Bone Morphogenetic Proteins 2 and 4 Are Selectively Expressed by Late Outgrowth Endothelial Progenitor Cells and Promote Neoangiogenesis

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Objective—Endothelial progenitor cells are currently identified either by their surface antigen expression or by their generation of early colonies in culture (CFU-Hill). Another population, endothelial colony-forming cells (ECFCs), has strong vessel-forming capacity but is less well characterized. Given the potential usefulness of CFU-Hill and ECFCs as cell therapy products, their thorough characterization is of major importance.

Methods and Results—CFU-Hill and ECFCs were expanded from human cord and adult blood. Bone morphogenetic proteins 2 and 4 (BMP2/4) were selectively expressed by ECFCs but not by CFU-Hill. The BMP pathway was involved in ECFC commitment and angiogenic potential in vitro. In vivo, BMP inhibition strongly reduced plug vascularization in bFGF-containing Matrigel plugs implanted in C57/B16 mice. Moreover, ECFC exposure to BMP increased their therapeutic potential in a nude mouse model of hindlimb ischemia. In amputation specimens from patients with critical leg ischemia who had received a local therapeutic injection of bone marrow mononuclear cells, newly formed vessels were strongly positive for BMP2/4, suggesting that endothelial cells involved in neovascularization have an ECFC-like phenotype.

Conclusion—BMP2/4 are a marker of ECFCs and play a key role in ECFC commitment and outgrowth during neovascularization. (Arterioscler Thromb Vasc Biol. 2008;28:2137-2143.)

Key Words: endothelial progenitor cells ■ bone morphogenetic protein ■ critical leg ischemia ■ cell therapy ■ neovascularization

Endothelial progenitor cells (EPCs) have been used for autologous angiogenic therapy and as a cardiovascular risk biomarker.1 Autologous mononuclear cells (MNCs) from bone marrow and peripheral blood have been tested for their angiogenic capacity in patients with ischemic diseases (for a review see1). However, the cell type responsible for the observed neovascularization and resulting clinical benefit is not known.

EPCs were initially defined as cells coexpressing the surface antigens CD34⁺, CD133⁺, and VEGFR2⁺. However, these antigens are also expressed on hematopoietic progenitor cells (HPCs), and two recent studies have shown that these cells give rise to hematopoietic cells in culture, but never to endothelial cells.2,3 Thus, EPC definition, quantification, and isolation is reaching a new level of complexity.

At least two populations of EPCs have been described.4 “Early” EPCs appear within 4 to 7 days of culture, are spindle-shaped, and express both endothelial and leukocyte markers. This cell population can be quantified as described by Hill.5 The number of these so-called “CFU-Hill” in peripheral blood has been proposed as a new biomarker, as it correlates negatively with cardiovascular risk factors.5 Endothelial colony-forming cells (ECFCs),6,7 also called “late” EPCs, develop after 2 to 3 weeks of culture and have the characteristics of precursor cells committed to the endothelial lineage. ECFCs are less well studied than “early” EPCs. By analogy with the terminology used for the hematopoietic lineage, the term colony forming unit-endothelial cells (CFU-ECs) should be restricted to endothelial colonies isolated in a 3D culture system such as methylcellulose.8,9 However, the term “endothelial progenitor cells” (EPCs) is controversial. Indeed, cells obtained in culture are already differentiated toward the endothelial lineage, and are thus referred to below as CFU-Hill and ECFCs rather than “progenitors”, based on the stringent criteria of Ingram and Yoder.10

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To better characterize CFU-Hill and ECFCs, we examined their expression of selected embryonic stem cell genes. We found that bone morphogenetic proteins (BMPs) are selectively expressed by ECFCs but not by CFU-Hill. In addition, we show that the BMP pathway augments the expansion, proliferation, and migration of ECFCs, as well as their angiogenic potential in vivo.

Methods

Culture of ECFCs, CFU-Hill, and CFU-ECs
ECFC culture and characterization have been described in detail elsewhere1,11–15 and the cell culture method is described in supplemental materials, (available online at http://atvb.ahajournals.org). CFU-Hill were cultured using the EndoCult Liquid Medium kit (StemCell Technologies) according to the manufacturer’s instructions. Recombinant noggin from R&D systems was added to ECFCs and CFU-Hill cultures on the first day of culture and was present in all freshly added media. Gene expression profiles of ECFCs and CFU-Hill were compared with those of human embryonic stem cells (hESC), kindly provided by Dr Michel Puce´at (UMR861, Evry, France). For clonogenic assay of endothelial colonies (CFU-ECs), MNCs were seeded (4 or 8 x 10^5 cells per well) in basic methylcellulose Stem alpha 1 (Stem alpha) with 100 ng/mL human recombinant VEGF (R&D Systems).

In Vitro Angiogenic Assays
All experiments were performed in duplicate and repeated with cells from at least three different ECFCs, CFU-Hill, CFU-EC or CFU-GM colonies. These experiments are described in detail in supplemental materials.

In Vivo Matrigel Plug
These experiments are described in detail in supplemental materials.

In Vivo Quantification of ECFC-Induced Neovascularization
Nude mice underwent surgery to induce unilateral hindlimb ischemia as previously described16 and were then randomly assigned to injection of ECFCs treated with BMP2 or BMP4 for 48 hours, or untreated ECFCs. After 1 week, the ischemic/normal limb blood flow ratio was determined by using a laser Doppler perfusion imaging system (Moor Instruments). Vessel density was evaluated by using high-definition microangiography (Trophy system) after 14 days of treatment, as previously described.16

Local Injection of BM-MNCs in Patients With Critical Leg Ischemia
The Optimization of Progenitor Endothelial Cells in the Treatment of Critical Leg Ischemia (OPTIPEC) clinical trial is a multicenter, phase I nonrandomized study. Patients were eligible for the protocol if they had critical leg ischemia associated with limited gangrene or a nonhealing ischemic ulcer and if they were not eligible for surgical revascularization or percutaneous angioplasty, or if such a procedure had little chance of success. The cell therapy protocol was similar to that initially published by Tateishi-Yuyama et al.17 Four patients treated with BM-MNCs were studied here; their clinical characteristics are described elsewhere.18

Results

BMP2/4 Are Selectively Expressed by ECFCs
CFU-Hill and ECFCs can be readily distinguished in culture on the basis of their morphological and clonogenic properties. To obtain CFU-Hill, MNCs were harvested and seeded onto fibronectin-coated tissue culture plates in Endocult medium. After 48 hours nonadherent cells were collected and replated in the same culture media for an additional 3 days on fibronectin-coated tissue culture plates. At day 5, CFU-Hill colonies were identified by the presence of elongated sprouting cells radiating from a central core of round cells (supplemental Figure IA). To obtain ECFCs, MNCs were harvested and seeded onto collagen-coated tissue culture plates in EGM2. In contrast to the CFU-Hill method, nonadherent cells were discarded daily and fresh EGM-2 medium was added to the cultures. ECFC colonies that originated from adherent cells (supplemental Figure IB) were identified between 7 and 30 days of culture. Differences in the culture methods and, particularly adherence/nonadherence to different substrates at early time points, suggest that ECFCs originate from an adherent cell and CFU-Hill from a nonadherent cell, and that the two cell types represent clonally distinct populations.16

However, selective markers are required to identify cells able to merge into neovessels. To better define ECFCs and CFU-Hill and to identify new markers differently expressed by these cells, we studied the expression of selected genes involved in stem cell biology by using real-time quantitative RT-PCR (Figure 1A). We compared their expression with that of human embryonic stem cells. We chose to explore the three classes of morphogens with distinct functions in early embryonic development, namely hedgehog proteins, wingless proteins, and bone morphogenetic proteins (BMPs). We also examined the expression of molecules with a role in hematopoietic stem cells (BMI-1), in the side population (ABCG-2), and finally Nanog and Oct4, which were recently shown to be expressed by a subset of ECFCs.19 Numerous stem cell gene mRNA were found in both CFU-Hill and ECFCs (Figure 1A). The three representatives of the hedgehog family were quantified: SHH and IHH was poorly expressed by both cell types, and DHH was expressed at high levels only by ECFCs from cord blood. High levels of BMP2/4 mRNA and protein were detected in ECFCs derived from both cord blood and adult blood, whereas no expression was detected in CFU-Hill (Figure 1A and 1B). Moreover, BMP2/4 were also expressed at high levels in hESC (Figure 1A). As BMP2/4 expression distinguishes ECFCs from CFU-Hill, we restricted the rest of this study to BMP2/4.

CFU-EC Obtained in Methylcellulose Do Not Express BMP2 or -4
The methylcellulose assay can be used to isolate progenitor cells. Precursors and mature cells do not survive in the three-dimensional medium, whereas progenitors give rise to colony-forming units (CFU) that can be counted. When VEGF alone was added to methylcellulose,9 small round colonies, corresponding to CFU-ECs, were observed after 14 days of culture. They consisted of a cluster of round cells in the center and lengthened cells at the periphery (Figure 2A). The endothelial phenotype of CFU-ECs was shown by the expression of von Willebrand factor (vWF) (Figure 2B), and by their ability to incorporate Di-Ac-LDL (supplemental Figure IIA). These cells were harvested, and their endothelial and hematopoietic gene expression was studied at the mRNA level. Overall, CFU-ECs showed a CFU-Hill profile, with strong CD14 positivity (Figure 2C, and supplemental Figure IIB). We further showed that CFU-EC had features of proliferative cells, despite their CFU-Hill phenotype. Indeed,
they strongly expressed Ki67, as observed with ECFCs and CFU-GM, whereas Ki67 expression was weak in CFU-Hill. This result was confirmed by the large proportion of cells in S-phase (supplemental Figure IIC). Importantly, CFU-ECs did not express BMP2 or 4. These results suggested that the cells obtained in methylcellulose alpha1/VEGF had the CFU-Hill phenotype but retained a proliferative capacity similar to that of CFU-GM. CFU-ECs can thus be considered as cells that derive from a population of circulating CD14+ endothelial progenitors.

**BMP Inhibition by Noggin Inhibits the Emergence of ECFCs**

Given the well-described role of BMP in regulating vasculogenesis, we investigated the effect of BMP pathway manipulation on the emergence of ECFC colonies. Noggin, a BMP antagonist that acts by binding to BMP, thereby inhibiting their binding to BMP receptors, significantly inhibited ECFC generation from cord and adult blood (respectively *P*<0.0001 and *P*<0.0003, Figure 3A). As expected, noggin had no effect on the number of CFU-Hill colonies, as counted with the Endocult system (*P*=0.78, supplemental Figure III).

**BMP2/4 Activation Enhances Angiogenesis Mediated by ECFCs In Vitro**

Flow cytometry showed that the three main BMP receptors (BMPR-1A, BMPR-1B, and BMPR-2) were expressed on more than 90% of ECFCs (supplemental Figure IV). Id genes

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**Figure 1.** BMP2 and 4 expression in CFU-Hill and ECFCs. A, Gene expression profile (RTQ-PCR) of AB-CFU-Hill, ECFCs from AB and CB and human embryonic stem cells (hESCs). Values above 1000 represent very strong gene expression. B, Expression of BMP2/4 protein by CFU-Hill (left) and ECFCs from AB (center) and CB (right) studied with standard immunohistochemical procedures.

**Figure 2.** Colony forming unit-endothelial cells (CFU-ECs) in methylcellulose have a CFU-Hill phenotype and do not express BMP2 or 4. A, CFU-EC colony (day 14) cultured from adult peripheral blood MNCs in methylcellulose containing only VEGF at 100 ng/mL. B, CFU-ECs express vWF. Inset: negative control. C, Gene expression profile of ECFCs, CFU-Hill, CFU-ECs, and CFU-GMs isolated from adult blood in methylcellulose.
are well-characterized BMP targets. We therefore studied the expression of the major Id gene (Id2) on BMP receptor activation in ECFCs. Id2 expression was induced by BMP2/4 treatment, and this effect was antagonized by noggin (supplemental Figure V).

To examine the effects of BMP2/4 on proliferation, ECFCs were treated with recombinant BMP2/4 for 72 hours. As shown in Figure 3B, BMP2 and BMP4 induced ECFC proliferation, the effect was significant because 1 ng/mL for BMP4 whereas it reached statistical significance only at 50 ng/mL for BMP2 (*P<0.008). Proliferation was also quantified by measuring H\(^3\) thymidine incorporation. BMP2 and 4 increased H\(^3\) thymidine incorporation, but only the effect of BMP4 reached significance (P=0.33 and 0.02 for BMP2 and BMP4, respectively; Figure 3C). A Boyden chamber model was used to test BMP chemoattraction of ECFCs. As shown in Figure 3D, a significant migratory effect of both BMP2/4 on ECFCs was observed (respectively *P=0.01 and *P=0.001). Finally, we examined the effects of 50 ng/mL BMPs on ECFC tube formation. In EBM2 medium (without any growth factors), few pseudotubes were observed in the absence of BMP. Both morphogens induced a strong and significant increase in tube formation, an effect that was inhibited by noggin (Figure 3E and 3F).

Effect of BMP on Angiogenesis In Vivo
We firstly used the Matrigel plug assay as a model of in vivo angiogenesis. Basic-FGF-containing plugs, with and without noggin (850 ng per plug), were implanted subcu-
Simultaneously in C57/B16 mice. The plugs were then recovered 14 days later and their blood vessel content was analyzed. Figure 4A clearly shows that the presence of noggin drastically reduced blood vessel formation. This effect was further quantified by measuring the hemoglobin content of the plugs, which was four times lower in the presence of noggin (*\(P<0.0001\), supplemental Figure VI). To obtain direct evidence that BMP2/4 facilitate ECFC-mediated angiogenesis, we investigated the in vivo effects of BMP2/4 treatment on ECFCs. ECFCs stimulated with BMP2 or BMP4 were intravenously injected into nude mice that had undergone femoral artery ligation. As shown in Figure 4B, injection of ECFCs stimulated by BMP2 or BMP4 improved foot perfusion by 25% on day 7, as compared to injection of nonstimulated ECFCs (*\(P=0.01\) and 0.0002 respectively for BMP4 and BMP2). The mice were then killed on day 14 and a microangiographic score was calculated. Intravenous injection of BMP4-stimulated ECFCs significantly raised the ischemic/nonischemic limb angiography score by 40% compared to control animals injected with untreated ECFCs (*\(P=0.04\)). The score was not improved by BMP2-treated ECFCs (*\(P=0.01\) compared to BMP4-treated ECFCs; Figure 4C). These results are in line with the stronger proliferation of BMP4-stimulated ECFCs compared to BMP2-stimulated ECFCs (Figure 3C).

**Therapeutic Neovascularization by Bone Marrow Mononuclear Cells in Critical Leg Ischemia Patients Involves BMP2/4-Positive Endothelial Cells**

Injection of bone marrow mononuclear cells into ischemic limbs increases collateral vessel formation. In the OPTIPEC trial, patients treated with BM-MNCs for critical leg ischemia had extensive neangiogenesis in distal tissues.18 All the newly formed vessels were functional (blood cells were present within their lumen) and were limited by a continuous endothelium expressing the endothelial cell markers CD34, and vWF, proliferative marker Ki-67 (supplemental Figure VII), but not leukocyte antigen CD45 (Figure 5). Moreover, newly formed vessels formed after treatment with BM-MNCs are surrounded by vascular smooth muscle cells, indicating improved vessel maturation. Here, we found that these cells also expressed BMP2/4 (Figure 5), confirming that neovessels derive from the ECFC lineage in this setting. These data also strongly suggest that CFU-Hill, which does not express BMPs, do not participate directly in neovascularization.

**Discussion**

Here we show that BMPs 2 and 4 are specifically expressed by ECFCs derived from adult and cord blood. BMPs are secreted growth factors that belong to the transforming growth factor (TGF) beta (TGF-\(\beta\)) superfamily and act via BMP type 1 (BMPR-1A and BMPR-1B), and type II (BMPR-2) serine/threonine kinase receptors. BMP2/4 appear to be the BMP group most strongly involved in vascular development.20

**BMP2/4 Are Markers of ECFCs**

We found that BMP2/4 are not expressed by CFU-Hill, contrary to ECFCs, whether these cells are derived from adult or cord blood, confirming the common ECFC lineage hypothesis forwarded by Ingram et al.6 We also show that the BMP pathway regulates ECFC commitment, as noggin addition to MNC cultures significantly attenuated ECFC growth. This suggests that human progenitor cell commitment to the endothelial lineage requires BMPs, as shown for the hematogoblast in ESC.21,22

Based on the method of quantification of hematopoietic progenitors CFU-GM by the clonogenic test in methylcellulose, we isolated progenitor cells from peripheral blood in the presence of VEGF and characterized the endothelial cell colony-forming units (CFU-ECs) thus obtained. This confirmed the monocytic origin of endothelial progenitors quan-
BMPs also act as proangiogenic factors, inducing ECFC proliferation, migration, and pseudotube formation in vitro and in vivo in a model of hindlimb ischemia. Recombinant BMP activated BMP receptors, as witnessed by Id2 gene activation, leading to increased proliferation, migration and pseudotube formation in vitro. Id has been shown to play an important role in mediating an angiogenic response during embryonic development and during post natal tumor angiogenesis.23 We cannot exclude the possibility that BMP2/4 promote angiogenesis by mechanisms other than direct ECFC stimulation. Indeed, BMP2/4 induce VEGF expression in osteoblasts.24 However, in our experimental conditions, BMP2/4 failed to stimulate the gene expression of VEGF, SDF-1 and angiopoietin 2 or their receptors in ECFCs (supplemental Table). Moreover, BMP2/4 may repress antiangiogenic factors. Indeed, another member of the BMP family, BMP1, has been implicated in the processing of a major basement membrane proteoglycan and in the release of an antiangiogenic factor.25 However, it is very difficult to measure at mRNA or protein level the expression of antiangiogenic factors because most of them are fragments of different molecules of the extracellular matrix: thrombospondin-1, endostatin, tumstatin, angiotatin, or 16-kDa N-terminal fragment of human prolactin. Other studies are needed to explore BMP interaction with other antiangiogenic factors that could be expressed or bound by ECFCs: CXCL4 (platelet factor 4), members of serpin superfamily like antithrombin or pigment epithelium derived factor (PEDF), or vasohibin. Indeed, such as observed with VEGF, BMP, could increase vasohibin synthesis, which functions as an endothelium-derived negative feedback regulator of angiogenesis.26 Finally, some BMP antagonists are probably secreted by ECFCs. Because BMPs have been shown to modulate synthesis of their own antagonist Noggin,27 we can hypothesize that modulation of BMP antagonist synthesis could have proangiogenic properties. BMP antagonists have been shown to activate endothelial cell receptors in a BMP-independent manner,28 thus BMP synthesis by ECFCs could also act by blocking potential antiangiogenic properties of these molecules.

BMP2/4 Are Expressed by Neovessels in Patients Who Receive Autologous BM-MNCs

We then examined whether BMP2/4 expression could be used to distinguish the origin of endothelial cells incorporated in human neovessels. We found that neovascularization observed in patients who had received autologous BM-MNCs by the intramuscular route involved cells expressing BMP2/4. However, it is unclear whether the highly proliferative cells contributing to neovessel formation in these conditions originate from the cell therapy product and/or from an endothelial “side population” of progenitors. These results are in line with those of Yoder et al, who found that ECFCs, contrary to CFU-Hill, formed functional human-murine chimeric vessels in a short-term xenograft model of blood vessel formation.7 Our clinical results suggest that neovascularization involves cells of the same lineage as ECFCs.

Conclusion

This study provides the first evidence that ECFCs express BMP2/4 morphogens. BMPs can be used as a marker of immaturity (also expressed by hESCs), as well as a marker of the ECFC lineage (not expressed by CFU-Hill), and finally as an angiogenic marker during ECFC commitment and expansion. Moreover, neovascularization observed in patients with critical leg ischemia treated with BM-MNCs involves BMP-expressing cells with an endothelial phenotype. This suggests that CFU-Hill, although able to promote angiogenesis...
through growth factor secretion, are not incorporated into functional new vessels in humans, as previously shown in mice. We propose a new classification for cells derived from EPCs in culture based on BMP2/4 expression: BMP-positive endothelial precursors correspond to ECFCs (late EPCs or BOECs), responsible for neovascularization, whereas BMP-negative endothelial precursors correspond to proangiogenic hematopoietic progenitor cells (CFU-Hill, early EPCs, circulating angiogenic cells, or CFU-EC).

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Disclosures

None.

References

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Bone morphogenetic proteins 2 and 4 are selectively expressed by late outgrowth endothelial progenitor cells and promote neoangiogenesis.

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SMADJA: BMP2/4 role in endothelial progenitor cells.

Expanded methods for on line publication

Preparation of mononuclear cells (MNC)
Cord or adult blood was diluted 1:1 with PBS, EDTA 0.2 mM and overlaid on Histopaque-1077 (Sigma-Aldrich, Saint-Quentin Fallavier, France). Cells were centrifuged at 1000 g for 20 min. MNC were collected and washed 3 times in PBS, EDTA 0.2 mM.

Culture of ECFC
Cord blood (CB) ECFC culture and characterization have been described in detail elsewhere. To obtain adult blood (AB) ECFC, MNC were plated in collagen (BD, Grenoble, France)-coated 6-well plastic culture dishes at a density of $5 \times 10^7$ per well with EGM2 medium (Lonza, Saint-Beauzire, France) composed of endothelial cell basal medium-2 (EBM2), 5% fetal bovine serum (FBS), and growth factors. ECFC colonies appeared between 7 and 21 days of culture and consisted of well-circumscribed cobblestone monolayers. Colonies were counted with an inverted
microscope at x20 magnification. Recombinant noggin from R&D systems (Lille, France) was added to ECFC cultures on the first day of culture and was present in all freshly added media.

**Culture of CFU-Hill**

CFU-Hill were cultured using the EndoCult® Liquid Medium kit (StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. MNC were resuspended in complete EndoCult® medium and seeded at 5x10^6 cells/well in fibronectin-coated tissue culture plates (BD Biosciences, Mountain View, CA, USA). After 48 h, nonadherent cells were collected and plated in Endocult® buffer at 10^6 cells/well of 24-well fibronectin-coated tissue culture plates for 3 days. Recombinant noggin (R&D systems) was added to CFU-Hill cultures in Endocult® buffer from the first day of culture.

**Culture of CFU-EC: clonogenic assay in methylcellulose**

Freshly isolated total MNC were washed with PBS, EDTA 2 mM then seeded (4 x10^5 cells per well) in methylcellulose plates (Stem alpha 1F, Stem alpha®, Saint-Genis l’Argentièreme, France) including stem cell factor, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, erythropoietin, interleukin 3, and interleukin 6. Plates were studied under phase-contrast microscopy, and granulocyte-macrophage colony-forming units (CFU-GM) were counted after 14 days of incubation. For clonogenic assay of endothelial colonies, MNC were seeded (4 or 8 x10^5 cells per well) in basic methylcellulose (Stem alpha 1, Stem alpha®) with 100 ng/mL human recombinant VEGF (R&D systems). Each culture was performed in duplicate. Colonies were counted with a phase-contrast microscope after 14 days of incubation at 37°C, and then harvested. After washing in PBS, the cells were either cytopun and incubated
with anti-von Willebrand factor antibodies (Dako), or frozen pending mRNA extraction for quantitative RT-PCR analysis. To assess the capacity of CFU-EC to incorporate Dil-acetylated low-density lipoprotein (Di-Ac-LDL), harvested cells were incubated with 10 µg/mL Di-Ac-LDL (Molecular Probes, Eugene) in EGM2 medium for 4 h at 37°C. Di-Ac-LDL uptake was measured by flow cytometry. In one set of experiments, CFU-EC were obtained along with CD14-positive cells, sorted from MNC by positive selection with anti-CD14 microbeads (Miltenyi Biotec, Paris, France) on a magnetic cell sorter (Miltenyi Biotec). Purity was >95%, as assessed by flow cytometry.

**Real-time quantitative RT-PCR**

The theoretical and practical aspects of real-time quantitative RT-PCR on the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems Courtaboeuf, France) are described in detail elsewhere.\(^5\) Primers for TBP and the target genes (sequences available on request) were chosen with the assistance of Oligo 5.0 software (National Biosciences, Plymouth, MN, USA).

**Immunohistochemistry**

Immunohistochemical studies were performed with standard procedures, using a three-step avidin-biotin method.\(^6\) Anti-CD45 was used as a leukocyte marker, anti-CD34 and anti-von Willebrand factor as endothelial cell markers, anti-Ki-67 as a marker of proliferation and anti-α-smooth muscle actin as a vascular smooth muscle cell marker (all from Dako, Trappes, France). Polyclonal anti-BMP antibody purchased from R&D systems recognizes both BMP2/4.
**Cell-cycle analysis**

Flow cytometric analysis was carried out in a fluorescence-activated cell sorter FACS (Beckman Coulter). To analyze the percentage of each cell-cycle phase \(10^4 \text{ cells/sample}\) DNA was stained by incubation with 50 µg/ml propidium iodide containing DNase-free RNase for 10 min at 4 °C.

**Tissue sampling**

Human amputation specimens were submitted to a standard dissection protocol. In the case of lower calf amputation, the gastrocnemius was removed below the site of bone marrow cell injection and along the tibial arteries. The forefoot tissues and arteries (plantar and toe arteries) were also sampled in every case. Between 15 and 20 paraffin blocks per sample were stained with hematoxylin and eosin.

**Cell proliferation assay**

The effect of BMP2/4 on ECFC proliferation was examined by measuring cell phosphatase activity based on the release of paranitrophenol (pNPP) (Sigma) measured at OD 405 nm (Fluostar optima; BMG labtech, Champigny-sur-Marne, France) after 72 h of incubation. DNA synthesis was also determined by measuring incorporation of 5'-[^3]H-thymidine ([^3]H-Tdr, Amersham, Les Ulis, France) with a Betamatic counter (1900 CA Packard) during 4 hours, after 72 h of incubation. Results are expressed as the increase in over control (EBM-2 without BMP). The values of thymidine incorporation of ECFC were expressed as count per minutes (cpm). EPC were activated in 1% FBS-EBM2 medium containing recombinant BMP2 or BMP4 (50 ng/mL, R&D systems) with and without noggin.
Migration assays

Boyden chambers (Costar, Avon, France) with 8-µm pore-size filters were used for migration assays, as previously described. The filters were coated with 0.2% gelatin to improve cell attachment. The lower compartment of the chamber was filled with EBM2 supplemented with 50 ng/mL recombinant BMP2, BMP4 or VEGF (all from R&D systems). Cells were harvested, resuspended in EBM2 without FBS at a density of 200 cells/µL and placed in the upper compartment of the Boyden device. After incubation at 37°C for 5 h, the filters were collected and cells adhering to the lower surface were fixed, colored with Giemsa and counted. Experiments were carried out in triplicate and were repeated with cells from three different ECFC colonies.

In vitro Matrigel tube formation assay

After 16 h of serum and growth factor privation, ECFC (3x10⁴ cells/well) treated with 50 ng/ml BMP2 or BMP4 in EBM2 medium were seeded on growth factor-reduced Matrigel (200 µl) (BD Biosciences) and cultured for 18 h at 37°C with 5% CO2. Capillary-like structures were sought by phase-contrast microscopy and networks formed by ECFC were quantified with Videomet software version 5.4.0.

In vivo Matrigel plug assay

Matrigel plugs were prepared on ice by mixing Matrigel (BD Matrigel Matrix) with recombinant bFGF (300 ng/mL, Peprotech, Rocky Hill, NJ, USA) in the presence or absence of noggin (850 ng per plug). Five hundred microliters of Matrigel was injected subcutaneously on the back of C57/Bl6 mice. After 14 days the Matrigel plugs were dissected, homogenized in lysis buffer and
disrupted with a Polytron (IKA-Werke, Staufen, DE). The hemoglobin concentration was measured in the supernatants with Drabkin’s reagent (Sigma, Saint Louis, Mo, USA).

**Statistical analysis**

Data are shown as means ± SEM. Significant differences were identified by ANOVA followed by Fisher’s protected least-significant-difference test. All statistical tests were performed using the Stat View software package (SAS, Cary, NC, USA). Differences with p values <0.05 were considered significant.

**Expanded results for on line publication**

**Characterization of endothelial cell populations (CFU-Hill and ECFC) derived from adult and cord blood**

The starting material used to isolate endothelial populations consisted of either mononuclear cells (MNC) from adult peripheral blood (AB) or CD34+ sorted cells from human umbilical cord blood (CB). To obtain CFU-Hill from adult blood, MNC were seeded in fibronectin-coated culture plates with Endocult® medium. After 48 h, non adherent cells were collected and replated for a further 3 days on fibronectin. On day 5, CFU-Hill colonies were identified as elongated sprouting cells radiating from a central core of round cells (Figure IA). These cells did not replicate in vitro and gradually disappeared 20 days after plating.

For ECFC culture, AB-MNC and CB-CD34+ cells, respectively, were seeded on collagen-coated culture plates and gelatin in endothelial growth medium (EGM2). Non adherent cells were discarded daily and fresh EGM2 medium was added. Colonies emerged from adherent cells between days 7 and 21. ECFC have a cobblestone appearance typical of the endothelial lineage
(Figure IB). They rapidly grow from several cells to a colony, become near-confluent monolayers, and show multiple population doublings. As expected, immunophenotyping showed that CFU-Hill and ECFC expressed the endothelial cell-surface antigens CD31 and VEGFR-2. Importantly, neither AB-ECFC nor CB-ECFC, contrary to CFU-Hill, expressed the hematopoietic cell-specific surface antigens CD45 and CD14.
Supplementary Table

Effect of BMP2 and 4 on the mRNA levels of VEGF, angiopoietin 2 and SDF-1 and their receptors

EPC were stimulated with BMP2 or 4 for 8 h after 16 h of serum and growth-factor privation. mRNA levels were measured by real-time RT-PCR and normalized to the TBP mRNA level (mean ± SEM, n=3), by comparison with unstimulated cells.

<table>
<thead>
<tr>
<th></th>
<th>BMP2</th>
<th>BMP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF-1</td>
<td>1.2 ± 0.3</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>CXCR-4</td>
<td>0.6 ± 0.3</td>
<td>1.2 ± 0.8</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>1.0 ± 0.5</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.9</td>
</tr>
<tr>
<td>ANG-2</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Tie-2</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>
Legends for supplementary figures

Figure I. CFU-Hill and ECFC morphology.
A- Representative phase-contrast photomicrograph of a CHU-Hill colony (day 5) cultured from adult peripheral blood MNC (mag. x20).
B- Representative phase-contrast photomicrograph of an ECFC colony (day 20) cultured from adult peripheral blood MNC (mag. x20).

Figure II: Characterization of Colony forming unit-endothelial cells (CFU-EC) in methylcellulose.
A. CFU-EC incorporation of Di-Ac-LDL (flow cytometry). Blue line: Ig control. Red line: positive incorporation of Di-Ac-LDL.
B. Flow cytometric analysis of VEGFR-2 (KDR), CD31, CD45 and CD14 on the CFU-EC. The red line histogram represents the control (IgG1).
C. Adult CFU-EC, CFU-Hill and ECFC were stained with propidium iodide, and analyzed with a flow cytometer. The mean ± SEM of percentage of cells in S phase of the cell cycle is shown.

Figure III: Noggin did not modify CHU-Hill formation
Quantification of CFU-Hill obtained from adult blood in the presence and absence of noggin. Bars represent the mean ± SEM of ten independent experiments (p=0.78)
**Figure IV: ECFC express BMP2/4 receptors**

Flow cytometric analysis of BMP receptors (from left to right: receptors 1A, 1B and 2) (red histogram) expression on the ECFC surface. The blue line histogram represents the control (IgG1).

**Figure V: BMP2/4 regulate ECFC Id2 expression**

BMP2/4 (50 ng/mL) induce the expression of Id2 at the mRNA level in ECFC. Noggin incubation with BMP2/4 inhibits Id2 expression.

**Figure VI: Angiogenic response to noggin in vivo in the Matrigel plug assay**

The Matrigel plugs were excised and angiogenesis was estimated by measuring the hemoglobin content in the Matrigel matrix. *p<0.0001 versus control.

**Figure VII: Quantification of Ki-67 in CFU-Hill, ECFC and newly formed neovessels observed in amputation specimens from patients having received local injections of BM-MNC for critical leg ischemia.**
Supplementary Figure I

A

B
Supplementary Figure II

A. CFU-EC: Di AcLDL

B. VEGFR-2 (KDR)  CD31  CD45  CD14

C. Phase S %

AB-CFU-EC  AB-CFU-Hill  AB-ECfc
Supplementary Figure III

Supplementary Figure IV

BMPR-1A  BMPR-1B  BMPR-2
Supplementary Figure V

![Bar chart showing normalized mRNA expression of Id2 under different conditions](image)

**BMP2**
- - + + - -

**BMP4**
- - - + +

**Noggin**
- - + - +

Supplementary Figure VI

![Bar chart showing Hb concentration](image)

- **FGF**
  - 1.5

- **FGF+noggin**
  - 

* indicates a significant difference.
Supplementary Figure VII

References


