations with the network model. We obtained samples from early EPCs and endothelial outgrowth cells, and the expression levels of TGF-β family members were evaluated in each respective stage. As confirmed by quantitative reverse transcriptase-polymerase chain reaction and flow cytometry analysis, ALK1 expression was significantly upregulated during EPC→EC differentiation (Figure 1C and 1D). Conversely, the expression of ALK5 was decreased. Previous reports have indicated that ALK1 is important in blood vessel formation during embryogenesis[14] and adult stages,[19] and that mice genetically deficient for ALK1 exhibit severe vascular malformation.[16] This suggests the possibility that activation of ALK1 signaling by specific ligands contributes to the differentiation and homing of stem/progenitor cells that participate in new vessel formation. BMP9, a high-affinity ligand for ALK1,[23,39] demonstrated a similar expression pattern, which was consistent with the increased expression of ALK1 (Figure 1C).

Table. GOBP and KEGG Pathways Enriched By Up-Pattern Regulators

GOBP and KEGG Pathways	Count	*P* Value
Focal adhesion	86	1.25E−47
Cell cycle	118	2.62E−44
Regulation of apoptosis	102	4.97E−31
ECM-receptor interaction	45	1.97E−29
Regulation of cell proliferation	85	6.06E−21
TGF-β signaling pathway	33	7.12E−16
ErbB signaling pathway	33	7.12E−16
Cell adhesion	66	2.94E−13
DNA repair	37	1.66E−11
Chromosome organization	47	5.87E−10
Embryonic development ending in birth	37	1.62E−09
Vasculature development	30	1.40E−08
Apoptosis	24	1.62E−08
p53 signaling pathway	21	1.99E−08
Neuron differentiation	40	7.13E−08
Wnt signaling pathway	31	1.33E−07
Regulation of actin cytoskeleton	38	2.20E−07
Chemokine signaling pathway	34	5.73E−07

ECM indicates extracellular matrix; ErbB, erythroblastic leukemia viral oncogene homolog; GOBP, gene ontology biological processes; KEGG, Kyoto Encyclopedia of Genes and Genomes; and TGF, transforming growth factor.

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Effect of BMP9 on EPC→EC Differentiation In Vitro

Next, we evaluated whether BMP9 actively participated in EPC→EC differentiation. We treated EPC cultures with BMP9 every 2 days, starting 7 days after the initial EPC isolation. endothelial outgrowth cells morphology began to appear on day 15 in BMP9-treated EPCs when compared with day 21 in unstimulated control EPC cultures (Figure 2A–2C). We then characterized differentiated colonies by assessing EC marker expression. We performed quantitative reverse transcriptase-polymerase chain reaction for CD31, KDR, and CD144 (Figure 2D) and further confirmed the results by fluorescence-activated cell sorter analysis (Figure 2E). BMP9 stimulation led to significant increases in the expression levels of CD31, KDR, and CD144 (Figure 2D and 2E), which suggested that BMP9 stimulation acts on EPCs to promote EC differentiation.

Effect of BMP9 on EPC Functionality In Vitro

EPC homing is a multistep process that involves initial cell adhesion to the microvascular wall, transmigration, and tissue invasion.[40] The EPC homing capacity enhances the functional integration of injected EPCs used in cell-based therapies. Therefore, we examined the effect of BMP9 on EPC migration using modified Boyden chamber assays. When compared with unstimulated EPCs, BMP9 stimulation increased EPC migratory capacity to a level that approached the extent obtained after stimulation with VEGF (Figure 2F), a known EPC chemokine.[41]

EPC adhesion to the extracellular matrix and mature ECs is important for regenerative activity during vasculogenesis.[40] Therefore, we examined the effect of BMP9 on EPC adhesion to fibronectin and human umbilical vein cells (HUVECs) using VEGF as a positive control,[41] and discovered that BMP9 increased EPC adhesion to fibronectin (Figure 2G) and HUVECs (Figure 2H). A prior report indicated that integrins are important for the homing of transplanted hematopoietic stem cells to bone marrow.[42] Thus, we investigated the contribution of α5- and β1-integrins, which have important roles in EPC adhesion and migration.[42] Interestingly, BMP9 treatment (10 ng/mL) for 10 days increased EPC integrin α5 and β1 mRNA expression levels (Figure 2I).

To gain additional insight into the effect of BMP9 on EPC angiogenic properties, we performed Matrigel angiogenesis assays. The coculturing of HUVECs with EPCs on a Matrigel matrix led to tubule network formation and the ability of EPCs to integrate into vascular networks in vitro.[9] When we cocultured HUVECs with EPCs, BMP9 stimulated greater tubule network formation (Figure 2J) as determined by tube length (Figure 2K) and number (Figure 2L). Furthermore, the number of EPCs incorporated into EPC/HUVEC tubule networks per total tubule area was significantly higher when cocultures were stimulated with BMP9 (Figure 2M).

Finally, to explore the effect of BMP9 stimulation on EPC viability, we measured cellular ATP levels after treatment with different doses of BMP9 during starvation for 48 hours, but no differences in EPC viability after BMP9 treatment were detected (Figure III in the online-only Data Supplement). Collectively, the data suggested that BMP9 increases EPC capacity in vitro by enhancing both migratory and adhesive activities rather than by affecting EPC viability.

Blocking Effect of ALK1 on BMP9-Induced EPC→EC Differentiation In Vitro

Having demonstrated that EPCs express ALK1 (Figure 1C and 1D), we investigated the role of ALK1 on BMP9-induced EPC differentiation. The transfection of EPCs with lentiviral constructs encoding constitutively active ALK1 (CA-ALK1) or dominant-negative ALK1 constructs resulted in increased ALK1 protein expression when compared with EPCs that were mock-transfected with virus alone (Figure IVB in the online-only Data Supplement). When we replenished the EPC media containing 10 ng/mL BMP9 every 2 days for 25 days, CA-ALK1–transfected EPCs exhibited EC-like morphology earlier than either dominant-negative ALK1– or mock-transfected EPCs (Figure 3A; Figure IVA in the online-only Data Supplement). The increase in the number of differentiated colonies was also greater in CA-ALK1–transfected EPCs than in dominant-negative ALK1– or mock-transfected EPCs (Figure 3B). As well, differentiated colonies were determined by EC using quantitative reverse transcriptase-polymerase chain reaction for CD31, KDR, and CD144 (Figure 3C; Figure IVC and IVD in the online-only Data Supplement). Taken together, the data suggested that BMP9 contributes to EPC→EC differentiation through ALK1 signaling. Interestingly, when we cultured transfected EPCs without BMP9, we noted an increase in the number of differentiated colonies in CA-ALK1–transfected EPCs (Figure IVE in the online-only Data Supplement), but to a lesser extent than BMP9-treated EPCs. The results indicated that ALK1

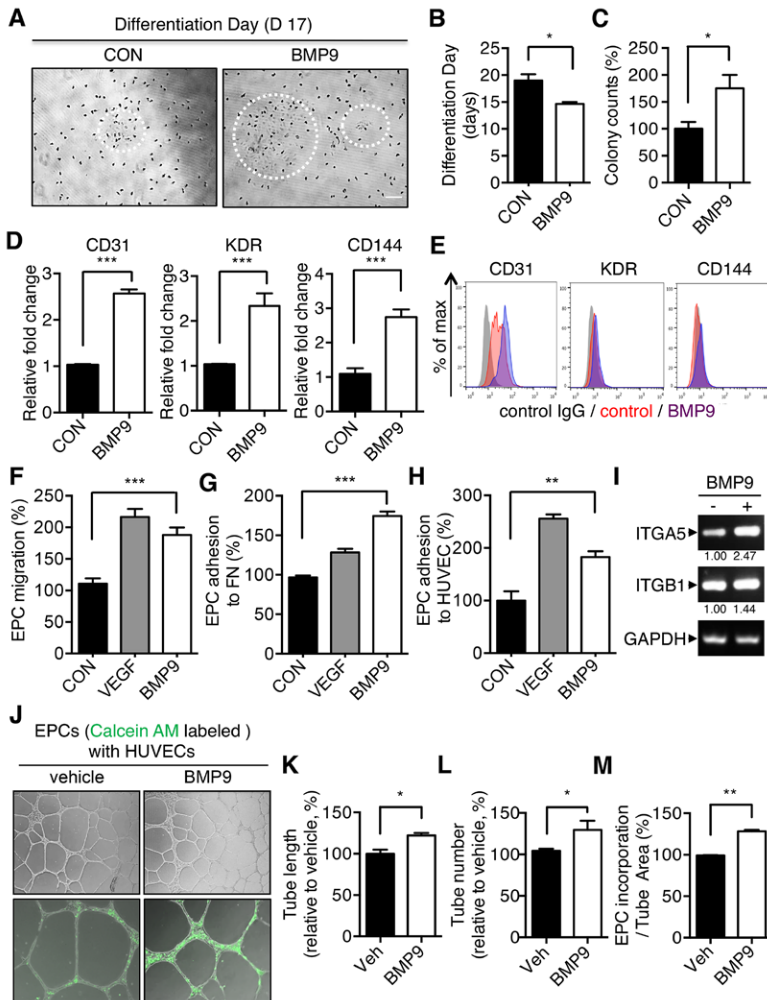

Figure 2. Bone morphogenetic protein 9 (BMP9) induces endothelial progenitor cell (EPC)–endothelial cell (EC) differentiation and endothelial progenitor cells (EPCs) angiogenic function in vitro. A–C, Morphological assessment of the differentiation of EPCs on day 17 using phase-contrast microscopy (**A**). Dashed lines indicate endothelial outgrowth cell colony. Scale bars, 150 μm. Quantitative analysis of the differentiation rate (**B**) and the number of colonies (**C**). *$P<0.05$ vs control. **D** and **E**, After exposure of EPCs to BMP9 (10 ng/mL) for 17 days, mRNA and protein levels of the endothelial markers *CD31*, *KDR*, and *CD144* were assessed by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (**D**) and fluorescence-activated cell sorter analysis (**E**). ***$P<0.001$ vs control. **F**, D7-EPC migration assays were performed using a modified Boyden chamber containing BMP9 (10 ng/mL) or vascular endothelial growth factor (VEGF; 20 ng/mL) in the lower chamber. Cell migration was assessed by counting cells in 8 random fields. ***$P<0.001$ vs control. **G** and **H**, Calcein AM–labeled D7-EPCs were seeded onto fibronectin (**G**) or human umbilical vein cell (HUVEC; **H**)–coated 96-well plates with BMP9 (10 ng/mL) or VEGF (20 ng/mL). After 1 hour in culture, wells were gently washed, and adhesion was quantified by counting attached EPCs using fluorescence microscopy. ***$P<0.001$, **$P<0.01$ vs control. **I**, EPCs were cultured with or without BMP9 (10 ng/mL), and integrin α5 and β1 mRNA levels were determined by RT-PCR. Densitometric band analyses are quantified using ImageJ software and are presented as the relative ratio of ITGA5 or ITGB1 to GAPDH. The relative ratio in untreated control cells is arbitrarily presented as 1.00 (**bottom**). **J**–**M**, D7-EPCs stained with calcein AM were cocultured with HUVECs atop Matrigel-coated 24-well plates in low-serum (1%) media with or without BMP9 (10 ng/mL). Representative images of complete tube formation (**J**, original magnification ×40 [**top**] and ×100 [**bottom**]), and quantification of tube length (**K**), tube number (**L**), and the number of EPCs that were incorporated into tube-like structures (**M**). *$P<0.05$, **$P<0.01$, n=5.

activation is required for EPC→EC differentiation, and that BMP9 may act endogenously through ALK1 in EPCs.

Blocking Effect of ALK1 on BMP9-Induced Angiogenic EPC Activity In Vitro

To investigate whether ALK1 affects BMP9-induced EPC angiogenic activity, we used a soluble chimeric ALK1 protein (hALK1-Fc) to competitively inhibit exogenous BMP9 signaling.[43] Treatment with hALK1-Fc reduced BMP9-induced Smad1/5/8 phosphorylation in EPCs to basal levels (Figure 3D) and decreased BMP9-induced EPC migration and adhesion (Figure 3E and 3F). In addition, hALK1-Fc treatment abrogated BMP9-induced increases in tube length and number (Figure VA–VC in the online-only Data Supplement) and decreased the number of EPCs incorporated into the vessel-like structures (Figure VD and VE in the online-only Data Supplement). These data suggested that ALK1 activation is related to the BMP9-induced angiogenic abilities of EPCs.

To demonstrate that the role of BMP9 in tube formation is primarily because of the effect of BMP9 on EPCs rather than on HUVECs, we primed EPCs with BMP9 for 24 hours before performing Matrigel angiogenesis assays. The coculturing of BMP9-primed EPCs with HUVECs on a Matrigel matrix resulted in increased tubule network formation (Figure 3G–3J). When EPCs were pretreated with hALK1-Fc, HUVECs cocultured with BMP9-primed EPCs on a Matrigel matrix exhibited reduced tubule formation as measured by length and number (Figure 3H and 3I). We found that hALK1-Fc pretreatment also reduced the number of BMP9-primed EPCs incorporated into tubules (Figure 3J). Collectively, our results demonstrated that the effect of BMP9 on tubule network formation is exerted solely on EPCs and is mediated by ALK1/Smad signaling.

Enhanced Neovascularization by Local Injection of BMP9 and EPCs in a Mouse Hindlimb Ischemia Model

Finally, we investigated whether injecting EPCs with BMP9 would enhance neovascularization in vivo using a mouse hindlimb ischemia model. After inducing ischemia by excising 1 femoral artery, athymic nude mice received an intramuscular injection of human EPCs (hEPCs), with or without BMP9, in the lower calf muscle of the ischemic limb. As additional controls, other groups of mice with hindlimb ischemia were injected with vehicle or BMP9 alone. Blood perfusion in the ischemic and nonischemic hindlimbs was assessed using laser-Doppler imaging. Mice coinjected with EPCs and BMP9 exhibited significantly enhanced recovery of hindlimb blood

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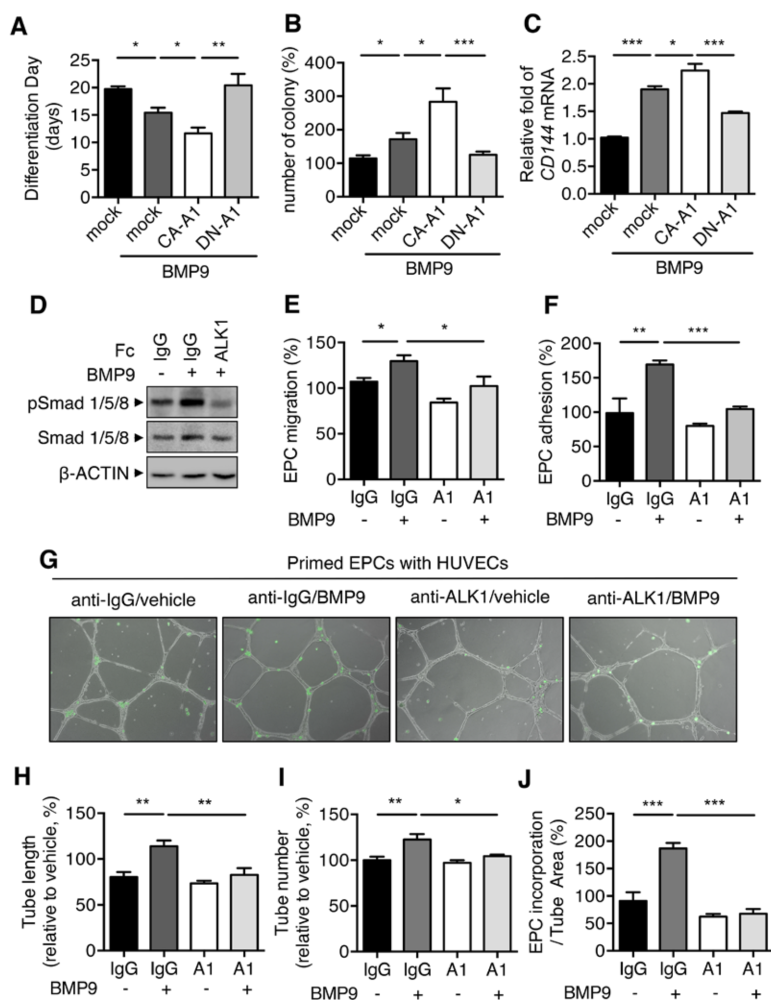

Figure 3. Bone morphogenetic protein 9 (BMP9)–induced endothelial progenitor cell (EPC) differentiation and vascularization is regulated by activin receptor-like kinase 1 (ALK1) signaling in vitro. **A**–**C**, D7-EPCs transfected with lentivirus encoding mock, constitutively active (CA)-ALK1 or dominant negative (DN-ALK1). We treated transfected EPCs with or without BMP9 (10 ng/mL) every 2 days for 25 days. Quantitative analysis of the differentiation rate (**A**) and the number of colonies (**B**). After exposure of EPCs to BMP9 (10 ng/mL) for 20 days, mRNA levels of the endothelial marker *CD144* were assessed by quantitative reverse transcriptase-polymerase chain reaction (**C**). **D**, Before 1-hour treatment with hFc-ALK1 (10 μg/mL), EPCs were incubated with BMP9 (10 ng/mL) for 2 hours. BMP9-induced Smad 1/5/8 phosphorylation was determined by Western blotting. **E**, D7-EPC migration assays were performed using a modified Boyden chamber with or without BMP9 (10 ng/mL) and hFc-ALK1 (A1; 10 μg/mL) in the lower chamber. Cell migration was assessed by counting cells in eight random fields. **F**, Calcein AM–labeled D7-EPCs were seeded onto fibronectin-coated 96-well plates with or without BMP9 (10 ng/mL) and hFc-ALK1 (A1; 10 μg/mL). After 1 hour in culture, wells were gently washed, and adhesion was quantified by counting attached EPCs using fluorescence microscopy. **G**–**J**) We pretreated D7-EPCs with or without hFc-ALK1 (A1; 10 μg/mL) for 1 hour before priming with BMP9 (10 ng/mL). BMP9-primed EPCs and HUVECs were seeded into Matrigel-coated wells. Representative images of complete tube formation (original magnification ×40 [**G**]) and quantification of tube length (**H**), tube number (**I**), and the number of EPCs incorporated into tube-like structures (**J**). $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

perfusion when compared with mice that were treated with EPCs, vehicle, or BMP9 only (Figure 4A and 4B). Four weeks after injection, the group injected with vehicle only exhibited extensive necrosis of the ischemic hindlimb, which resulted in a high rate of limb loss and necrosis (Figure VI in the online-only Data Supplement). Intramuscular injection of EPCs reduced the rate of limb loss when compared with the vehicle or BMP9-only injection groups, and the effects of the coadministration of EPCs and BMP9 on limb salvage remained significantly enhanced (Figure 4A and 4B). Next, we examined the histology of hindlimb skeletal muscle sections from mice euthanized 28 days after EPC injection. Histological examinations revealed significantly more CD31-positive capillary ECs in muscle sections from mice that received EPCs and BMP9 versus mice that were injected with EPCs alone or with vehicle or BMP9 only (Figure 4C and 4D). Collectively, the data indicated that blood flow recovery and new vessel formation in ischemia-damaged muscle were increased by local injection of EPCs with BMP9.

To evaluate the impact of BMP9 on EPC-mediated revascularization further, we also compared the effects of BMP9-primed EPCs on neovascularization in the mouse hindlimb ischemia model. Cord blood–derived EPCs primed for 24 hours with or without BMP9 were intramuscularly injected into ischemic limbs. The injection of BMP9-primed EPCs improved limb perfusion when compared with vehicle-primed EPCs (Figure 4E and 4F). Histologically, we also observed more capillaries in ischemic limbs from mice that received BMP9-primed EPCs (Figure 4G and 4H). Hence, our results conclusively demonstrated that BMP9 enhances the effects of injected EPCs on increased blood perfusion and vessel density in ischemic hindlimbs in mice.

Engraftment of Transplanted EPCs With BMP9 Into Vascular Structures in Ischemic Limbs

To determine the contribution of EPCs implanted with BMP9 on neovascularization, we assessed EPC incorporation. The presence of hEPCs in ischemic limbs after transplantation was assessed using reverse transcriptase-polymerase chain reaction analysis of human GAPDH, and the results revealed that GAPDH was specifically expressed in the EPC-injected group (Figure 5A). We verified EPC engraftment using DiI-acLDL–labeled (Figure 5B; Figure VIIA in the online-only Data Supplement) and calcein AM–labeled EPCs (Figure 5C–5E; Figure VIIB in the online-only Data Supplement) and performed immunohistochemical labeling for human nuclear antigen (Figure 5D). The formation of capillary networks was confirmed by CD31-positive ECs in ischemic muscles (Figure 5B and 5C, arrowheads). Furthermore, numerous hEPCs were detected and frequently colocalized with CD31-positive mouse ECs in BMP9/EPC-injected mice (Figure 5B

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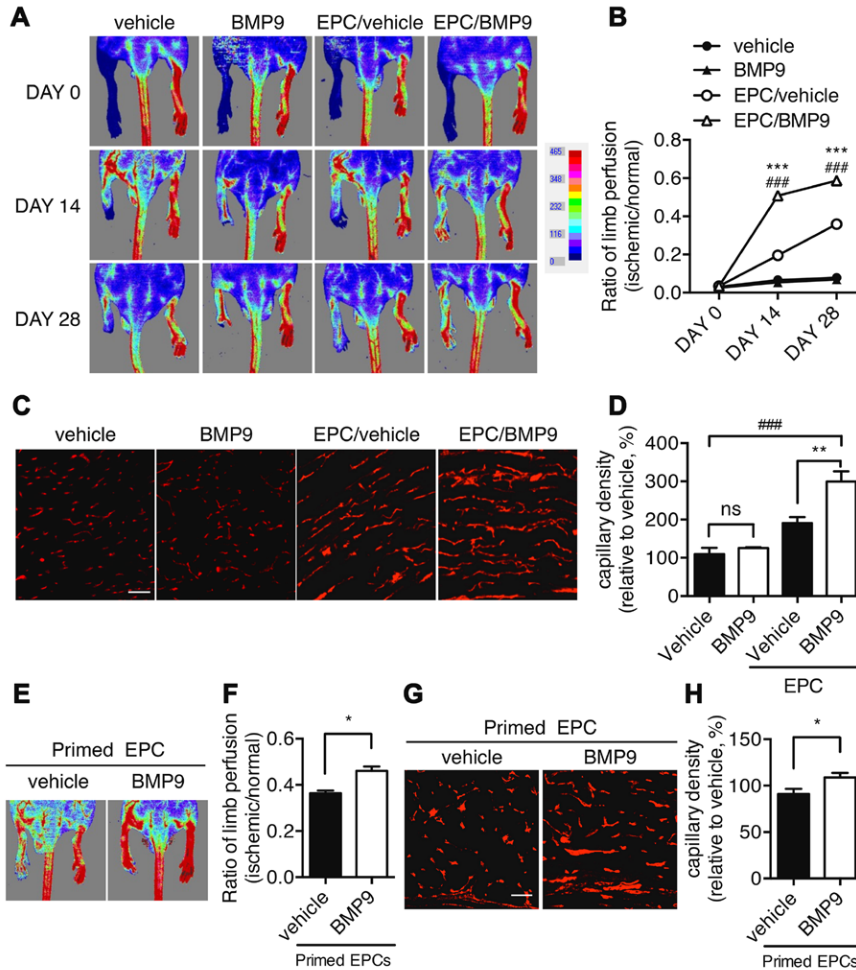

Figure 4. Bone morphogenetic protein 9 (BMP9) improves blood flow in ischemic mouse hindlimbs after human endothelial progenitor cell (hEPCs)–BMP9 transplantation. **A**–**D**, After unilateral hindlimb ischemia surgery, ischemic calf muscles were injected with EPCs alone, EPCs plus BMP9 (100 ng), BMP9 alone (100 ng), or vehicle. Representative examples of laser-Doppler images (**A**) and quantification of hindlimb blood flow (**B**). ###$P<0.001$, BMP9-treated vs vehicle-injected group; ***$P<0.01$, BMP9-treated vs hEPC-injected group; n=9 animals per group. **C**, Representative figures immunolabeled with antimouse CD31 (red). Scale bar, 50 µm. **D**, Quantitative analysis of capillary density. ###$P<0.001$, BMP9-treated vs vehicle-injected group; **$P<0.01$ BMP9-treated vs EPCs-injected group; n=10 animals per group. **E**–**H**, After unilateral hindlimb ischemia surgery, ischemic calf muscles were injected with BMP9-primed EPCs. EPCs were primed BMP9 (10 ng/mL) for 24 hours, before injection. Representative examples of laser-Doppler images at 28 days (**E**) and quantification of hindlimb blood flow (**F**). *$P<0.05$, n=5 animals per group. **G**, Representative figures immunolabeled with antimouse CD31 (red). Scale bar, 50 µm. **H**, Quantitative analysis of capillary density. *$P<0.05$, n=10 animals per group.

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and 5C, arrows). Human nuclear antigen staining with CD31 confirmed the presence of blood vessels comprised transplanted hEPCs (arrows) and mouse ECs (Figure 5D). In addition, incorporated calcein AM–labeled EPCs were stained with KDR, which revealed endothelial lineage 1 month after EPC injection (Figure 5E). Taken together, BMP9 helped the transplanted hEPCs to successfully engraft into mouse tissue and subsequently induced vascular network formation by improving both the incorporation of EPCs into the vasculature and the EPC→EC differentiation.

Mechanistic Link Between ALK1 and BMP9-Enhanced EPC Neovascularization in a Mouse Hindlimb Ischemia Model

To assess whether ALK1 is related to EPC function in vivo and in vitro, we induced hindlimb ischemia in mice and treated the animals with hALK1-Fc. As expected, BMP9 treatment with the transplantation of EPCs in the ischemic limbs led to a dramatic improvement in hindlimb blood perfusion (Figure 6A and 6B) and elevated vessel densities (Figure 6C and 6D). However, in hALK1-Fc–treated BMP9/EPCs transplanted into mice, we observed decreased blood perfusion (Figure 6A and 6B) and vessel density (Figure 6C and 6D). Furthermore, immunofluorescence labeling demonstrated that a greater number of hEPCs were incorporated into CD31-positive mouse ECs in the BMP9/EPC group, but hALK1-Fc treatment reversed this effect and resulted in outcomes similar to the EPC group (Figure 6E and 6F). These findings were consistent with our in vitro data, and suggested that the in vivo angiogenic capacity of EPCs is because of, at least in part, ALK1 pathway signaling in these cells.

Discussion

In the current study, we determined that BMP9 is a novel regulator of EPC-mediated neovascularization and demonstrated

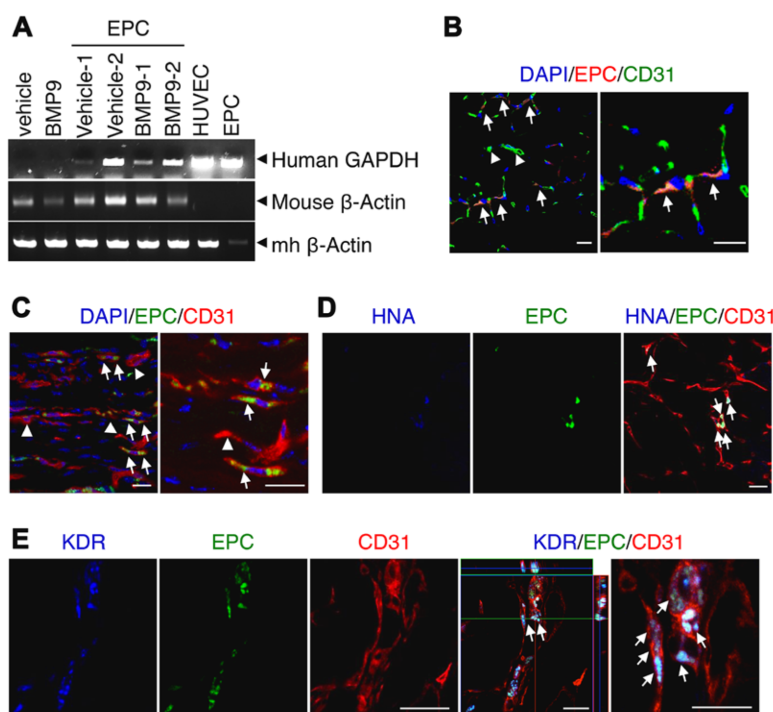

Figure 5. Bone morphogenetic protein 9 (BMP9) increases endothelial progenitor cell (EPC) incorporation and differentiation into ECs in vivo. **A**, reverse transcriptase-polymerase chain reaction for human GAPDH showing the presence of human cells in mouse ischemic tissues. In vitro cultured human umbilical vein cell (HUVEC)s and human EPC (hEPC) cDNA are positive controls. **B** and **C**, The formation of capillary networks between CD31-positive human cells (transplanted hEPCs, arrow) and CD31-positive mouse cells (mouse ECs, arrowheads) was observed in ischemic limb tissues 1 month after transplantation. **B**, Transplantation of DiI-acetylated low-density lipoprotein–labeled hEPCs (red) with BMP9 in ischemic limb. Vasculature was labeled with an anti-CD31 antibody (green), and nuclei were stained with DAPI (blue). Scale bars, 20 μm. **C**, Transplantation of calcein AM–labeled hEPCs (green) with BMP9 in ischemic limb. Vasculature was labeled with an anti-CD31 antibody (red), and nuclei were stained with DAPI (blue). Scale bars, 20 μm. **D**, Calcein AM–labeled hEPCs (green) were costained with human nuclear antigen (HNA, blue) and incorporated into the vascular endothelium (red) in the ischemic muscle. Scale bar, 20 μm. **E**, Transplanted hEPCs were differentiated into ECs that stained with kinase insert domain receptor (KDR). From **left** to **right**, First through third panels, calcein AM–labeled hEPCs (green) were costained with the human EC marker, KDR (blue), and were incorporated into the vascular endothelium (red) in ischemic tissue. Scale bar, 20 μm; fourth panel, a 3-dimensional-reconstructed merged confocal image. KDR-positive transplanted hEPCs (arrows) were observed in mouse ECs. Scale bar, 20 μm; fifth panel, an enlarged confocal image. Scale bar, 20 μm.

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that BMP9 significantly increased EPC→EC differentiation and the angiogenic activities of EPCs. BMP9 also improved neovascularization in a mouse hindlimb ischemia model by increasing vessel density and ultimately improving blood perfusion. We determined that the effects of BMP9-induced EPC neovascularization were mediated by ALK1, which is expressed in EPCs. Furthermore, both short- and long-term stimulation of BMP9 could induce EPC incorporation into neovascular structures, which indicates diverse therapeutic applications for patients with ischemic vascular diseases.

Accumulating evidence suggests that EPCs have therapeutic potential in promoting the re-endothelialization of damaged vessel walls and the neovascularization of ischemic tissues.[4,13] The contributions of EPCs to neovascularization are thought to be mediated through paracrine effects by the secretion of angiogenic cytokines and direct involvement of de novo differentiated ECs after EPC incorporation into ischemic tissues.[13] Several factors, including VEGF and stromal-derived factor-1, have been shown to promote neovascularization by enhancing EPC utility in the ischemic sites in addition to the direct angiogenic actions of EPCs.[7,8] Although the ways and extents to which these molecules affect EPCs vary, the recruitment of EPCs to the ischemic site, which involves motility and adhesion to the neovascular area, is a commonality. Accordingly, the results of the in vitro assays in this study demonstrated that BMP9 increases EPC chemotaxis and adhesion. Circulating EPCs are mobilized both endogenously in response to tissue ischemia and exogenously by cytokines in ischemic tissues.[4,11] The possibility that cytokines could mobilize EPCs has been implied by several previous observations. VEGF mobilizes EPCs from BM, and stromal-derived factor-1 stimulates local accumulation of transplanted EPCs, thereby resulting in enhanced neovascularization in a mouse hindlimb ischemia model.[7,41] Here, we demonstrated that BMP9 exerts a chemotactic effect on EPCs in a manner similar to that of VEGF. These observations suggested that BMP9-mediated signals can enhance EPC recruitment to ischemic sites. Multiple integrins have also been implicated in EPC mobilization, homing, and differentiation. In particular, integrin $\alpha5\beta1$ is expressed in EPCs and is involved in the homing of EPCs to denuded vessels, where it acts as a fibronectin receptor.[42] Interestingly, stimulation of EPCs with BMP9 increased adhesion to fibronectin and HUVECs by increasing integrin $\alpha5\beta1$ expression. Consequently, local BMP9 administration might improve EPC homing to ischemic sites by increasing their migration and adhesion. To this

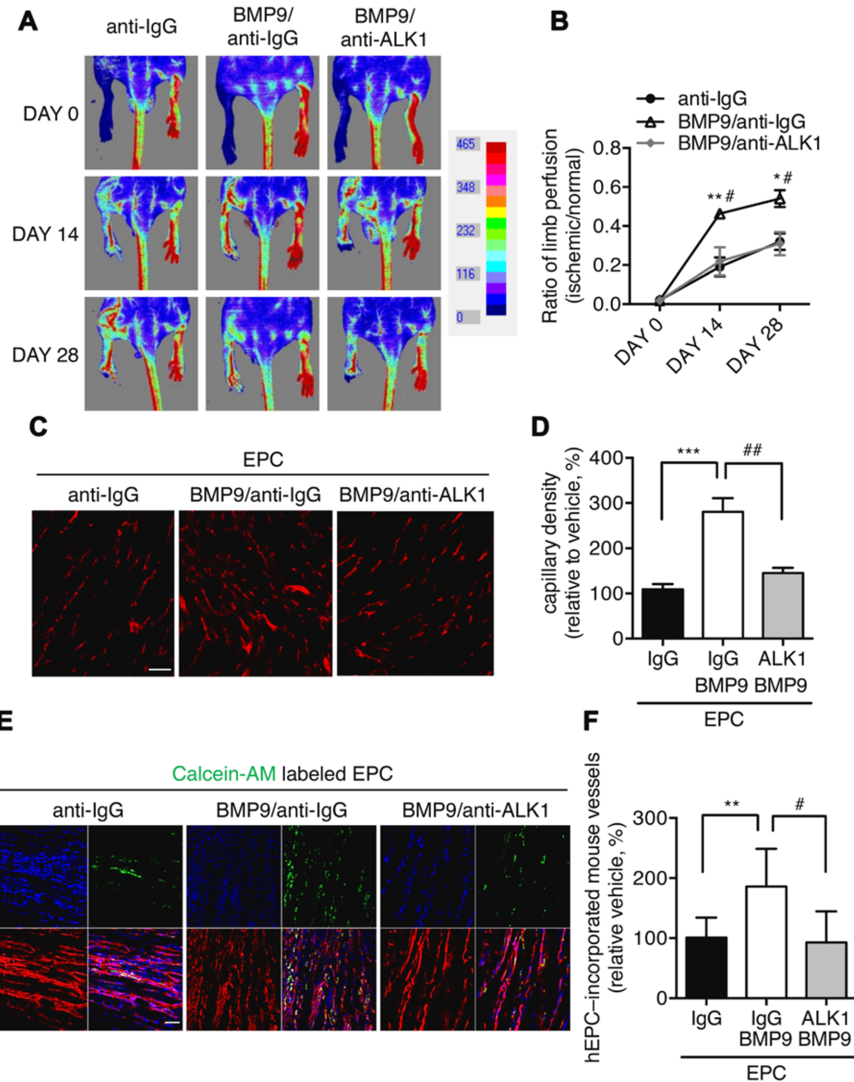

Figure 6. Bone morphogenetic protein 9 (BMP9)–induced endothelial progenitor cell (EPC) neovascularization is regulated by activin receptor-like kinase 1 (ALK1) in the mouse hindlimb ischemia model. After unilateral hindlimb ischemia surgery, ischemic calf muscles were injected with EPCs with or without BMP9 and hFc-ALK1. Representative examples of laser-Doppler images (**A**) and quantification of hindlimb blood flow (**B**). $^{**}P<0.01$, $^{*}P<0.05$, BMP9-treated EPCs vs EPCs group; $\#P<0.05$, BMP9-treated EPCs vs hFc-ALK1-treated EPCs group; n=5 animals per group. **C**, Representative figures of antimouse CD31 immunolabeling (red). Scale bar, 50 μm. **D**, Quantitative analysis of capillary density. $^{***}P<0.001$, $\#\#P<0.01$, n=7 animals per group. **E**, Confocal analysis showed that Calcein AM–labeled EPCs (green) were incorporated into the vascular endothelium (red) in ischemic muscle. Scale bar, 50 μm. **F**, Bar graph showing the relative density of human EPC (hEPC)–incorporated mouse vessels in ischemic regions. $^{**}P<0.01$, $\#P<0.05$. n=7 animals per group.

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end, we found that intramuscular injections of BMP9 and EPCs combined significantly improved blood flow recovery and capillary density in ischemic muscle versus the injection of only EPCs in the mouse hindlimb ischemia model. Interestingly, when we administered local injections of mouse or human BMP9 only, no improvement was observed. This result suggested that BMP9 only affects EPCs and may not be sufficient to mobilize EPCs from bone marrow. Nevertheless, coadministration of EPCs with BMP9 did lead to improvements. A sufficient number of EPCs were recruited to the local BMP9 injection sites and were synchronized by activation of adhesion molecules in the integrin family.

An alternative approach involves priming, which is the short-term, protein-based, ex vivo stimulation of EPCs before therapeutic injection. EPC pretreatment with mobilizing factors initiates an activation program within EPCs; thus, the therapeutic issue of EPC engraftment might circumvent the insufficient cell numbers and low efficiency of EPC incorporation.[9,12] We demonstrated that BMP9-primed EPCs repaired mouse limb ischemia by increasing capillary density, which subsequently improved blood flow. This result was further supported by our in vitro observation that priming EPCs with BMP9 increased the number of EPCs incorporated into tubules in Matrigel angiogenesis assays. Thus, BMP9 priming could enable EPCs to overcome the limitation of the duration of their effects on EPC-mediated neovascularization. Overall, we optimized the conditions of EPC-based therapy using co- and pretreatment with BMP9, which facilitated the multistep process of EPC recruitment, adhesion, and incorporation into vasculature.

The order of signals required to initiate EPC differentiation to ECs and to activate EPCs to participate in neovascularization remains unclear. To address the fundamental questions on EPC biology and the associated underlying molecular mechanisms, global gene expression profiling of cord blood–derived EPCs was performed to identify genes involved in EPC differentiation. The resulting profiles indicated a requirement for TGF-β signaling in EPC differentiation and provided a comprehensive understanding of EPC molecular mechanisms, which could decrease the associated risks and increase the efficacy of EPC-based therapies. Previous studies have demonstrated that TGF-β family members function in the maintenance and differentiation of ES, somatic stem cells, and cancer stem cells.[17,18] The activation of the TGF-β/activin/nodal branch through Smad2/3 signaling is important for human ES to retain their undifferentiated state, whereas the differentiation of human ES results in increased Smad1/5 phosphorylation and localization to the nucleus.[17] Combined with our results, which described Smad1/5 phosphorylation in EPCs after BMP9 treatment, the existing data suggest a compelling role for Smad 1/5 signaling in the differentiation of hEPCs and human ES.

TGF-β signaling is highly regulated to control the balance between activating and resting signals during angiogenesis in ECs.[20] Recent studies have demonstrated that TGF-β can bind ALK1 and ALK5, which are 2 distinct type 1 receptors that exert opposite effects in ECs.[20,21] In accord with previous studies, ALK1 and ALK5 expression levels exhibited opposite patterns in our system as the level of ALK1 was increased, whereas ALK5 decreased during EPC→EC differentiation. The ratio of ALK5:ALK1 on ECs is important for determining whether the condition of the endothelium is activated or quiescent. ALK1 is upregulated at sites of active angiogenesis during mouse embryogenesis and stimulates EC migration and proliferation. ALK5, however, is more widely expressed, and inhibits EC migration and proliferation.[44] Similarly, we examined the expression of ALK1 and ALK5 during EPC→EC differentiation and found that the activation of ALK1 induced EPC→EC differentiation, EPC adhesion, and migration. Increasing evidence indicates that BMP9 binds ALK1 with high affinity, and that ALK1 is abundantly expressed in the vascular system, including in ECs.[22] This report describes ALK1 expression on EPCs and demonstrates both in vitro and in vivo that BMP9 can activate EPCs through ALK1. Defective ALK1 signaling reduces EPC differentiation and angiogenic functional abilities, even in the presence of BMP9 stimulation. CA-ALK1 expression after lentiviral transfection significantly accelerated EPC→EC differentiation versus dominant-negative ALK1 and mock transfectants. Recently, soluble ALK1 was used to block circulating BMP9. The hALK1-Fc conjugate binds to both BMP9 and BMP10, but not to other TGF-β family members (ie, TGF-β1, TGF-β2, and TGF-β3), and exhibits a powerful antiangiogenic ability that blocks vascularization in tumor angiogenesis.[43,45] In accord with previous results, hALK1-Fc/BMP9 coadministration blocked Smad1/5 phosphorylation and decreased both tube formation and the number of EPCs incorporated into neovessel-like structures in Matrigel angiogenesis assays. Likewise, tube formation induced by BMP9-primed EPCs was reduced by pretreatment with hALK1-Fc. We also observed that BMP9-induced EPC neovascularization in ischemic limbs was ALK1 dependent. Thus, we think that the interaction of BMP9 and ALK1 is specifically involved in EPC-based neovascularization, and that the BMP9/ALK1-mediated signal is a novel molecular target for the modulation of EPC-based therapeutic neovascularization.

Knowledge of the cues that specify cell types is important because the introduction of inappropriate cells into organs during stem/progenitor cell-based therapies decreases transplantation safety and efficacy. For instance, the unnecessary introduction of hematopoietic cells results in undesired side effects, such as the formation of dilated hemorrhage-prone vessels and edema.[13,46] In our mouse hindlimb ischemia model, the vasculature-incorporated EPCs differentiated and acquired endothelial phenotypes, including KDR expression. This observation suggested that EPCs not only integrated into blood vessels but also differentiated into mature, functional ECs after BMP9 stimulation. There is still a discrepancy for isolation and definition of true EPCs. Interestingly, after the repeated washing process of MNC cultures, we found that umbilical cord blood–derived adherent cells on fibronectin-coated dish did not have CD45 expression. On the contrary, we have collected cells from the supernatant and found cells with high CD45 expression. Identical results were obtained with EPCs on collagen-coated dish. $CD45^{high}$ cells adhered weakly to fibronectin or collagen-coated culture dish and washed out by soup. Therefore, CD45− functional EPCs remained adherent. These umbilical cord blood–derived $CD45^{-}$ cells generated endothelial outgrowth cells with identical morphological and phenotypic characteristics as described by Timmermans et al.[47] Endothelial outgrowth cells derived from the true EPC contribute more directly to neovascularization.[48] Overall, true EPCs transplantation with BMP9 is expected to improve safety and efficacy of cell therapy.

In summary, our results delineated relationships involved in BMP9/ALK1, EPC→EC differentiation, and EPC-mediated neovascularization. It is debatable whether BMP9 functions as an angiogenic factor[43,49] or as an antiangiogenic factor in ECs.[24,39] However, our results extended the angiogenic role of BMP9 through ALK1 signaling in EPC biology, and demonstrated that BMP9 improves angiogenesis by increasing EPC functional incorporation into developing neovasculature and enhances vasculogenesis by inducing EPC→EC differentiation. Several studies involving animal models have shown that EPCs that differentiated into mature ECs accounted for ≈25% of ECs in newly formed vessels.[50,51] However, we suggest that BMP9 could improve this low efficacy by promoting the adhesion of integrin-induced EPCs to injured vascular sites, incorporation of EPCs into vessels, and the differentiation of EPCs into functional ECs, all of which eventually accelerate neovascularization. In addition, both short- and long-term administration of BMP9 could have clinical implications for EPC-mediated neovascularization. Accordingly, BMP9/ALK1 should be considered a potential molecular target for modulating EPC-based therapeutic neovascularization in ischemic vascular diseases.

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Disclosures

None.

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Significance

Peripheral arterial occlusive disease can lead to critical limb ischemia, which causes gangrene and eventually necessitates amputation of the limb. Therefore, more effective revascularization strategies that stimulate blood vessel growth are necessary to treat patients with critical limb ischemia. Given the angiogenic roles of EPCs in pathophysiological conditions, cell therapy with EPCs has been used to treat vascular diseases such as limb ischemia. Here, we found that BMP9 stimulated multiple steps of EPC homing and differentiation in vitro and promoted both EPC incorporation into the neovascular area and differentiation into EC, which resulted in enhanced angiogenesis in hindlimb ischemia. The effects of BMP9 are primarily because of their binding of ALK1 expressed on EPCs and the blockage of ALK1 signaling impaired neovascularization. Thus, our findings suggested that the use of BMP9 might be significant in EPC-based neovascularization strategies used to treat ischemic vascular diseases.

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