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Sonic Hedgehog signaling regulates vascular differentiation and function in human CD34 positive cells

Vasculogenic CD34$^+$ cells with Sonic Hedgehog

Kurando Kanaya[a], Masaaki Ii[c,*], Teiji Okazaki[a], Toru Nakamura[b], Miki Horii-Komatsu[e], Cantas Alev[f], Hiroshi Akimaru[f], Atsuhiko Kawamoto[e], Hidetoshi Akashi[a], Hiroyuki Tanaka[a], Michio Asahi[c], Takayuki Asahara[d,**]

[a] *Department of Surgery, Kurume University School of Medicine, Fukuoka, Japan*
[b] *Department of Gastroenterology, Kurume University School of Medicine, Fukuoka, Japan*
[c] *Department of Pharmacology, Faculty of Medicine, Osaka Medical College, Osaka, Japan*
[d] *Department of Regenerative Medicine Science, Tokai University School of Medicine, Kanagawa, Japan*
[e] *Group of Vascular Regeneration Research, Institute of Biomedical Research and Innovation, Kobe, Japan*
[f] *RIKEN Center for Developmental Biology, Kobe, Japan*

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Abstract Identification of pivotal factors potentially present in the in situ environment and capable of influencing the function of CD34$^+$ cells, which can be used for autologous cell therapy, is of paramount interest. SHh is one of the morphogens essential for embryonic vascular development as well as postnatal neovascularization, and the activation of SHh signaling with angiogenic and vascular differentiation responses in CD34$^+$ cells by SHh treatment differed depending on the G-CSF treatment or the background disease. SHh enhanced the migration, proliferation, adhesion, and EPC colony forming capacities of G-CSF mobilized CD34$^+$ cells, increasing the vasculogenic/angiogenic potential for neovascularization. An increase in the differentiation potential of CD34$^+$ cells toward vascular lineages was demonstrated with SHh treatment involving TGFβ signaling pathway. The SHh-activated G-CSF mobilized CD34$^+$ cells directly contributed to vascular regeneration while non-activated CD34$^+$ cells showed a lower regenerative capacity in a mouse ischemic hindlimb model. SHh signaling regulates human CD34$^+$ cell fate and function, and may potentiate the therapeutic effect of G-CSF mobilized CD34$^+$ cells on ischemic diseases.

Abbreviations: SHh, Sonic Hedgehog; EPC, endothelial progenitor cell; EPC-CFA, endothelial progenitor cell-colony forming assay; EC, endothelial cell; VSMC, vascular smooth muscle cell; PTCH, patched receptor; SMO, smoothened; HUVEC, human umbilical vein endothelial cells; G-CSF, granulocyte colony-stimulating factor; HSC, hematopoietic stem cell.

* Correspondence to: M. Ii, Department of Pharmacology, Faculty of Medicine, Osaka Medical College, 2-7, Daigaku-machi, Takatsuki, Osaka 569-8686, Japan.
** Corresponding author.
E-mail addresses: masaii@art.osaka-med.ac.jp (Masaaki), asa777@is.icc.u-tokai.ac.jp (T. Asahara).

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Introduction

Hedgehog (Hh) proteins are crucial regulators of organ development during embryogenesis. Sonic Hedgehog (SHh) is a prototypical morphogen known to regulate epithelial/mesenchymal interactions during embryonic development of limbs, lung, gut, hair follicles, and bone (Johnson and Tabin, 1997; St-Jacques et al., 1998, 1999). Patched (PTCH) and Smoothend (SMO) are known receptors of SHh, and PTCH inhibits SMO activation of the downstream Gli family transcription factors, which results in the activation of target genes (Bhardwaj et al., 2001; Cohen, 2003). Several recent observations have suggested an involvement of SHh in postnatal neovascularization (Kanda et al., 2003; Kusano et al., 2004, 2005; Mackie et al., 2012; Renault et al., 2010). In addition, exogenous administration of SHh has been shown to induce angiogenesis and accelerate repair of the ischemic myocardium and skeletal muscle (Kusano et al., 2004; Pola et al., 2003). Other reports also demonstrated that SHh gene therapy could significantly accelerate wound healing by inducing arteriogenesis (Asai et al., 2006) or restore nerve function in diabetic neuropathy by promoting angiogenesis (Kusano et al., 2004).

Yamazaki et al. showed that PTCH receptor expression was observed in cultured human peripheral blood-derived endothelial progenitor cells (EPCs) but not in mature endothelial cells (Yamazaki et al., 2008), suggesting that SHh might have a certain effect on human EPCs and $CD34^+$ cells. Indeed, recent studies demonstrated that a combined therapy of SHh gene transfer and mouse cultured EPC transplantation enhanced angiogenesis in ischemic skeletal muscle (Palladino et al., 2012) and that genetically modified human $CD34^+$ cells with SHh preserved cardiac function increasing angiogenesis in the ischemic myocardium following myocardial infarction (Mackie et al., 2012). Based on these evidences, we hypothesized that activation of the SHh pathway during development might regulate the fate of human EPCs, $CD34^+$ cells, and promote vasculogenic activities in ischemic tissues in situ.

CD34 has been recognized as one of the best-characterized human hematopoietic stem cell-related cell surface antigens defined to date (Sutherland et al., 1993). Its expression is down-regulated during hematopoietic development and it is not found on mature peripheral blood cells. In fact, the function of CD34 was reported to be an adhesion molecule that allows stem cells to remain in the proper stromal environment for hematopoiesis in the bone marrow (Gordon et al., 1987), and transplantation of bone marrow- or cord blood-derived $CD34^+$ cells was shown to reconstitute ablated bone marrow following lethal irradiation as part of therapeutic approaches aiming for the cure of hematological disorders involving leukemia (Link et al., 1996), malignant lymphoma (Tomblyn et al., 2007), and refractory anemia (Korbling et al., 1995). On the other hand, putative endothelial cell (EC) progenitors or angioblasts were isolated from adult human peripheral blood for the first time by selection on the basis of expression of the cell surface antigen $CD34^+$ and recognized as EPCs in 1997 (Asahara et al., 1997). Since then, a number of preclinical animal studies (Iwasaki et al., 2006; Jujo et al., 2010; Wang et al., 2010) and clinical studies (Kawamoto et al., 2009; Manginas et al., 2007) have indicated the therapeutic potential of EPCs including peripheral or bone marrow (BM)-derived $CD34^+$ cells for vascular regeneration in the ischemic limb or ischemic heart disease. The several lines of evidences accumulated in these studies clearly supported the therapeutic efficacy of $CD34^+$ cell transplantation in ischemic tissues, however, a clear and distinct mechanism of action for the direct or indirect $CD34^+$ cell contribution to neovascularization remains to be established.

Previous studies identified concurrent dose-dependent effects of $CD34^+$cells on the incorporation into vascular endothelial cells and the vascular recovery of ischemic tissues following transplantations (Iwasaki et al., 2006). However, other studies have demonstrated that the major positive effect of $CD34^+$ cell transplantation on vascular regeneration in ischemic tissues was due to the production of various stem/progenitor chemokines, angiogenesis-related cytokines, angiogenic growth factors, anti-inflammatory cytokines, and survival factor, promoting resident EC proliferation and migration (Ii et al., 2005). We and others have experienced difficulties in regard to the commitment of $CD34^+$ cells into totally differentiated endothelial cells in vitro. Several publications also questioned the involvement of EPCs in new blood vessel formation during tumor angiogenesis or in ischemic diseases, by showing perivascular lodging cell void of endothelial lineage profiles but expressing smooth muscle cell lineage markers (Rajantie et al., 2004).

A possible unifying explanation for these diverse and to some extent controversial aspects of EPC biology may be the current lack of knowledge about the pivotal factors present in the in situ environments of EPCs, depending on as well as influencing the molecular make-up and interactions of the transplanted cells with the recipient tissues, and being thus possibly involved in the induction and specification of the cell fate and regenerative function of EPCs and $CD34^+$ cells in ischemic tissues. We hypothesize that SHh may be one of these pivotal players present in situ and possibly involved in the regulation of the fate of EPCs, promoting the differentiation of $CD34^+$ cells and EPCs into regenerative vascular cells under ischemic conditions. To evaluate the possible effects of SHh on $CD34^+$ derived EPCs, we designed and performed a series of in vitro and in vivo experiments.

Methods

Human recombinant Sonic Hedgehog (SHh) protein

Human recombinant SHh protein, N-terminus (Cat #: 1314-SH-025) was purchased from R&D Systems, Inc. (MN). Human SHh cDNA encodes a 45 kDa precursor protein. An autocatalytic reaction yields a 19 kDa amino-terminal domain SHh-N protein containing cholesterol and palmitate, and a 25 kDa carboxy-terminal domain SHh-C protein. The N-terminal domain retains all known signaling capabilities, while the C-terminal domain is responsible for the intramolecular processing, acting as a cholesterol transferase. SHh can act as both a short-range contact dependent factor and as a long-range, diffusible morphogen.

Cells

G-CSF mobilized human peripheral blood $CD34^+$ cells were purchased from ALL CELLS (Emeryville, CA) and used for

both in vitro and in vivo studies as indicated with the label of "Hv-GmCD34". We also used G-CSF mobilized human peripheral blood $CD34^+$ cells collected from patients with Burger's disease who enrolled in a clinical trial regarding intramuscular transplantation of autologous and G-CSF-mobilized $CD34^+$ cells in patients with intractable critical limb ischemia (CLI) in Institute for Biomedical Research and Innovation (IRBI, Kobe, Japan) (Pt-GmCD34) and fresh human peripheral blood $CD34^+$ cells were isolated from healthy volunteers (Hv-CD34) and diabetic volunteers for in vitro experiments. This cell collection was performed in accordance with an approval of institutional ethical committee of IRBI. All cells were kept in cryopreservation until the experiments were performed. Cells were thawed and incubated in 5% FBS/EBM2 supplemented with growth factors (EGM2-MV BulletKit, Lonza) for 24 h for recovery of cell activity. Cells were further incubated with SHh protein at following doses: 0, 0.2, 1.0 and 5.0 μg/mL for 24 h under a hypoxic condition (37 °C in 5% O2 and 5% CO2). The cells pre-treated with SHh were used for in vitro and in vivo experiments.

Cell transplantation in a hindlimb ischemia model

Athymic nude mice (age 8–10 week and weighting 17–22 g) were used for all experiments. Mice underwent surgery to induce severe unilateral hindlimb ischemia. The animals were anesthetized by intraperitoneal injection of Nembutal™ (pentobarbital, 10 mg/kg) (Sigma-Aldrich, Tokyo, Japan) followed by subcutaneous injection of buprenorphine (0.05 mg/kg/day) for 3 days after surgery. Under sufficient anesthesia, the left iliac artery and vein, the deep femoral and circumflex arteries and veins, and the entire left superficial artery and veins (from just below the deep femoral arteries to the popliteal artery and vein) were ligated and cut. $CD34^+$ cells were pre-incubated with SHh (1 μg/mL) in 5% FBS/EBM2 supplemented with growth factors (EGM2-MV BulletKit, Lonza) for 24 h under a hypoxic condition (37 °C in 5% O2 and 5% CO2). The SHh-treated $CD34^+$ cells (1×10^5 cells) were locally injected to lower limb muscles dividing into 4 sites. All surgical procedure was approved by RIKEN institutional animal use and care committee (Animal protocol #: AH16-06-17).

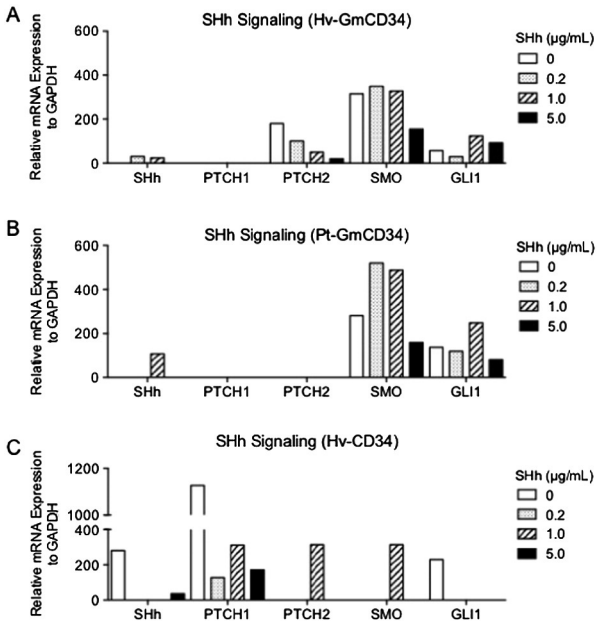


Figure 1 SHh signaling-related gene expressions in SHh-treated human $CD34^+$ cells. Human $CD34^+$ cells isolated from healthy volunteer with (A, Hv-GmCD34) or without G-CSF administration (C, Hv-CD34) and patient of Burger's disease with G-CSF administration (B, Pt-GmCD34). The mRNA expressions of SHh, PTCH1, PTCH2, SMO, and GLI1 were examined in each type of cells treated with the indicated concentrations of SHh protein following 24 h in culture by real-time RT-PCR. The relative mRNA expression levels of the target genes were normalized to those of GAPDH and the representative data are presented following the triplicated analyses.

Statistical analysis

All values are presented as mean ± SEM. Statistical comparisons between 2 groups were performed by Student *t* test. Multiple groups were analyzed by one-way ANOVA followed by appropriate post hoc test (Tukey procedure) to determine statistical significance. All in vitro experiments were repeated at least in triplicate and analyzed. The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

The other methods are described in detail in Supplementary material.

Results

SHh activates SHh signaling in G-CSF mobilized (Gm) $CD34^+$ cells

We first performed a series of pilot experiments for the selection of a suitable cell type in this study. We screened for SHh signaling among Gm-$CD34^+$ cells obtained from a healthy volunteer (male, 42 y.o.) (Hv-GmCD $CD34^+$ cells), Gm-$CD34^+$ cells obtained from a patient with inflammatory vascular disease (male, 39 y.o., Burger disease) (Pt-GmCD $CD34^+$ cells), and non-GmCD34^+ cells obtained from a healthy volunteer (male, 44 y.o.) (Hv-$CD34^+$ cells) by quantitative real-time PCR (qPCR) analysis. SHh signaling was already activated with SHh, PTCH1, and GLI1 mRNA upregulations in Hv-$CD34^+$ cells (0 μg/mL in Fig. 1C), while little activation of SHh signaling was observed in Hv-Gm $CD34^+$ cells and Pt-Gm $CD34^+$ cells with only high SMO and low GLI1 mRNA expressions. Interestingly, no PTCH1 mRNA expression was detected in both Hv-Gm $CD34^+$ cells and Pt-Gm $CD34^+$ cells, and PTCH2 mRNA expression was detected only in Hv-Gm $CD34^+$ cells (0 μg/mL in Fig. 1A and B), suggesting that PTCH2 but not PTCH1 may play a role as a receptor for SHh in HV-GmCD34^+ cells. The SHh treatment induced no mRNA expressions of SHh and GLI1 at any dose of SHh in Hv-$CD34^+$ cells, in contrast, SHh and GLI1 mRNA expressions were upregulated by SHh (1 μg/mL) treatment in Hv-GmCD34^+ cells and Pt-Gm $CD34^+$ cells

(Fig. 1A and B), suggesting that SHh signaling is activated by SHh stimulation in Gm CD34⁺ cells.

In the regulation of angiogenic/vasculogenic growth factors by SHh, the treatment with SHh upregulated VEGF-A mRNA expression regardless of the cell sources (Fig. 2A, B, and C) and VEGF-B and PDGF-A mRNA expressions in Pt-GmCD34⁺ cells and Hv-CD34⁺ cells (Fig. 2A and B). The ANG1 mRNA expression was upregulated by SHh (1 μg/mL) only in Pt-GmCD34⁺ cells (Fig. 2B), and SHh did not affect the mRNA expressions of PDGF-B and HGF in all cell types. For the assessment of vascular differentiation of CD34⁺ cells by SHh, CD31 mRNA was highly expressed only in GmCD34⁺ cells (Fig. 2D and E), while not only CD31 but also eNOS, calponin, and SM α-actin (α-SMA) were slightly expressed or not expressed at all in all type of cells (Fig. 2D, E, and F). Although SHh treatment exhibited a little effect on CD31 mRNA expression, SHh upregulated eNOS and αSMA in GmCD34⁺ cells (Fig. 2E and F) and did eNOS, calponin, and αSMA in non-GMCD34⁺ cells (Fig. 2F). Similar gene expression patterns in response to SHh treatment were observed in other individuals (n = 2) in each group (data not shown).

Thus, we decided to use Hv-GmCD34⁺ cells rather than Pt-GmCD34⁺ cells in the following series of pilot experiments for the reasons of; 1) SHh signaling activation in response to exogenous SHh treatment with PTCH2 receptor, 2) insufficient number of cells with the same lot/person for all experiments in this study, and 3) unevenness depending on the background diseases in the patients' cells compared with the healthy volunteers' cells.

SHh promotes CD34⁺ cell functional activities

To elucidate the direct effects of SHh on Hv-GmCD34⁺ cells, we performed a series of in vitro assays. The proliferation activity of the SHh-treated CD34⁺ cells was examined. SHh significantly increased proliferation activity in a dose dependent manner (Fig. 3A). To assess the CD34⁺ cell adhesion activity, the cells were incubated with SHh in fibronectin-coated culture dishes for 48 h. The number of adhered CD34⁺ cells was significantly increased in a dose dependent manner (Fig. 3B). Next, we evaluated the effect of SHh on CD34⁺ cell migration. The migratory response of CD34⁺ cells toward different concentrations of SHh was assessed by modified Boyden's chamber assay. The effect of SHh on CD34⁺ cell migration peaked at 1 μg/mL while a higher concentration of 5 μg/mL resulted in less effect (Fig. 3C). Finally, EPC colony forming assay (Masuda et al., 2012) with CD34⁺ cells was performed to evaluate in vitro angiogenic activity. SHh significantly increased the formation of total colonies including small type and large type (Supplemental Fig. 1S-A) with CD34⁺ cells at a dose of 1 μg/mL but not of 5.0 μg/mL (Fig. 3D). CD34⁺ cells with large colony forming capacity represent high angiogenic capacity compared to those with small colony forming capacity (Masuda et al., 2012). Even though the colony forming EPCs are in a minor population, they will be distributed in neovasculature producing a variety of growth factors/cytokines that can promote angiogenesis with resident endothelial cells. The paracrine effect is a major function of CD34⁺ cells. Interestingly, the EPC colony forming capacity of CD34⁺ cells isolated from volunteers with diabetes (DM) was lower than that with healthy volunteers (non-DM) (Supplemental Fig. 1S-B and -C). SHh treatment (1 μg/mL) significantly increased the number of large EPC colonies in non-DM group (Supplemental Fig. 1S-B)

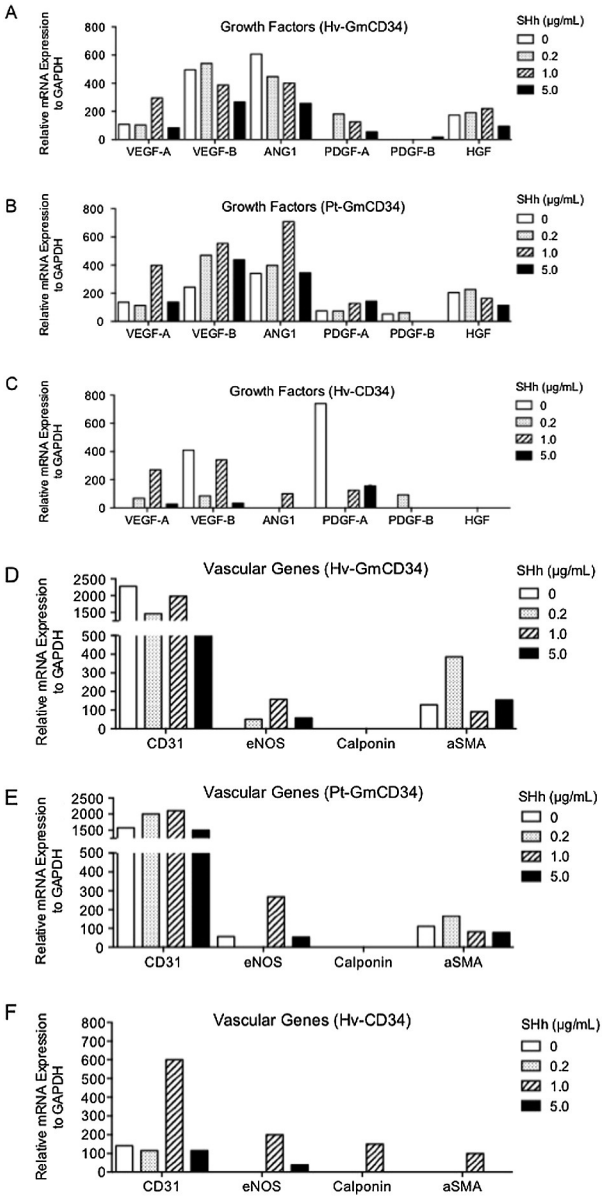


Figure 2 Angiogenic growth factor and vascular cell-related gene expression levels in SHh-treated human CD34⁺ cells. Human CD34⁺ cells isolated from healthy volunteer with (A and D, Hv-GmCD34) or without G-CSF administration (C and F, Hv-CD34) and patient of Burger's disease with G-CSF administration (B and E, Pt-GmCD34). The mRNA expressions of growth factor, VEGF-A, VEGF-B, angiopoietin 1 (ANG1), PDGF-A, PDGF-B, and hepatocyte growth factor (HGF) and vascular gene, CD31, eNOS, calponin, and smooth muscle α-actin (aSMA) were examined in each type of cells treated with the indicated concentrations of SHh protein following 24 h in culture by real-time RT-PCR. The relative mRNA expression levels of the target genes were normalized to those of GAPDH and the representative data are presented following the triplicated analyses.

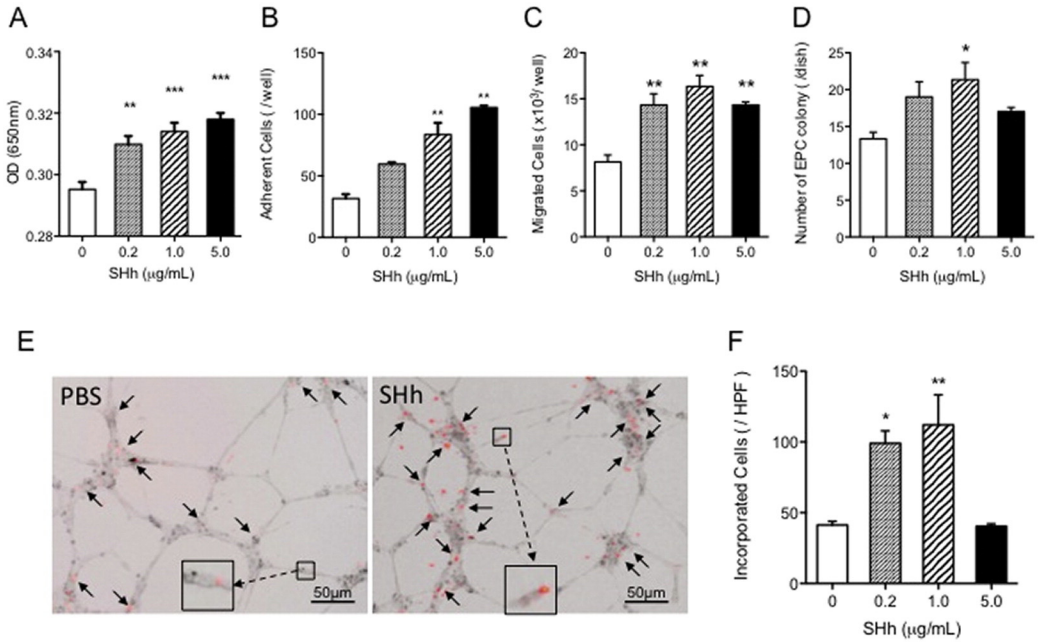


Figure 3 The promotional effect of SHh on functional activities of human G-CSF mobilized CD34$^+$ cells. A, The proliferation activity of CD34$^+$ cells was expressed as optical density (OD) measured at 650-nm wavelength with MTS dye. (n = 6) B, the adhesion activity of CD34$^+$ cells was expressed as the number of attached cells on fibronectin coated plastic dishes after 48 h in culture. (n = 3) C, the SHh-treated or non-treated CD34$^+$ cell migration was induced by SDF-1α (100 ng/mL), the number of migrated cells was evaluated as the migration activity after 48 h in culture. D, EPC-Colony forming assay (n = 3). The colony forming activity was expressed as the number of SHh-treated or non-treated CD34$^+$ cell-derived colonies in methylcellulose-based special medium after 18 days in culture. E, Vasculogenesis assay with HUVECs and SHh-treated CD34$^+$ cells. The DiI (red fluorescence)-labeled SHh-treated or non-treated CD34$^+$ cells and HUVECs were cultured for 24 h on Matrigel™ coated chamber slides. The DiI-labeled CD34$^+$ cells co-localized with cord-like structures formed by HUVECs were visualized under a phase contrast/fluorescent microscope. (E) Black arrows indicate incorporated CD34$^+$ cells. Square boxes indicate high magnification of selected area. F, Quantification of incorporated CD34$^+$ cells into cord-like structures of HUVECs. The vasculogenic activity of CD34$^+$ cells was assessed by the frequency of CD34$^+$ cell incorporation into cord-like structures. *$P < 0.05$; **$P < 0.001$; and ***$P < 0.0001$ vs. 0 μg/mL of SHh.

and small EPC colonies in the DM group (Supplemental Fig. 1S-C), suggesting that background disease such as DM influences the response of CD34$^+$ cell function to SHh treatment.

Finally, in order to examine whether SHh promotes incorporation of CD34$^+$ cells into cord-like structures made of HUVECs, tube formation assay was performed by co-culturing DiI-labeled CD34$^+$ cells with HUVECs on Matrigel. The CD34$^+$ cells co-localized with the formed cord-like structures of HUVECs and were visualized under a phase contrast/fluorescent microscope (Fig. 3E). The number of incorporated CD34$^+$ cells (red) into the cord-like structure was significantly greater in the SHh-treated group at doses of 0.2 and 1.0 μg/mL than that in the control group, however, high dose of SHh (5.0 μg/mL) showed no effect (Fig. 3F), suggesting that SHh increased the vasculogenic or pro-angiogenic activity of CD34$^+$ cells at a low/mid concentration but not at a high (5.0 μg/mL) concentration.

CD34$^+$ cells show differentiation potential into endothelial and smooth muscle lineages by SHh

To confirm the differentiation potential of CD34$^+$ cells into the endothelial and smooth muscle lineages by SHh treatment, protein expressions of CD31 and eNOS for EC differentiation and calponin and SMα-actin for VSMC differentiation were examined by fluorescent immunocytostaining. SHh significantly increased calponin and SMα-actin positivity in CD34$^+$ cells under SMC differentiation culture condition (Fig. 4A and C). On the other hand, although there was no significant change in CD31 positivity with SHh versus without SHh in CD34$^+$ cells, eNOS positivity was significantly increased in CD34$^+$ cells by SHh under EC differentiation culture condition (Fig. 4B and D). These findings suggest that SHh can promote CD34$^+$ cell differentiation into the endothelial lineage and vascular smooth muscle lineage under specific cell differentiation conditions.

TGFβ signaling pathway may be involved in SHh-induced CD34$^+$ cell fate in vascular differentiation

To explore the mechanism for which SHh promoted vascular geneupregulation in CD34$^+$ cells, we examined a series of signaling molecule gene expressions with SHh treatment focusing on TGFβ/BMP signaling pathway by qPCR analysis (Table 1S). SHh (1.0 μg/mL) upregulated mRNA expressions of not only TGFβ1 and its receptor TGFβR2 but also the downstream molecules of TGFβ signaling pathway, AlK5, Smad2, Smad3, and Smad4. In contrast, little

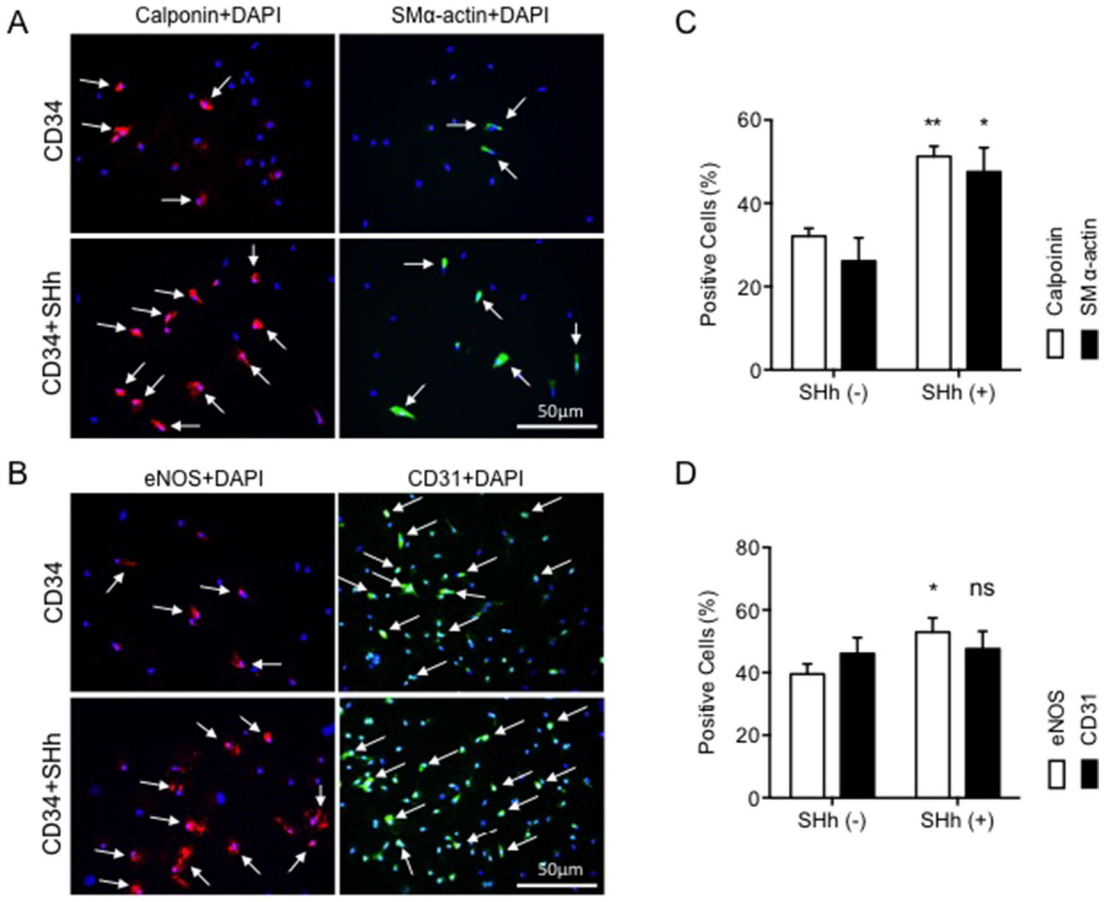


Figure 4 Immunocytochemistry for vascular protein expressions in human G-CSF mobilized CD34$^+$ cells with or without SHh treatment. The protein expressions of, calponin/SMα-actin (VSMC marker) (A) and eNOS/CD31 (EC marker) (B) were examined in CD34$^+$ cells with or without SHh treatment (1.0 μg/mL) following 14 days in culture under VSMC differentiation condition and EC differentiation condition, respectively. Arrows indicate each staining (red, calponin and eNOS, green, SMα-actin and CD31)-positive cells. The percent of staining-positive cells was calculated and averaged (C and D). ns, not significant, *$P < 0.05$ and **$P < 0.001$ vs. 0 μg/mL of SHh.

effect of SHh treatment on BMP2/BMPR2 (BMP receptor 2) and the downstream molecules of TGFβ/BMP signaling pathway ALK1, Smad1, and Smad5 were observed (Fig. 5A and Supplemental Fig. 2S). Consistent with the mRNA upregulation of TGFβ signaling pathway, mRNA expressions of a number of vascular transcription factors, Ets2, Fli1, TEL, SCL, Sp1, Runx1, CBF1, ZEB2, and Id2 were also upregulated in a quite similar dose response manner of SHh (Fig. 5B). The SHh-induced protein upregulations of key molecule, TGFBR2, Smad 3, and Smad 4 were further confirmed by Western blotting (Supplemental Fig. 3S). These results allowed us to speculate that SHh-induced vascular differentiation is mediated by TGFβ signaling pathway rather than BMP signaling pathway in CD34$^+$ cells.

We next performed a TGFβ blocking study to test whether the TGFβ signaling pathway is critical for SHh-induced vascular differentiation and growth factor production in CD34$^+$ cells. Although the upregulated CD31 gene expression by SHh was not canceled by TGFβ inhibitor (SB525334) significantly, other vascular marker genes of eNOS, calponin, SM alpha actin (aSMA), and growth factor VEGF-A were significantly reduced by TGFβ signaling inhibition (Fig. 5C), suggesting that TGFβ signaling is, at least in part, involved in SHh-induced vascular differentiation and VEGF-A production in CD34+ cells.

SHh promotes the effect of CD34$^+$ cells on blood perfusion recovery following HLI

Based on the results in vitro, we have tested the hypothesis that SHh might enhance the effect of CD34$^+$ cells on blood perfusion recovery in ischemic hindlimbs. We transplanted human peripheral blood-derived CD34$^+$ cells with or without SHh-pretreatment via intramuscular injection, and PBS instead of cells was used as a control.

Serial measurements of blood perfusion in hindlimbs by LDPI were performed at days 0, 7, 14, 21, and 28. LDPI disclosed striking differences in blood perfusion on and after day 14 post-induction of HLI (Fig. 6A). The blood perfusion ratio of ischemic/non-ischemic hindlimbs in the SHh-CD34$^+$ group significantly increased compared with the control group, while the CD34$^+$ group did not show a significant increase of the blood perfusion ratio after 14 days post-HLI

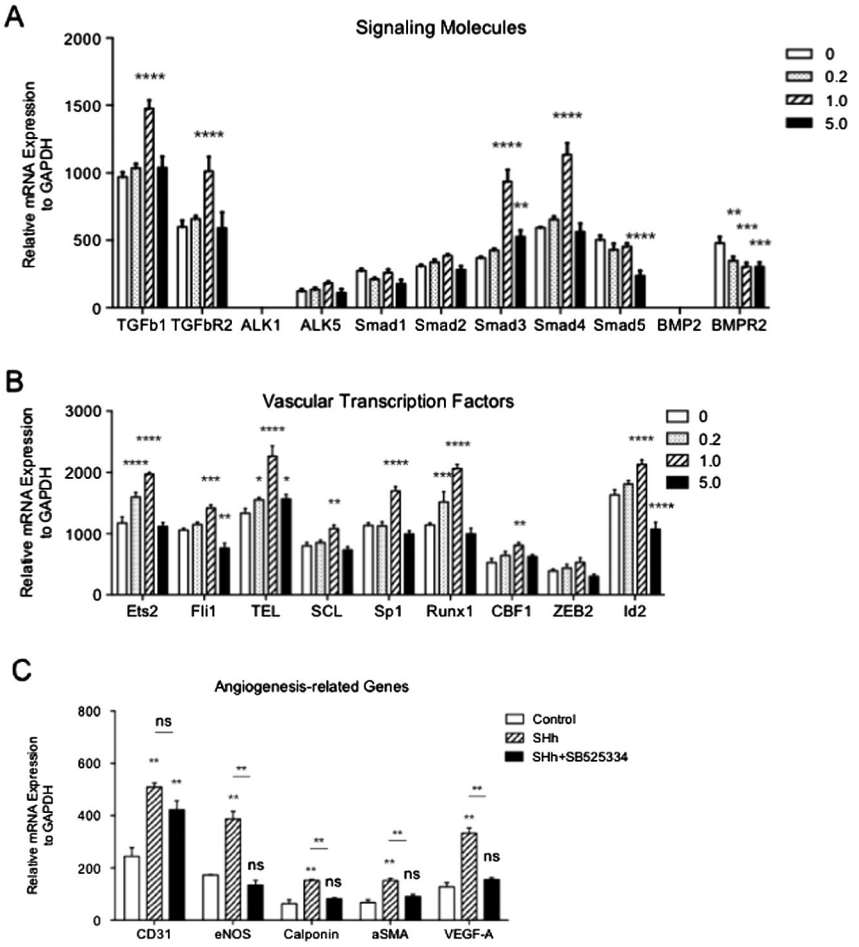


Figure 5 TGFβ-Smad/BMP-Smad signaling-related gene and vascular gene expressions in SHh-treated human G-CSF mobilized CD34⁺ cells. The mRNA expressions of TGFβ1/BMP2, downstream molecule of TGFβ/BMP including the receptors, (A) vascular transcription factor, (B) and vascular genes (C) were examined in CD34⁺ cells treated with the indicated concentrations of SHh protein in the presence or absence of TGFb antagonist SB525334 (5 μM) following 24 h in culture by real-time RT-PCR. The relative mRNA expression levels of the target genes were normalized to those of GAPDH and the representative data are presented following the triplicated analyses (n = 3 in each group, *P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001). Ets2: v-ets avian erythroblastosis virus E26 oncogene homolog 2, Fli1: Friend leukemia virus integration 1, TEL: Ets variant 6, SCL: stem cell leukemia, Sp1: specificity protein 1, Runx1: Runt-related transcription factor 1, CBF1: CRT/DRE binding factor 1, ZEB2: Zinc finger E-box binding homeobox 2, Id2: inhibitor of DNA binding 2, dominant negative helix-loop-helix protein.

surgery. The SHh-CD34⁺ group also exhibited a significant increase of the blood perfusion ratio of ischemic/non-ischemic hindlimb compared with the CD34⁺ group at 21 days post-HLI surgery (Fig. 6B). Although the endogenous mouse tissue-derived SHh mRNA expression was upregulated 3 days following HLI (Fig. 6C), the low expression levels and the delay of expression timing may exhibit less effect on transplanted CD34+ cells. These findings suggest that pre-treatment of SHh promoted the angiogenic/vasculogenic activity of CD34⁺ cells in ischemic hindlimbs resulting in significant blood perfusion recovery.

SHh promotes the effect of CD34⁺ cells on angiogenesis and vasculogenesis in ischemic hindlimb

To provide histological evidence of improved blood perfusion in ischemic hindlimbs by SHh treated CD34⁺ cell transplantation, histological assessment of capillaries in the ischemic muscle was performed. The staining with the EC markers isolectin B4 and Ulex lectin was performed on skeletal muscle sections retrieved from ischemic hindlimbs after HLI surgery to quantify capillary density as an index of neovascularization (Fig. 7A). The SHh-CD34⁺ group exhibited significantly increased capillary density compared with the control group, whereas the CD34⁺ group did not on day 14 and day 28 after HLI surgery. Only on day 14 after surgery, there was a significant difference between the SHh-CD34⁺ group and the CD34⁺ group (Fig. 7B).

To confirm whether the transplanted CD34⁺ cells incorporated into vascular structures in the ischemic limb muscles, we performed double-immunofluorescent staining of human mitochondria (hMtCd)/lectin (Fig. 7C) and hMtCd/SMα-actin (Fig. 7D). Double-positive cells of hMtCd/lectin and hMtCd/SMα-actin were more frequently observed in the SHh-CD34⁺ group than the CD34⁺ group. However, the frequency of

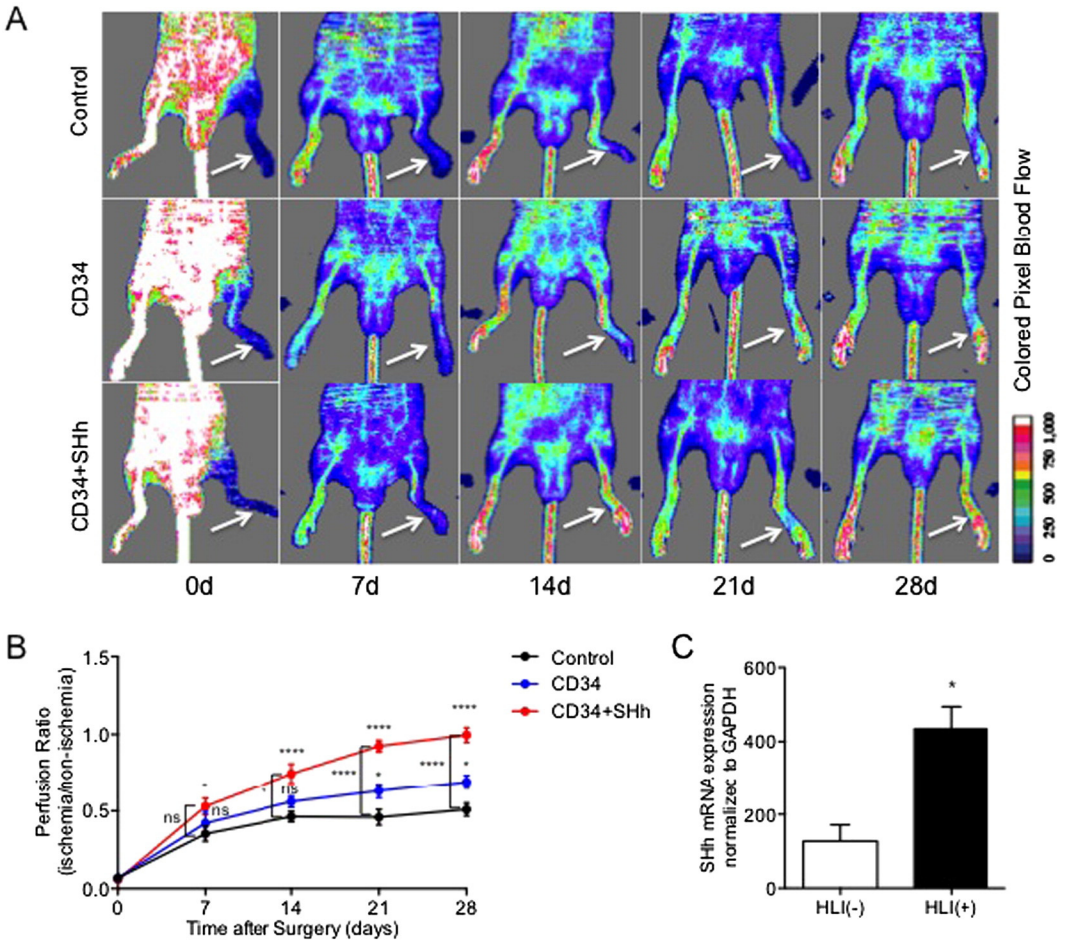
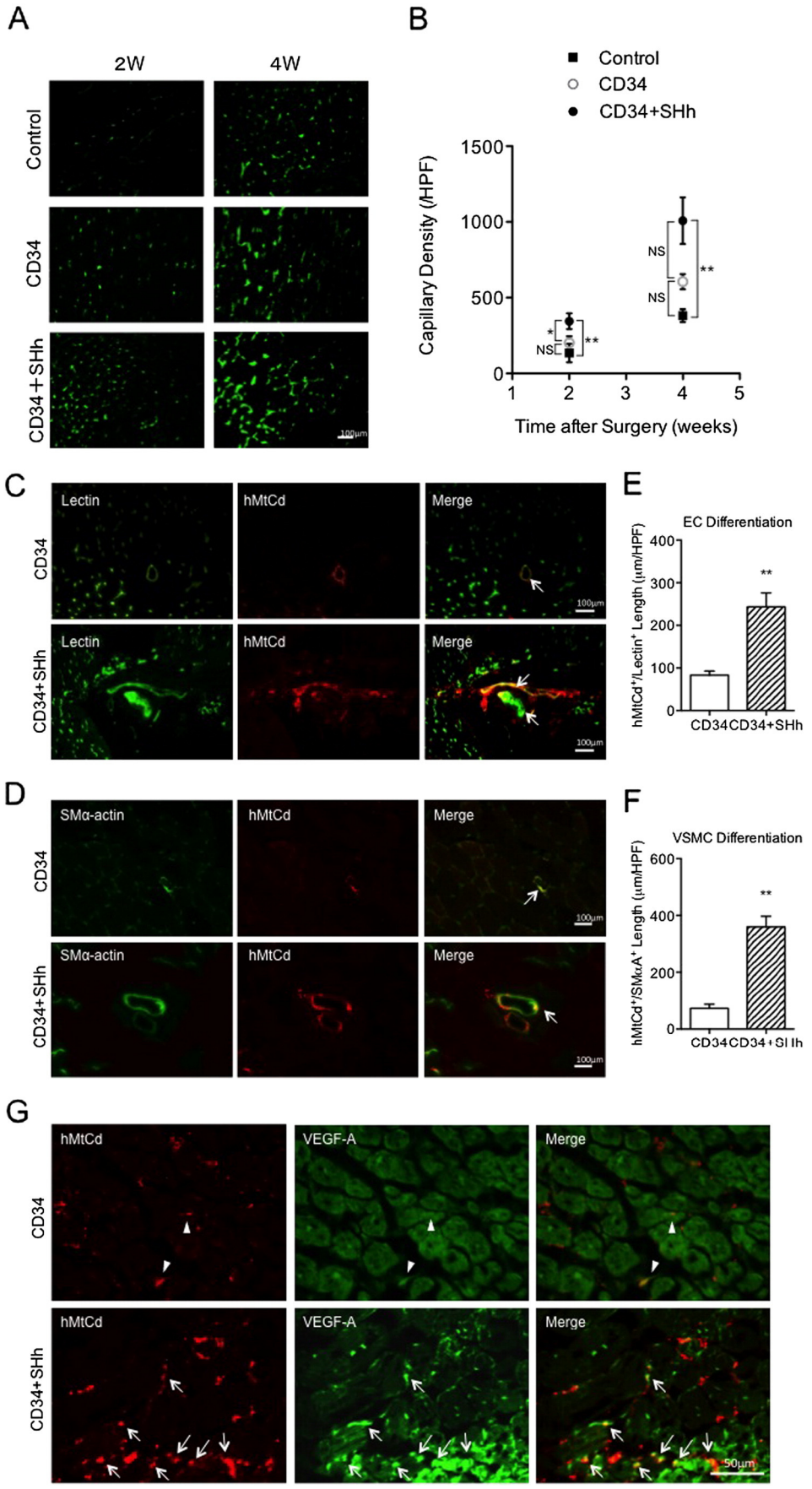


Figure 6 Blood perfusion recovery in ischemic hindlimbs transplanted with SHh-treated human G-CSF mobilized $CD34^+$ cells. A, Representative blood perfusion images assessed by Laser-Doppler perfusion imaging system immediate, 7, 14, 21, and 28 days following hindlimb ischemia treated with PBS (Control), $CD34^+$ cells (CD34), and SHh-treated $CD34^+$ cells (CD34 + SHh). Purple/blue, low blood perfusion; green/yellow, mid blood perfusion; and red/orange, high blood perfusion. Arrows indicate ischemic hindlimbs with each treatment. B, Quantification of blood perfusion in ischemic hindlimbs. The relative blood perfusion ratio of ischemic limb (left) to intact limb (right) was calculated and averaged in each group. (n = 8 in each group) C, SHh gene expression in mouse muscle tissue with (HLI+) or without HLI (HLI−). SHh mRNA expressions in hindlimb were analyzed by real-time RT-PCR 3 days following surgery, and the expressions were normalized to GAPDH (n = 4). ns, not significant; *$P < 0.05$; and ****$P < 0.0001$ vs. control.

hMtCd/SMα-actin positive cells was lower than that of hMtCd/lectin positive cells. Indeed, the perimeters of both $hMtCd^+$/$Lectin^+$ and $hMtCd^+$/SMα-$actin^+$ were significantly greater in the SHh-$CD34^+$ group than that in the $CD34^+$ group (Fig. 7E and F, respectively). Finally, we examined the VEGF-A protein expression of one of the upregulated genes by SHh (1.0 μg/mL)

Figure 7 Histological assessment of vascularity in ischemic hindlimb muscles transplanted with SHh-treated G-CSF mobilized $CD34^+$ cells. A, Fluorescent immunostaining with FITC-isolectin B4 for detection of capillaries (green) was performed on ischemic muscle sections in each group 2 weeks and 4 weeks after surgery and treatment with PBS (Control), $CD34^+$ cells (CD34), and SHh-treated $CD34^+$ cells (CD34 + SHh). B, Quantification of vascularity in ischemic hindlimb muscles. The capillary density was calculated by counting capillaries stained in green (A) in high power field (HPF) and averaged in each group 2 weeks and 4 weeks after surgery. *$P < 0.01$ vs. CD34 and **$P < 0.001$ vs. control. (n = 6 in each group) double fluorescent immunostaining for human mitochondria (hMtCd, red) and Lectin (C) or SM α-actin (green) (D) was performed on sections of $CD34^+$ cell (CD34) and SHh-treated $CD34^+$ cell (CD34 + SHh) transplanted ischemic hindlimb muscles 2 weeks after surgery. Merged images indicate co-localization (arrows) of $hMtCd^+$ cells (red) and $Lectin^+$ capillaries (green) or SM α-$actin^+$ arterioles (green) in the SHh-treated $CD34^+$ cell transplanted ischemic hindlimb muscle. Quantification of $hMtCd^+$/$Lectin^+$ perimeter (length) (E) and $hMtCd^+$/SM α-$actin^+$ perimeter (length) (F) in ischemic tissue. Six sections from 3 mice were examined by morphometry and averaged. HPF, high power field (200×). (G) Double fluorescent immunostaining for transplanted SHh-treated $CD34^+$ cells vs. non-treated $CD34^+$ cells in ischemic hindlimb tissue. Human $CD34^+$ cells were visualized with an antibody for human mitochondria (hMtCad) in red and the VEGF-A expression was shown in green. Arrowheads ($CD34^+$ cells) and arrows (SHh-treated $CD34^+$ cells) indicate double positive cells for hMtCd and VEGF-A.


A
2W
4W
Control
CD34
CD34+SHh
100μm
B
Control
CD34
CD34+SHh
Capillary Density (/HPF)
1500
1000
500
0
1
2
3
4
5
Time after Surgery (weeks)
NS
**
*
NS
**
NS
C
Lectin
hMtCd
Merge
CD34
CD34+SHh
100μm
D
SMα-actin
hMtCd
Merge
CD34
CD34+SHh
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EC Differentiation
hMtCd+/Lectin+ Length (μm/HPF)
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in cultured CD34+ cells (Fig. 2A) in the transplanted SHh-treated cells in ischemic tissue. Fluorescent double immunostaining clearly indicated that transplanted SHh-treated CD34+ cells (hMtCd+ cells) expressed VEGF-A while the transplanted non-treated CD34+ cells faintly expressed it in ischemic tissue (Fig. 7G).

These findings suggest that SHh promoted the effect of CD34+ cells on not only neovascularization with angiogenesis but also vasculogenesis/arteriogenesis via differentiation into vascular lineages producing VEGF-A protein in ischemic tissue.

Discussion

Originally, human bone marrow-derived CD34+ cells were defined as hematopoietic stem/progenitor-rich cells, while endothelial progenitor cells (EPCs), which play an important role in post-natal angiogenesis/vasculogenesis in ischemic tissues, have also been shown to be associated with CD34+ cells (Asahara et al., 1997). CD34+ cells in the human peripheral blood have recently been used as a tool for therapeutic angiogenesis of ischemic diseases, with several lines of evidence accumulated in clinical/animal studies underlining the therapeutic efficacy of CD34+ cell transplantation. However, some previous reports described that the preferential mechanism of CD34+ cell action is likely an indirect contribution to angiogenesis, namely, a paracrine effect by production of a variety of cytokines and angiogenic growth factors rather than a direct contribution of these cells to angiogenesis/vasculogenesis in ischemic tissues (Wang et al., 2010). In this study, we assessed whether human CD34+ cells could commit to the vascular lineage upon activation of SHh signaling, and have demonstrated that 1) Hedgehog signaling was activated by SHh stimulation in G-CSF mobilized- (Gm-) CD34+ cells, 2) SHh promoted the functional activity of Gm-CD34+ cells including their paracrine effects, 3) SHh promoted Gm-CD34+ cell commitment to vascular lineage, exhibiting a vasculogenic activity both in vitro and in vivo, and 4) TGFβ signaling pathway might be involved in the SHh-induced vascular differentiation of Gm-CD34+ cells.

Pola et al. discovered that SHh triggered neovascularization through SHh/PTCH signaling as an indirect angiogenic agent specifically present in mesenchymal cells i.e. fibroblasts for the first time (Pola et al., 2001), and that the inhibition of SHh signaling was sufficient to decrease ischemia-induced local angiogenesis and upregulate VEGF in skeletal fibroblasts (Pola et al., 2003). Also, Renault et al. reported that SHh does not activate Gli-dependent transcription in ECs because of the absence of enhanced PTCH mRNA expression in SHh treated ECs (Renault et al., 2010). A notable finding of this study is that human CD34+ cells do indeed express a receptor for SHh, PTCH, and that the response to SHh protein was also confirmed by upregulation of mRNA expression of not only the downstream molecule Gli1 but also SHh and SMO, suggesting that SHh induced and activated the SHh signaling pathway in G-CSF stimulated CD34+ cells but not non-stimulated CD34+ cells via an autocrine mechanism. The angiogenic growth factor gene and vascular gene expressions are relatively low in non-mobilized CD34+ cells compared with G-CSF mobilized CD34+ cells, and the response to SHh treatment was small except for VEGF-A and eNOS. A possible reason for the small response to SHh in CD34+ cells is due to the short time (24 h) treatment with SHh in vitro, while the transplanted SHh-treated CD34+ cells exhibited pro-angiogenic effect, perhaps, with long term exposure to endogenous SHh production in ischemic tissue, or only a small subpopulation of CD34+ cells might be endothelial lineage.

Based on the evidence that SHh is one of the notochord-derived morphogens which plays a crucial role during embryonic vascular development (Carmeliet and Tessier-Lavigne, 2005) and has also been shown to be involved in postnatal neovascularization, we hypothesized that the SHh signaling pathway might promote Gm-CD34+ cell function and increase differentiation into the vascular lineages. To explore the direct effects of SHh on Gm-CD34+ cells, we performed a series of experiments in vitro and in vivo. In contrast to recent studies in which there are no direct effects of SHh on cellular responses, such as proliferation, migration, and serum deprived survival, in cultured ECs (Kanda et al., 2003; Pola et al., 2001), we could demonstrate a direct effect of SHh on GM-CD34+ cell functions including an increase in proliferation, adhesion, migration, tube formation, and an upregulation of SHh signaling-related genes. We further confirmed the SHh-induced differentiation potential of GM-CD34+ cells into ECs and VSMCs with marker expressions of eNOS/CD31 and calponin/SMα-actin, respectively. In hematopoietic stem cells (HSCs) including CD34+ cells, it has been reported that Notch and Wnt signaling played a role in the self-renewal and Smad signaling with TGFβ negatively regulated the growth while Smad signaling with bone morphogen (BMPs) regulated the development (Blank et al., 2008). We therefore focused on these signaling to explore underlying mechanism for SHh-induced vascular differentiation of Gm-CD34+ cells. Apart from critical signaling pathway in HSCs, TGFβ-Smad signaling pathway was highly activated and SHh (1.0 μg/mL) pre-treatment further upregulated the signaling synchronized with a variety of vascular transcription factor gene expressions. Organ including vascular network development is regulated by differential SHh concentration (Scherz et al., 2007). Although it would be difficult to translate the in vitro concentration of SHh to in vivo settings, we assumed that 1.0 μg/mL of SHh would be critical in the contribution of endogenous CD34+ cells to vascular development or post-neonatal angiogenesis. Also, SHh-induced TGFβ1 in CD34+ cells might directly promote angiogenesis (Ferrari et al., 2009) and arteriogenesis (van Royen et al., 2002) influencing resident ECs and VSMCs in ischemic tissue. On the other hand, BMP-Smad signaling pathway was downregulated (Fig. 5) and Notch/Wnt signaling pathway was not affected (data not shown) by SHh (1.0 μg/mL) pre-treatment in CD34+ cells.

For in vivo experiments, we utilized transplantation of SHh-pretreated Gm-CD34+ cells rather than the co-administration of Gm-CD34+ cells (EPCs) and SHh protein or SHh gene transfer (Palladino et al., 2012) into ischemic hindlimbs, and could detect both EC marker and VSMC marker expressing cells, possibly derived from transplanted Gm-CD34+ cells in the microvasculature sprouting toward the center of healing during histological analysis. This morphogenetic effect of transplanted Gm-CD34+ cells was likely attributed to their direct contribution to vasculogenesis/arteriogenesis (Supplemental Fig. 4S, left side) as well as an indirect paracrine effect via secreted pro-angiogenic growth factors/cytokines from the Gm-CD34+

cells activated by SHh (Supplemental Fig. 4S, right side). Therefore, even though the number of transplanted Gm-CD34$^+$ cells (10^5 per hindlimb) was lower than that in previous reports (Madeddu et al., 2004; O et al., 2011; Zhou et al., 2007), the pre-treatment of Gm-CD34$^+$ cells with SHh could exhibit immediate paracrine effect at the time of transplantation and direct contribution to neovascularization differentiating into ECs and SMCs by endogenous SHh protein production from ischemic tissue in late phase, resulting in sufficient outcome compared with non-treated Gm-CD34$^+$ cells. Although SHh expression in normal adult organs/tissues is extremely limited, it is expressed constitutively in perivascular and interstitial tissues, and induced incidentally in ischemic tissues (Kusano et al., 2005; Pola et al., 2003). This indicates that constitutive or inducible SHh expression may play a key role during cell fate decision and functional regulation of circulating CD34$^+$ cells (EPCs) in situ. As recent papers demonstrated, resident vascular progenitors are located in adventitia tissues and can contribute to vascular repair or remodeling by promoting a vasculogenic zone in the adventitia (Ergun et al., 2011). The induced SHh expression in the perivascular area could encourage the recruitment of circulating CD34$^+$ cells, which in turn could increase their vasculogenic capacity and enhance vascular repair by the local enrichment of growth factors, and an increase in the paracrine effect on resident ECs (Supplemental Fig. 5S, right side). The adventitia as a putative source for SHh could thus foreordain CD34$^+$ cells into an endothelial phenotype while promoting the smooth muscle phenotype in the outer vascular tissue leading to neovascularization (Supplemental Fig. 5S, left side).

Conclusions

SHh signaling was activated only in human G-CSF mobilized (Gm-) CD34$^+$ cells but not in normal CD34$^+$ cells and the trend appeared to be evident in the patient's Gm-CD34$^+$ cells. The SHh signaling activation enhanced certain cell functions that are essential for neovascularization including vasculogenesis/arteriogenesis demonstrating an increased differentiation potential of these cells into ECs/VSMCs, with the activated CD34$^+$ cells contributing to vascular regeneration in ischemic tissues. Since Gm-CD34$^+$ cells with activated SHh signaling are used in ongoing clinical trials of autologous CD34$^+$ cell transplantation therapy for non-option patients with ischemic cardiomyopathy (Losordo et al., 2011), critical limb ischemia including Burger disease and ASO (Kawamoto et al., 2009; Kudo et al., 2003), the modification/pre-treatment of Gm-CD34$^+$ cells with SHh may make sense and therefore enhance the therapeutic outcome. In terms of inducing stable vascular regeneration in ischemic tissues, not only angiogenesis with just ECs but also arteriogenesis with both ECs and pericytes would be critical aspects of a successful Gm-CD34$^+$ cell transplantation. Thus, an efficient direct induction system for human Gm-CD34$^+$ cell differentiation into ECs and pericytes/VSMCs would be required in Gm-CD34$^+$ cell transplantation for ischemic diseases. Although further pre-clinical studies will be warranted, our findings may give rise to a novel and simple strategy for the next generation-CD34$^+$ cell therapy in patients with ischemic diseases.

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Disclosures

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.01.003.

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SUPPLEMENTAL MATERIAL

Supplemental Methods

2.1 EPC-Colony Forming and Cell Differentiation Assays

The EPC colony forming assay (EPC-CFA) with SHh-treated $CD34^+$ cells was performed according to the procedures established in our laboratory.(Masuda et al., 2011) In brief, SHh-treated $CD34^+$ cells (1 x 10^3/well [35mm dish]) were suspended with 30% FBS/IMDM and cultured in methylcellulose-containing medium MethoCult SF H4236 (StemCell Technologies) with 100 ng/mL SCF (Wako), 50 ng/mL VEGF (Wako), 20 ng/mL interleukin-3 (IL-3, Wako), 50 ng/mL basic fibroblast growth factor (bFGF, Wako), 50 ng/mL epidermal growth factor (EGF, Wako), 50 ng/mL insulin-like growth factor-1 (IGF, Wako) and 2 U/mL heparin (SIGMA) for 18 days.

To assess the differentiation capacity of $CD34^+$ cells into endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), cells were either cultured in 5% FBS/EBM2 supplemented with growth factors (EGM2-MV BulletKit, Lonza) for 7 days and were cultured in 10% FBS/DMEM (Invitrogen) supplemented with TGFβ1 (2 ng/mL) for 14 days at 37˚C in 5% CO2 with or without SHh protein at a dose of 1.0 μg/mL, respectively. The cell differentiation status was evaluated by quantitative real-time RT-PCR analysis of EC-related and VSMC-related gene expression levels and fluorescent immunocytochemistry using antibodies for CD31 (Abcam, 1:200), eNOS (BioWorld, 1:200), calponin (Abcam, 1:200), and SMα-actin (Sigma, 1:500). Formation of de novo vascular-like structures was evaluated by co-culture of the above described two types of cells with Matrigel™ (BD Biosciences) and incubation with 5% FBS/EBM-2 supplemented with growth factors (EGM2-MV BulletKit, Lonza) for 24 hours at 37˚C in 5% CO2.

2.2 Cell Function Assay

Proliferation activity of the SHh-treated $CD34^+$ cells was examined with the use of CellCouterKit-8 (DOJINDO, Tokyo, Japan) according to the manufacturer's instructions. Briefly, subconfluent cells ($CD34^+$ cells: 10000 cells/well) were reseeded on 96-well flat-bottomed plates with 100 μl of growth medium (EGM2-MV BulletKit, Lonza). Then, cells were incubated in the presence of SHh (0, 0.2, 1.0 and 5.0 μg/mL) for 48 hours at 37˚C in 5% CO2. The absorbance at 570-nm wavelength was recorded with the use of a 96-well ELISA plate reader (SPECTRA MAX 190, Japan Molecular Device).

Migration activity was evaluated with a modified Boyden's chamber assay as described previously.(Ii et al., 2006) Briefly, the polycarbonate filter (5- μ m pore size) (Transwell®, Corning) was placed between upper and lower chambers. The SHh-treated cell suspensions (5×10^4 cells/well) were placed in the upper chamber, and the lower chamber was filled with 0.5% FBS/EBM2 medium (Lonza) with growth factors alone or with SDF-1α (100ng/mL). The cells were incubated for 24 hours at 37˚C in 5% CO2. Migration activity was evaluated as the total number of migrated cells on the lower

chamber-side polycarbonate filter in each well.

Adhesion activity was evaluated by counting the number of cells on extracellular matrix-coated culture dishes as described previously.(Ii et al., 2006) Cells ($1x10^5$/well) were plated on fibronectin-like protein (Sigma) coated 96-well culture dishes with 5% FBS/EBM2 supplemented with growth factors (EGM2-MV BulletKit, Lonza) and incubated for 48 hours at 37˚C in 5% CO2. After fixation with 2% PFA/PBS for 15 minutes at 4˚C and nuclear staining with 4',6-diamidino-2-phenylindole (DAPI: Wako Pure Chemical Industries Ltd., Osaka, Japan), attached cells were counted under a fluorescent microscope (BioZero, KEYENCE, Tokyo, Japan).

Endothelial tube formation was assessed with the use of Matrigel™ (BD Biosciences) as described previously.(Asai et al., 2006) Briefly, the SHh-treated $CD34^+$ cells ($1x10^3$ /well) and human umbilical endothelial cells (HUVECs) ($1.25x10^4$ /well) were seeded on plastic chamber slides (μ-Slide Angiogenesis™, Ibidi GmbH) coated with 10 μL of Matrigel™ with 50 μL of 5%FBS/EBM2 supplemented with growth factors (EGM2-MV BulletKit, Lonza), and incubated for 24 hours at 37˚C in 5% CO2. The $CD34^+$ cell assisted-tube formation activity of HUVECs was examined under phase-contrast microscopy (BioZero, KEYENCE, Tokyo, Japan).

2.3 Quantitative Real-time RT–PCR Analyses

After 24 hour-incubation in 5% FBS/EBM2 supplemented with growth factors (EGM2-MV BulletKit, Lonza) with or without SHh protein (1.0 μg/mL), $CD34^+$ cells ($5x10^5$ /dish) were plated onto 35mm culture dishes and incubated for 48 hours at 37˚C in 5% CO2. For TGFβ signaling inhibition study, TGFβ1 antagonist, SB525334 (5μM), was added in the presence of SHh protein (1μg/mL). Total RNA was extracted from $CD34^+$ cells using a RNeasy Mini Kit (QIAGEN Science, Hilden, Germany.), and reverse transcription were performed using PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara Biochemicals, Kyoto, Japan.) according to the manufacturer's instructions. For quantitative RT-PCR, the converted cDNA samples (2 μL) were amplified in triplicate by a real-time PCR machine (ABI Prism7000, Applied Biosystems, Foster City, CA, USA) in a final volume of 10 μL using SYBR Green Master Mix reagent (Applied Biosystems, Foster City, CA, USA) with gene-specific primers and TaqMan probes. (**Table 1S**) Melting curve analysis was performed with Dissociation Curves software (Applied Biosystems) and the mean cycle threshold (Ct) values were used to calculate gene expressions with normalization to human GAPDH.

2.4 Physiological Assessment of Ischemic Hindlimbs

To estimate limb perfusion 0,7,14,21,and 28 days after ischemia induction, the ischemic (left) to normal (right) limb blood flow ratio was measured with a LASER Doppler imager (MoorLDI-Mark2: Moor Instruments). After two LASER Doppler images were recorded, the average perfusion for the

ischemic and non-ischemic limbs was calculated on the basis of colored histogram pixels.

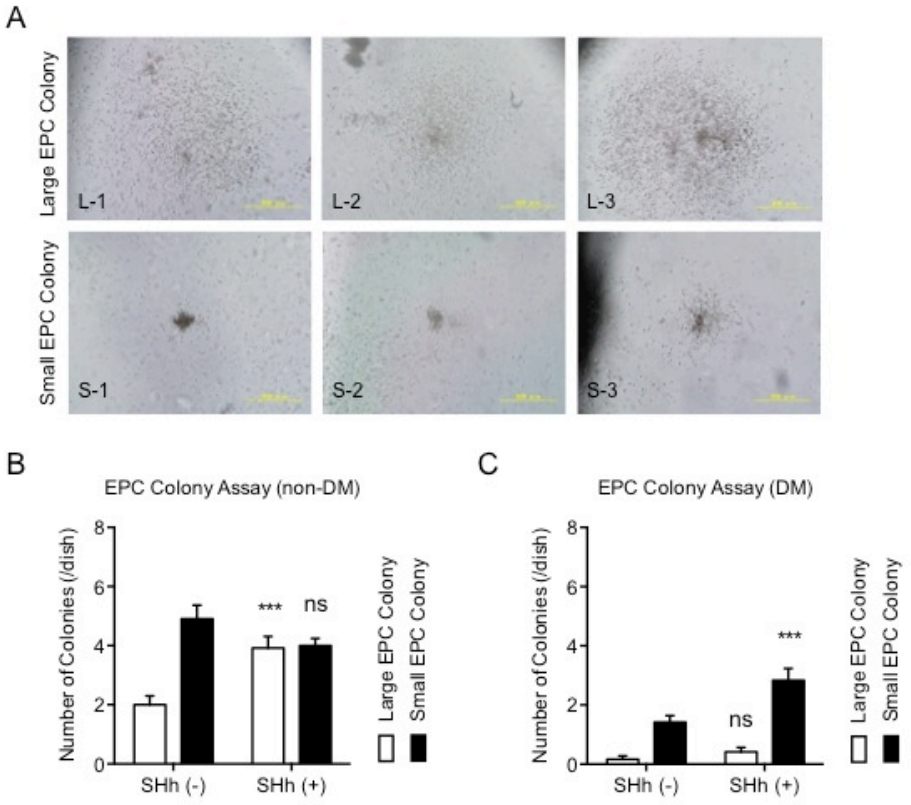
2.5 Histological Assessment of Ischemic Hindlimb

Tissue sections from the lower calf muscles of ischemic and healthy limbs were harvested on days 14 and 28. For immunohistochemistry, tissues were embedded in OCT compound (Miles Scientific, Elkhardt, IN) and snap frozen in liquid nitrogen. Frozen sections of 6-μm thickness were mounted on silane-coated glass slides, air-dried for 1h, and counterstained with a mixture of FITC-isolectin B4 (Vector, B-1205, 1:200) and FITC-Ulex lectin (Invitrogen, 121141, 1:200) for capillary density. After time points, 30 fields (x100 magnification) were counted for each of the 6 animals. For assessment of the transplanted Gm-$CD34^+$ cell differentiation into vascular lineage and angiogenic growth factor production, double-fluorescent immunostaining with an antibody for human mitochondria (hMtCd: Abcam, 1:100) developed with an immunogen of recombinant full length protein, corresponding to amino acids 1-192 of human mitochondrial ribosomal protein L11, produced in E.coli, and isolectin B4/Ulex lectin (Vector/Invitrogen, 1:200) or SMα-actin (Sigma, 1:500) or VEGF-A (SantaCruz, 1:50). Double-positive cells were counted in 5 selected high power fields (HPF) and averaged for quantitative analysis.

2.6 Western blot analysis

Cell pellets were resuspended in RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing a protein inhibitor cocktail (Sigma, Tokyo, Japan), and the protein concentration was determined with the DC protein assay kit (BIO-RAD Laboratories, Hercules, CA, USA). Proteins were loaded onto NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA), electrophoresed, and electrotransferred to Fluorotrans Membrane (Pall Life Science, Ann Arbor, MI, USA). Following electrotransfer, the membrane was blocked (5% of milk in phosphate-buffered saline with 0.05% Tween-20), and incubated overnight at 4°C with each primary antibodies: TGF-b receptor type II (Millipore, Billerica, MA, USA), Smad3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Smad4 (Cell Signaling Technology, Danvers, MA, USA), and b-actin (Sigma Chemical Co., St. Louis, MO, USA). Visualization of the protein signal was achieved with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare UK Ltd.,Buckinghamshrine, UK) and enhanced chemiluminescence Western blot analysis system (ImmunoStar LD; Wako Pure Chemical Industries, Ltd, Tokyo, Japan) using LAS 4000 mini (Fuji-film, Tokyo, Japan).

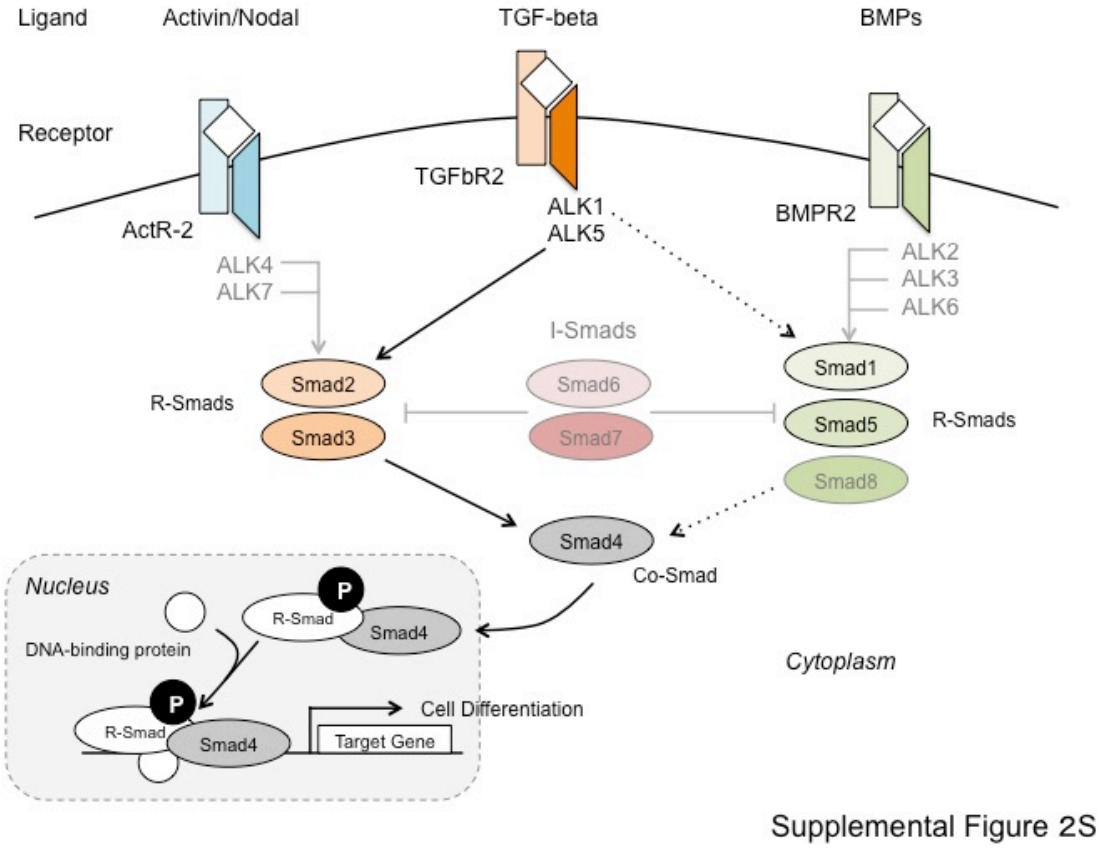
Supplemental Figures and Figure Legends



Supplemental Figure 1S

Supplemental Figure 1S. EPC colony forming assay with diabetic and non-diabetic peripheral blood mononuclear cells

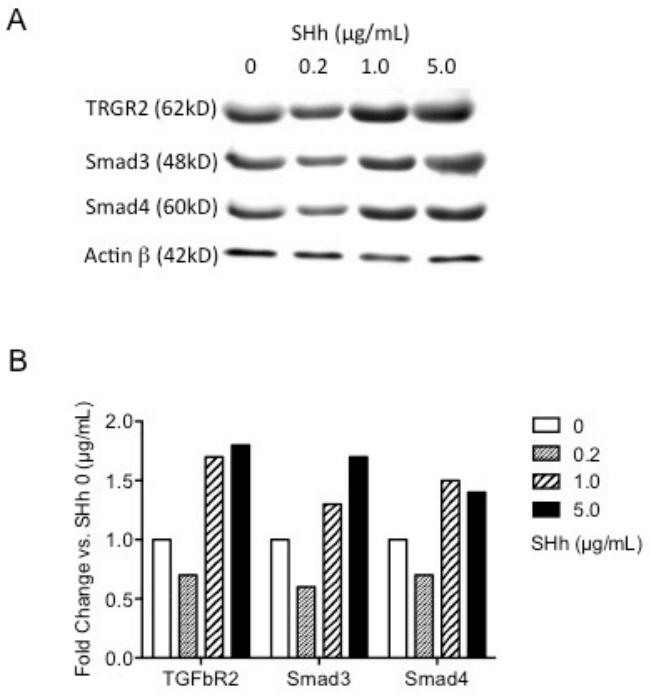
EPC colony forming assay for assessment of human $CD34^+$ cell angiogenic/vasculogenic capacity. Large EPC colonies (A, upper panels) and small EPC colonies (A, lower panels) were counted and quantified (B and C) after 14 days in culture under EPC differentiation condition. The assays were performed using $CD34^+$ cells isolated from non-diabetic (B, non-DM) and diabetic (C, DM) volunteers with (SHh (+)) or without SHh (1.0 μg/mL, SHh (-)) treatment. ns and ***, $P<0.001$ vs. SHh (-).



Supplemental Figure 2S

Supplemental Figure 2S. Smad signaling pathway in hematopoietic stem cells.

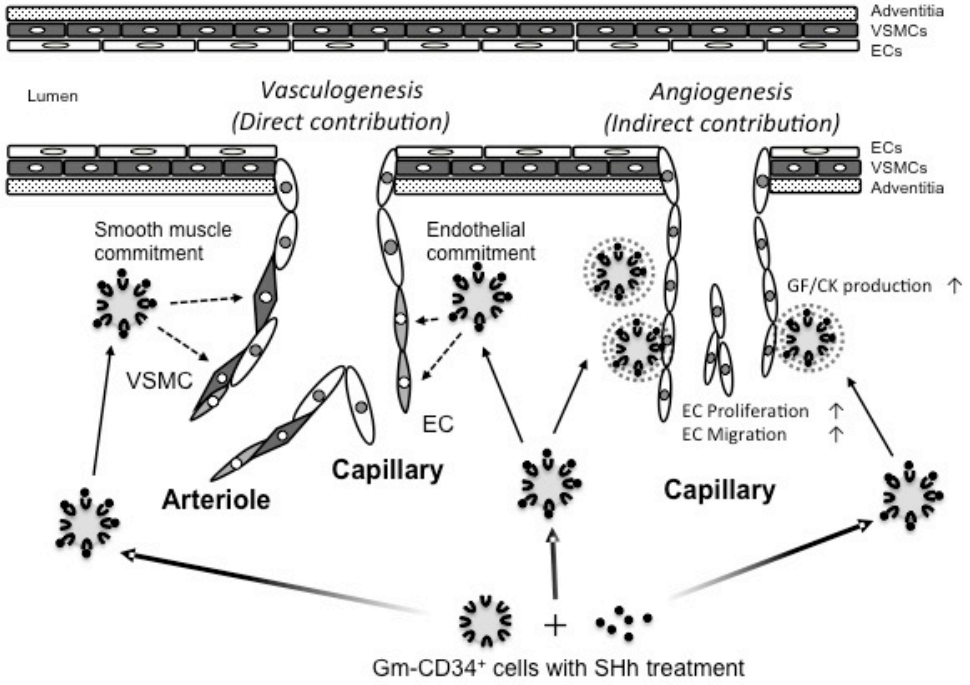
TGF-β family members bind and signal through 2 types of serine/threonine kinase receptors, type I and type II, both of which are necessary for signal transduction. The Smad proteins are activated by ligand binding through phosphorylation by type I receptors. Three groups of Smads have been identified: receptor-activated Smads (R-Smads), commonpartner Smads (Co-Smads), and inhibitory Smads (I-Smads). TGF-β and activin signal via R-Smad2 and 3, whereas BMP signals are transduced through R-Smad1, 5, and 8. Phosphorylated R-Smads subsequently associate with the Co-Smad4, creating a complex that translocates to the nucleus where target gene transcription is modified. The I-Smads, Smad6 and Smad7, function in a negative feedback loop to prevent activation of R-Smads. Commonly used alternative names include: ALK2/Activin type I receptor, ALK3/BMP type IA receptor, ALK4/Activin type IB receptor, ALK5/TGF-β type I receptor, and ALK6/BMP type IB receptor. P indicates phosphorylation. (Blank, et al. Signaling pathways governing stem cell-fate. Blood, 2008;111:497.)



Supplemental Figure 3S

Supplemental Figure 3S. Western blot analysis for TGFb/Smad signaling molecules.

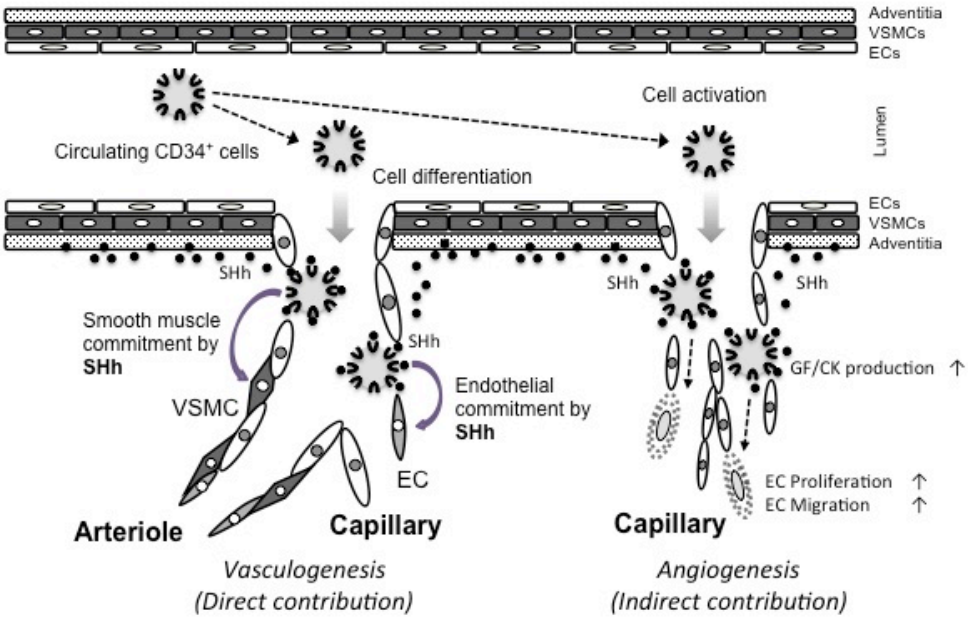
The protein expressions of TGFBR2, Smad3, Smad4, and Actin β were analyzed and detected as immunoblots in human CD34+ cells incubated with SHh at the indicated concentrations for 24 hours. (A) The blots were quantified by densitometrical analysis. Actin β was used as an internal control for the quantification of each protein expression. (B)



Supplemental Figure 4S

Supplemental Figure 4S. Overview of the effect of the transplanted SHh-treated Gm-CD34$^+$ cell functions in vasculogenesis/angiogenesis in ischemic tissue.

The transplanted SHh-treated Gm-CD34$^+$ cells reside in vasculature in ischemic tissue. A subpopulation of SHh-treated Gm-CD34$^+$ cells differentiate into endothelial cells and smooth muscle cells resulting in a co-operative direct contribution to vasculogenesis. (left side in Figure) SHh-treatement promotes the proliferation activity, migration activity and the GF/CK production capacity of the Gm-CD34$^+$ cells thus also contributing indirectly to angiogenesis. (right side in Figure) GF: growth factor, CK: cytokine, VSMC: vascular smooth muscle cell, EC: endothelial cell.



Supplemental Figure 5S

Supplemental Figure 5S. Overview of the effect of SHh on circulating $CD34^+$ cell fate and functions in vasculogenesis/angiogenesis in adult ischemic tissue.

Adventitia-derived SHh-stimulation increases the gene expression levels of SHh and its receptor Ptc1 as well as its downstream molecule Gli1 in recruited $CD34^+$ cells to ischemic tissue. A subpopulation of $CD34^+$ cells differentiate into endothelial cells and smooth muscle cells when treated with SHh resulting in a co-operative direct contribution to vasculogenesis. (left side in Figure) SHh promotes the proliferation activity, migration activity and the GF/CK production capacity of the recruited $CD34^+$ cells thus also contributing indirectly to angiogenesis. (right side in Figure) These effects of SHh on $CD34^+$ cells can be attributed to the autocrine reconstitution of the SHh signaling pathway triggered by an initial SHh exposure in situ. GF: growth factor, CK: cytokine, VSMC: vascular smooth muscle cell, EC: endothelial cell.

Supplemental Tables and supporting information

Table 1S. Specific primers for quantitative real-time RT-PCR amplifications.

Gene Name	Primer Sequences (5'-3')		TaqMan Probe
	Forward	Reverse	
SHh	CAGCGACTTCCTCACTTTCC	CGCGTCTCGATCACGTAGA	ACGACGGCGCCAAGAAGGT
PTCH1	AGTTAGTGTTGGAGCCATCAGAAA	GGAAAACCTAAAACTCTCTCCATCAG	TGTTTCATCTTGCTGACCTTGTGACACTC
PTCH2	GCTTGCTGGTTCCCACTTTG	CAGGAGCGTGAGCACTGTCA	TTCATTGTAAGGTACTTCTTTGCGGCG
SMO	CCTTCCATAGCCTCCAAACATG	TCTATGGCTTCCTGCTGTCTGA	TCCCAAGGCCCCACTTTCAAGA
GLI1	CTCCTCCCGAAGGACAGGTAT	ATAGCCACAAAGTCCAGCTGAGT	AACCCCCTGGACTCTCTTGATCTTGA
VEGF-A	GCTCTCTTATTTGTACCGGTTTTTGT	GCAGAGCTGAGTGTTAGCAAAATTA	CTCTCTCCCTGATCGGTGACAGTCAC
VEGF-B	AGGTGACACATGGCTTTTCAGA	GTTCCCCCACTGGGATATAGC	TCAGCAGGGTGACTTGCCTCAGA
ANG1	TTTTTCTATTCTTGGTGGCTTCTTC	GTAGGAACACAAAAGGACAAAATACTCA	TAGCAGGTAAGCCTCTCCTTCTAAAAACTT
PDGF-A	TGCATTGCCGCTTCTGTCT	ACTGGCAGCAGAGGGAGTGA	TGTTGTTGGTGTGCCCTGGTGC
PDGF-B	CCAAATAGGAGGGAGACTGTGGTA	GCAGAGGACTTTGGGAAATGG	AGGGAGGCAACACTGCTGTCCA
HGF	CCCACTTGTTTGTGAGCAACA	GACGATTTGGAATGGCACATC	AATGAGAATGGTTCTTGGTGTCATTGTTCC
CD31	CCACATACACTCCTTCCACCAA	TTGCCCTGGATCTCCTCTTG	TGGAACATCCTTGGAAATTGGAAGAGC
eNOS	CAGCAACGCTACCACGAAGA	TGCGTATGCGGCTTGTCA	ATTTTCGGGCTCACGCTGCG
SMA	CCTTCCAGCAGATGTGGATCA	AAGCATTTGCGGTGGACAAT	AACAGGAATACGATGAAGCCGGGC
Calponin	GACCCTCCGCTCTGTAGTGCTA	TGCTCAGTGCGTCCTTTGG	AGGGTCCAACATAGAGCCGGGTGT
TGFb1	TTGGAATGAGTCATGCCATATGTAG	GGCAGGGTTAGAAAACTTCTAAAGAC	TGCTGTAGATGGCAACTAGAACCTTTGAGT
TGFbR2	CGCCTAGAAATTCCACTTGCA	GAGGTCAATGGGCAACAGCTA	CGTAGGGCATGCTGATACCATCCC
ALK1	TGTGTCTTCCACCATCCTCATG	TTCCGAGCCTTGCACAATG	TGGCACTTTTCTAGGCCTGTCTCCC
ALK5	TTGGAATGAGTCATGCCATATGTAG	GGCAGGGTTAGAAAACTTCTAAAGAC	TGCTGTAGATGGCAACTAGAACCTTTGAGT
SMAD1	GTTCTGCAGCTGGTTAATTCATGT	CAAGCACTCCATATAACTGGTATTG	CTGTGAGAGCAAATGAATAATTCCTGC
SMAD2	GAGGAAATACATGGCCTTTGATG	CTGGCTTCTCGAGCAGAACAG	TCTGGCGTCTACTGCATTTCCCAG
SMAD3	GTGGCTTTTTGGCTCAGCAT	ACACGCGGCCACTTGTTT	CAGAAACACCAAACCAGGCTGGC

SMAD4	GGAGAGGAAGGGATGAAACCA	AGCCCGTGAGTCCTTCTATCAA	TGCTGTTGCAAAGGCTGCTTGTC
SMAD5	CGTCCCTCATTAATAGTGCCTTCT	GAAGGGAAAATCTGACCCTAAAAGA	AACAAAACTCCAGTAAGGCCAAAGAATCC
BMP2	TCTCCAAAGAACCCAGTTTTCTAAC	CAGAGGGTGGGCAGAAAACA	TGCCCAACACGCAGCAAAATTATG
BMPR2	TTTTCTGAATGGACAGGAGAAACA	CGAGTATTTTCTAGCCTTTCTTTTTCC	AAGCTACGGAGTATTCACTTCTGAGGATGC
ETS2	GGAACATCTTGCTGCCAAAAG	GGCAATTTCACGTGGTTCAA	ATCCTAGGCAGTGGCTCATTGTATGTGA
FLI1	GCTTTGGAAATGCGTGTAACAG	CCTCCTCTCCACAAGGGAAAGT	CAATAATCACAGCTCTGGGAAAAACAACG
TEL	GGGAGGGAGGCACCATAATC	TGATGCAGTTGGGTCAAAAGAG	CTCCCTAAAACCCACAGAAGACTAACCTGA
SCL	CAACTCTTTCGGCCTTTTGG	GTCTTCAGCAGAGGGTCACGTA	TGGGTCTGGCCGTACTTGTGATTTC
Sp1	CTTCCCCTTCCTAAGTCTGTCATC	GGGTTTTTAGGCACCAGAGATTG	TCTGGAAGGGATGGGTGGTGCT
Runx1	CCCCCACCTAGGGTCTATTTG	ACGCACGAATTTTCAGGATGT	TGGCAGTTATTGGGTTTGGTCACAA
CBF1	TTCTGTGCCAGACTTATGACTTTGT	TCCTGAAAGGGTTAAACAGAAACC	TTCAAGCACTGTAATGTGGGATGGATG
ZEB2	GGGCTTTTAGTCACTGAAATATGCA	CAAAGCTAAGCCTTCAGTCTGAATC	AGATGAAGAAAATACATGGCTTGTGCCC
Id2	GGGGAGCGAAAACGTTAAAATCA	ATTCACGCTCCACCTTTGAAA	TTGCCCAATCTAAGCAGACTTTGCCTT