Sonic hedgehog derived from human pancreatic cancer cells augments angiogenic function of endothelial progenitor cells

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Hedgehog signaling is important in the pathogenesis of pancreatic cancer. Several recent observations suggest the involvement of sonic hedgehog (SHH) in postnatal neovascularization. We identified a novel role for SHH in tumor-associated angiogenesis in pancreatic cancer. Immunohistochemical analysis revealed that patched homolog 1 (PTCH1), both a receptor for and transcriptional target of hedgehog signaling, was expressed in a small fraction of endothelial cells within pancreatic cancer, but not in normal pancreatic tissue. When endothelial progenitor cells (EPC) isolated from human peripheral blood were cultured with supernatant from SHH-transfected 293 cells or pancreatic cancer cells, mRNA levels of vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 and angiopoietin-1 were significantly increased, whereas no such induction was observed in human umbilical vein endothelial cell (HUVEC) and human dermal microvascular endothelial cell (HMVEC). HUVEC tube formation was stimulated when cocultured with EPC, and preconditioning EPC with supernatant from KP-1 N pancreatic cancer cells highly expressing SHH significantly enhanced the effect. The effect was partially attenuated by specific inhibition of SHH with cyclopamine or a neutralizing antibody. These findings suggest that tumor-derived SHH can induce angiogenesis, and this is mediated by its effects on EPC specifically. Targeting SHH would be a novel therapeutic approach that can inhibit not only proliferation of cancer cells but also EPC-mediated angiogenesis. (*Cancer Sci* 2008; 99: 1131–1138)

Pancreatic cancer is the fifth leading cause of cancer deaths in Japan, and its overall 5-year survival rate is only 9.3%. Hypovascularity is a typical characteristic of pancreatic cancer by diagnostic imaging.[1] However, a previous report has shown that the intratumoral microvessel density (MVD) of pancreatic ductal adenocarcinomas as measured by CD31 immunohistochemistry was equivalent to that of gastric and colon cancer.[2] In addition, several studies identified MVD and the levels of VEGF expression as clinically significant and independent prognostic factors for advanced pancreatic cancer.[3–5] These studies imply that the neovasculature in pancreatic cancer may still play a critical role in its malignant behavior.

Hedgehog signaling is essential for proper pattern formation and morphogenesis during embryogenesis.[6–8] The interaction of hedgehog protein with its receptor, Patched-1 (Ptch), leads to activation of transcription factor Gli, which induces downstream target genes including *Ptch* and *Gli* themselves.[9] Among the three types of hedgehog genes, *sonic hedgehog* (*Shh*), *Indian hedgehog*, and *desert hedgehog*, the role of the *Shh* pathway in blood vessel formation has been well studied in several animal models. Overexpression of *Shh* can cause hypervascularization of neuroectoderm,[10] and an impaired vascularization in lung was observed in *Shh*-deficient mice.[7] In addition to the role on embryonic vasculogenesis, *Shh* can also induce postnatal neovascularization. In adult mice, the *Ptch1* gene is normally expressed in cardiovascular tissue and exogenous *Shh* can strongly stimulate angiogenesis by primarily acting on fibroblasts to induce VEGF and angiopoietin-1 (Ang-1).[11] It has been also reported that *Shh* gene therapy may have significant potential to enhance wound healing by inducing arteriogenesis and restoring nerve function in diabetes.[12,13]

Aberrant activation of the SHH pathway has been demonstrated in human pancreatic cancer as well as in number of other types of cancer.[14–17] Blocking SHH has a prominent antigrowth effect in animal models, indicating that it acts in an autocrine manner.[17,18] In pancreatic neoplasms, the SHH pathway is activated during the early stages of carcinogenesis, such as Pan-INs in mice,[18] and intraductal papillary mucinous neoplasms in humans.[19] In addition, SHH plays a role in metastasis of pancreatic cancer,[20] and blocking the hedgehog pathway by cyclopamine increases the cytotoxic effect of radiotherapy and chemotherapeutic agents.[21] Thus, SHH is considered to be a new therapeutic target for pancreatic cancer.[18,20] However, little is understood about the role of SHH during tumor-associated angiogenesis.

Circulating BM-derived EPC were first described by Asahara *et al*. in 1997.[22] EPC can be isolated from adult peripheral blood and have the capacity to differentiate into endothelial cells *in vitro* and *in vivo* to support postnatal vasculogenesis. EPC are mobilized from the BM in response to ischemia and VEGF,[22,23] and the number of circulating EPC has been shown to increase in patients with various cancers.[24,25] Their contribution during the development of the tumor vasculature has been demonstrated by xenograft models.[26,27] Recently, Asai *et al*. have identified the novel role of *Shh* on BM-derived EPC in neovascularization during wound healing.[12] However, *Shh* has not been considered to be a direct stimulator of mature endothelial cells such as HUVEC.[11,28] These studies led us to speculate that SHH secreted from pancreatic cancer cells may play a role in the development of neovasculature by stimulating BM-derived EPC. We therefore tried to clarify the above hypothesis in the present study.

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Abbreviations: Ang-1, angiopoietin-1; BM, bone marrow; EC, endothelial cell; EPC, endothelial progenitor cell; HMVEC, human dermal microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; MNC, mononuclear cell; PTCH1, patched homolog 1; SDF-1, stromal cell-derived factor; SHH, sonic hedgehog; VEGF, vascular endothelial growth factor.

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Materials and Methods

Tissue preparation. Pancreatic ductal adenocarcinoma tissues were obtained as surgical discards as part of an institutional review board–approved protocol at the Asahikawa Medical Collage Hospital and Asahikawa Kosei General Hospital (four males, five females; mean age 68.5 ± 8.4 years). Freshly removed tissue specimens were fixed with formalin and embedded in paraffin for histological analysis.

Cell culture. Eight human pancreatic adenocarcinoma cell lines, PK1, PK8, MIAPaca2 (Cell Resource Center for Biochemical Research, Tohoku University, Sendai, Japan), Panc1, BxPC3 (from American Type Culture Collection [ATCC], Manassas, VA, USA), KP-1 N, KP-2, and KP-3 (from Japan Cancer Research Resource Bank [JCRB], Osaka, Japan) were used in this study. Cell lines from the stomach (MKN28, from JCRB), colon (SW480, from ATCC), liver (HepG2, from ATCC), and lung cancer (NCI-H1975, from ATCC) were also utilized. HUVEC and normal HMVEC (adult) (HMVEC-d Ad), purchased from Cambrex (Walkersville, MD, USA), were cultured in the endothelial cell basal medium (EGM2 for HUVEC; and EGM2-MV for HMVEC-d Ad) and early passages of cells (up to the 6th passage) were utilized.

Human SHH plasmid. For construction of the human SHH plasmid (phSHH), the 600-bp amino-terminal domain of human SHH coding sequence[12] was subcloned into pIRES2-EGFP (BD Biosciences Clontech, Mountain View, CA, USA). The mature forms of SHH are 19 kDa proteins that interact with heparin through an N-terminal basic domain and are tethered to the cell membrane through cholesterol and fatty acyl modification.[11,29] HEK-293 cells (293 cells) were transfected with phSHH expression vector or empty vector (EV) using Arrest-In transfection reagent (Open Biosystems, Huntsville, AL, USA) according to manufacturer's protocol and conditioned medium was collected 2 days later to stimulate endothelial cells.

Western blotting for SHH. Thirty μg of protein extracts from pancreatic cancer cells were resolved on a 12% NuPAGE Bis-Tris polyacrylamid gel (Invitrogen, Carlsbad, CA, USA), transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), and immunoblotted with anti-SHH (H-160, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin antibodies (1:5000; Sigma, St. Louis, MO, USA). Immunoreactive proteins were visualized using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Waltham, MA, USA).

Peripheral blood mononuclear cell isolation and EPC culture. Peripheral blood from healthy volunteers was mainly utilized as a source of human EPC. In addition, MNC collected by therapeutic apheresis from patients with ulcerative colitis[30] were also used for experiments that required large numbers of EPC. Informed consent was obtained from each patient in accordance with the guidelines of the Ethics Committee of Asahikawa Medical Collage. Briefly, MNC were isolated by density gradient centrifugation using Histopaque-1977 (Sigma) and cultured in EBM-2 with supplements (EGM2-MV BulletKit; Clonetics, San Diego, CA, USA) but without hydrocortisone on human fibronectin (Sigma)–coated dishes.[23,31] Attached cells were obtained by 4–7 days culture and utilized as human EPC for the *in vitro* studies. More than 95% of attached cells show endothelial characteristics as demonstrated by acetylated low-density lipoprotein (acLDL; Biomedical Technologies, Stoughton, MA, USA) uptake and Ulex Europaeus agglutinin (UEA)-lectin (Vector Laboratories, Burlingame, CA, USA) binding (**Supplementary Material 1**). In addition, expression of typical endothelial marker platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and CD105, and weak expression of CD34 but not c-Kit, was identified in the attached MNC by flow cytometry (all antibodies were purchased from Beckman Coulter, Fullerton, CA, USA; **Supplementary Material 1**). There was no significant difference in viability or levels of angiogenic cytokines in EPC between healthy volunteers and patients with ulcerative colitis (data not shown).

Subsequent to overnight culture in serum starved medium (0.5% fetal bovine serum [FBS] in EBM2), human EPC were stimulated by conditioned medium (CM) from SHH-transfected 293 cells or pancreatic cancer cell lines, and then used for gene expression analysis and tube formation assays. CM was prepared by culturing cells in serum-free medium (Ultraculture, Cambrex, Charles City, IA, USA) for 48 h, and a centrifugal filter device (Amicon ultra-15, 5 kDa; Millipore) was utilized to concentrate the CM from pancreatic cancer cells.

Quantitative real-time polymerase chain reaction (PCR) assay. Total RNA was extracted using the RNeasy Protect Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The 18S rRNA served as endogenous control. Primer sequences for SHH, PTCH1, VEGF, SDF-1, Ang-1, glioma-associated oncogene homolog 1 (GLI1), hedgehog interacting protein (HIP), and 18S rRNA are summarized in the Table 1. PCR cycles were 1 min at 95°C, followed by 40 cycles with annealing temperature, 55°C. A fluorogenic SYBR Green (Takara, Otsu, Shiga, Japan) and LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany) were used for real-time quantification. The results were presented as parameter threshold cycle (C_T) values. ΔC_T was the difference in the C_T values derived from the specific gene being assayed and 18 S rRNA, whereas $\Delta\Delta C_T$ represented

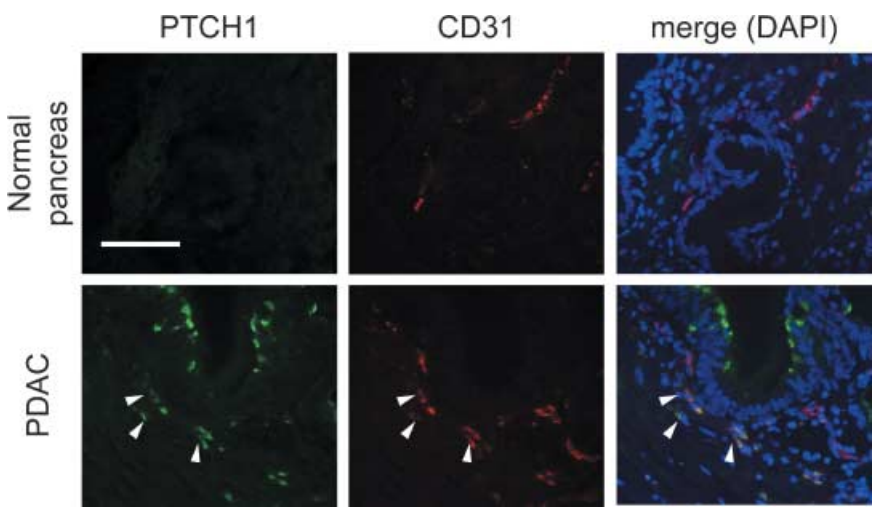

Fig. 1. Aberrant activation of the sonic hedgehog (SHH) signaling pathway in tumor-associated endothelial cells. Immunohistochemistry for patch homolog 1 (PTCH1) (green) and CD31 (red) in human pancreatic adenocarcinoma (PDAC) specimens. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI). In normal pancreatic tissues, PTCH1 was completely absent (top panel). In addition to abnormal epithelium in pancreatic cancer tissues, PTCH1 expression was also observed in small fraction of tumor associated endothelial cells (bottom panel). Note the colocalization of PTCH1 and CD31 immunostaining as indicated by arrow heads. Scale bar, 100 μm.

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Table 1. SYBR green real-time quantitative polymerase chain reaction primer sequences

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
SHH	CCAATTACAACCCCGACATC	CAGTTTCACTCCTGGCCACT
PTCH1	ACAAACTCCTGGTGCAAACC	AAGCGCTGGGATTAATGATG
GLI1	ATCAGGGAGGAAAGCAGACT	TGTCTGTATTGGCTGCACTC
HIP	GCTACGTGTTTGGAGATCGT	AGTGGTTTTTCTTGCCACTG
VEGF	AAGGAGGAGGGCAGAATCAT	ACACAGGATGGCTTGAAGATG
SDF-1	AAACTGTGCCCTTCAGATTG	CCAGGTACTCCTGAATCCAC
Ang-1	CATCTGGAACATGTGATGGA	TCTCCGACTTCATGTTTTCC
18S	CGTCTGCCCTATCAACTTTC	ATGTGGTAGCCGTTTCTCAG

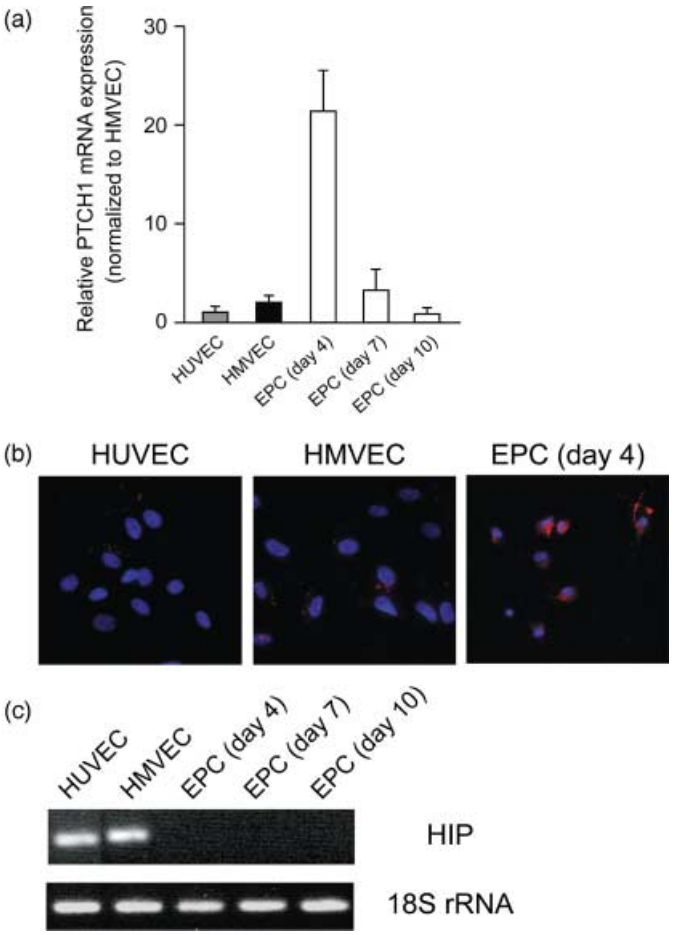

Fig. 2. Patch homolog 1 (PTCH1) and hedgehog interaction protein (HIP) expression in endothelial cells. (a) Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of PTCH1 mRNA expression in human endothelial progenitor cells (EPC) and mature endothelial cells (EC), and human umbilical vein endothelial cells (HUVEC) and HMVEC (human dermal microvascular endothelial cell). The level of PTCH1 mRNA in EPC (average of five individuals) was significantly higher than that of HUVEC (three clones) and HMVEC (two clones), but the expression in EPC gradually decreased during long-term culture. Values are shown as the average of three independent experiments. (b) Immunostaining for PTCH1 (red) in cultured EPC, HUVEC, and HMVEC. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI). PTCH1 protein is highly expressed in EPC, but weakly expressed in HUVEC and HMVEC. (c) HIP mRNA was clearly demonstrated by RT-PCR in HUVEC and HMVEC, but completely absent in EPC.

the difference between the paired samples, as calculated by the formula $\Delta\Delta C_T = \Delta C_T$ of a sample-ΔC_T of a reference. The amount of target, normalized to an 18S and relative to a reference, was expressed as $2^{-\Delta\Delta CT}$.

Tube forming assay. Human EPC isolated from peripheral blood were cultured for 48 h in the presence of CM from pancreatic cancer cells with or without cyclopamine (Biomol, Plymouth Meeting, PA, USA) or MAB4641, a neutralizing antibody against SHH (R&D systems, Minneapolis, MN, USA). One thousand HUVEC and 2.0×10^3 DiI-acLDL labeled EPC were suspended in EBM-2 containing 2% FBS and seeded onto growth factor-reduced matrigel (354230; Becton Dickinson, Franklin Lakes, NJ, USA) in 96-well plates. Eight hours later, capillary morphogenesis was examined under a phase-contrast and fluorescent microscope (IX70; Olympus, Tokyo, Japan). The number of branch points per HPF was counted, and the length of capillaries was quantified using ImageJ software 1.38.

Immunofluorescence staining. For immunofluorescence staining of PTCH1 and CD31 in human pancreatic cancer tissue, sections were incubated with mouse anti-CD31 (Dako, Carpinteria, CA, USA) at room temperature for 20 min, followed by Alexa Fluor 594 $F(ab')_2$ fragment of goat antimouse IgG (1:1000; Molecular Probes, Carlsbad, CA, USA) as a secondary antibody for 30 min. The sections were then incubated with rabbit anti-PTCH1 antibody (1:100; Research Diagnostics, Concord, MA, USA) at 4°C overnight, followed by Alexa Fluor 488 $F(ab')_2$ fragment of goat antirabbit IgG (1:1000) as a secondary antibody for 20 min. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) (50 ng/mL; Sigma), and sections were mounted in aqueous mounting medium. Images were obtained using a fluorescent microscope (BX-51, Olympus) and confocal microscope (LSM5; Zeiss, Jena, Germany).

Cultured EC were also stained with anti-PTCH1 antibody (1:200, Research Diagnostics) at room temperature for 1 h and followed by Alexa Fluor 488 $F(ab')_2$ fragment of goat antirabbit IgG (1:1000; Molecular Probes) for 20 min. Nuclei were counterstained with DAPI and images were examined with a fluorescent microscope.

Statistical analysis. All results are expressed as mean ± standard deviation unless otherwise noted. Statistical significance of differences was determined using a two-tailed Student's *t*-test.

Results

Activation of the SHH signaling pathway in tumor-associated endothelial cells. PTCH1 is a receptor for SHH as well as a transcriptional target,[9] and therefore, it serves as a marker of Hh pathway activation.[9,18,32] We first immunohistochemically examined PTCH1 expression in human pancreatic tissue from nine cases with invasive ductal adenocarcinoma. Aberrant expression of PTCH1 in concert with SHH in human pancreatic cancer has been demonstrated.[18] In addition to PTCH1 protein expression throughout the abnormal epithelium in pancreatic cancer tissues as described previously, stromal expression was also observed (Fig. 1). The majority of the stromal PTCH1 staining colocalized with CD31 immunostaining (Fig. 1, **Supplementary Material 2**), and PTCH1 expression was observed in a small fraction ($6.8 \pm 2.4\%$) of CD31 positive tumor-associated EC in all nine cases tested. In contrast, PTCH1 was completely absent in normal pancreatic tissue, including epithelial cells or EC. These results indicate an intrinsic stromal activation of the hedgehog pathway in pancreatic cancer, and suggest the involvement of SHH signaling in tumor-associated angiogenesis.

Prior studies have indicated that mature EC express both SHH receptor PTCH1 and the negative regulator, hedgehog-interacting protein (HIP).[28] We quantified the expression levels of PTCH1 and HIP mRNA in mature EC and EPC isolated from

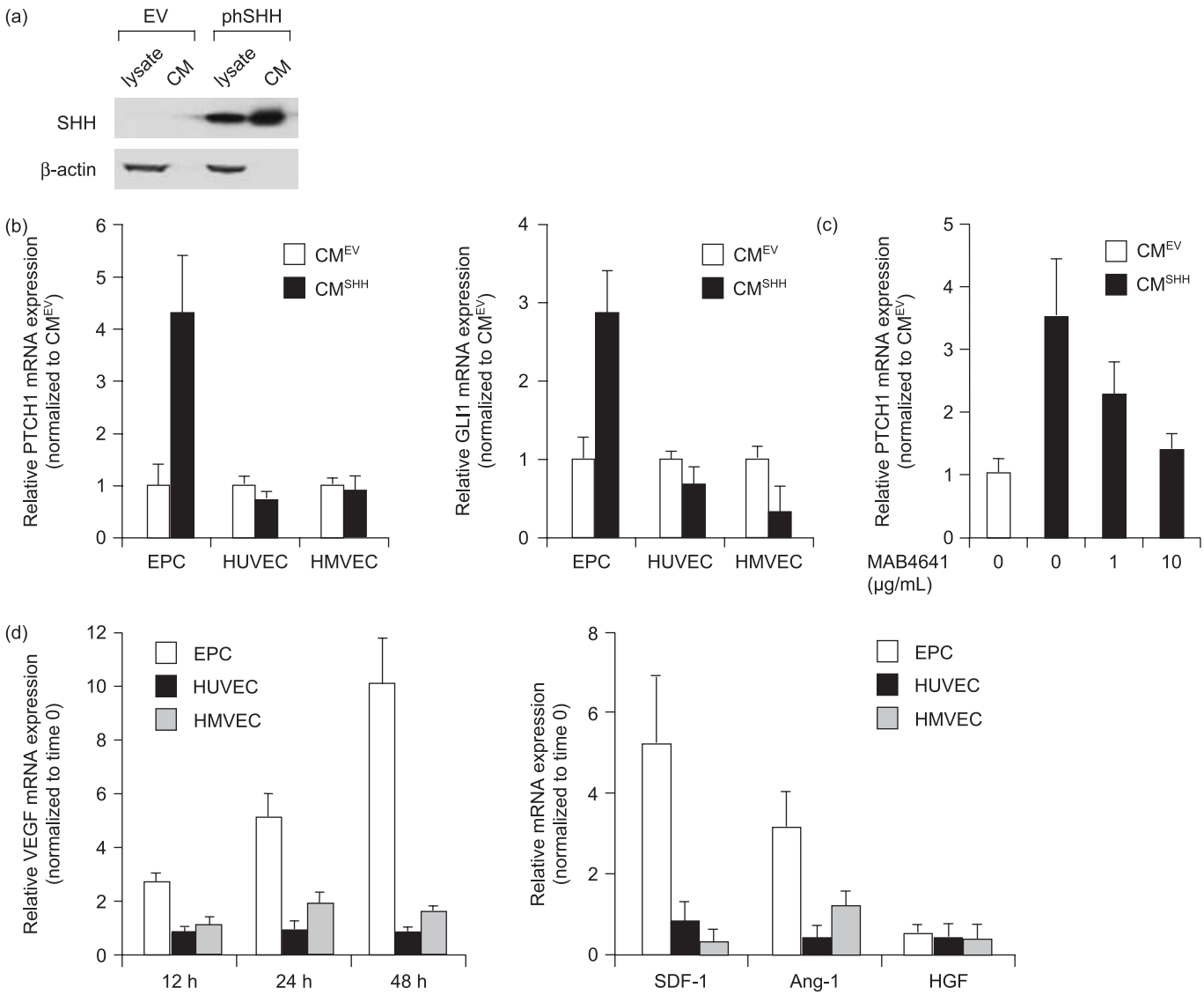

Fig. 3. Induction of angiogenic cytokines in human enodthelial progenitor cells (EPC) by sonic hedgehog (SHH). (a) Western blotting shows secreted SHH protein in conditioned medium of 293 cells transfected with a phSHH (CM^{SHH}). (b) Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of PTCH1 and GLI1 mRNA utilizing human EPC, human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMVEC) cultured with CM^{SHH}. PTCH1 and GLI1 mRNA were strongly induced by CM^{SHH} in EPCs, but not in HUVEC and HMVEC. (c) Blocking SHH by neutralizing antibody MAB4641 significantly attenuated the induction of PTCH1 by CM^{SHH}. (d) CM^{SHH} strongly induced vascular endothelial growth factor (VEGF) mRNA in EPC, but not in HUVEC and HMVEC. Other pro-angiogenic factors SDF-1 and Ang-1, but not HGF, were also up-regulated in the presence of CM^{SHH} in EPC, but no such induction was observed in HUVEC and HMVEC. Values are shown as the average of three independent experiments.

adult human peripheral blood. The PTCH1 mRNA level in EPC was significantly higher than that in HUEVC and HMVEC, but it gradually decreased during culture, indicating that PTCH1 is preferentially expressed in immature EC (Fig. 2a). Immunostaining for PTCH1 demonstrated strong PTCH1 protein expression in EPC but weak expression in HUVEC and HMVEC (Fig. 2b). In contrast, expression of HIP was clearly observed in HUVEC and HMVEC, but completely absent in EPC (Fig. 2c). These data led us to speculate that SHH may induce angiogenesis in pancreatic cancer and that this effect was mediated through EPC.

SHH up-regulates PTCH1 and induces angiogenic cytokines in EPC. SHH has been considered to induce angiogenesis indirectly through the production of fibroblast-derived angiogenic cytokines.[11] However, recently, a direct effect of hydrophobic modified SHH protein on mice BM-derived EPC was described.[12] It was evident that SHH can directly induce proliferation, enhance adhesion, and increase migration and tube forming activity of EPC. In the current study, we sought to determine whether the cell secreted form of SHH can also promote tumor angiogenesis in a paracrine setting. First, secreted SHH protein was prepared by transfecting phSHH into 293 cells (Fig. 3a). PTCH1 and GLI1 mRNA were strongly induced in human EPC isolated from peripheral blood when cultured with CM from SHH-transfected 293 cells (CM^{SHH}) (Fig. 3b), indicating that EPC can respond to secreted SHH. However, the up-regulation of PTCH1 and GLI1 by CM^{SHH} was not observed in mature EC, HUVEC, and HMVEC, consistent with previous reports.[11] The induction of PTCH1 (Fig. 3c) and GLI1 (data not shown) was effectively blocked by the presence of neutralizing antibody to SHH in a dose-dependent manner, indicating that the effect of CM^{SHH} was specifically mediated through SHH, and MAB4641 can sufficiently block SHH signaling.

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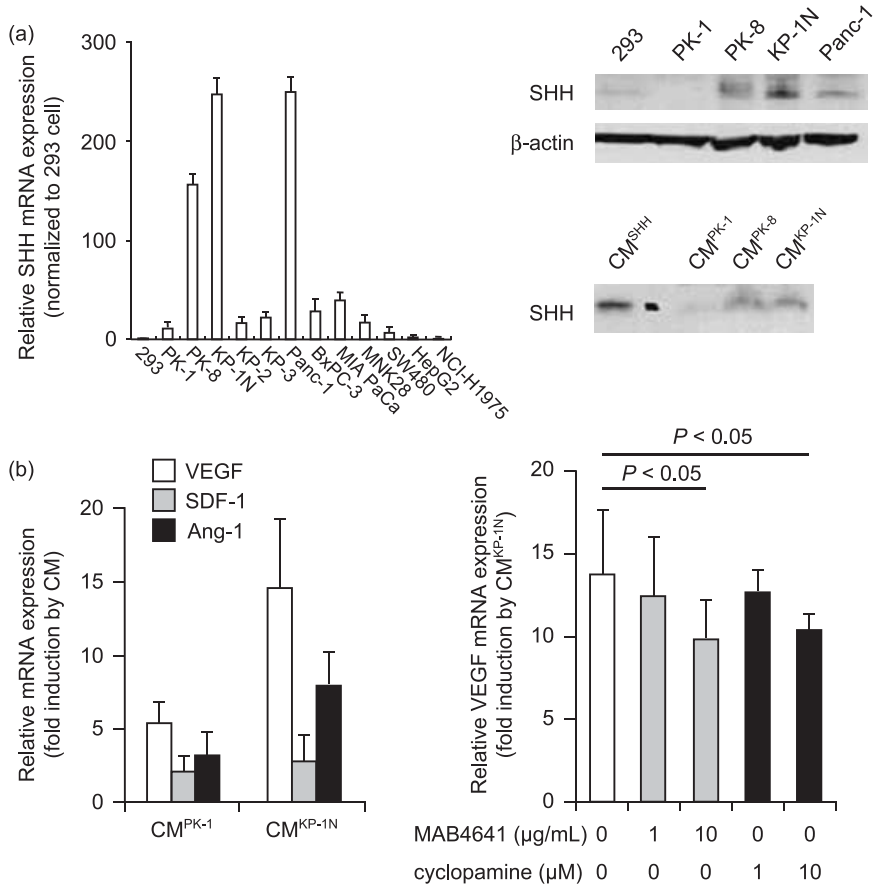

Fig. 4. Expression of sonic hedgehog (SHH) mRNA in panel of pancreatic cancer cell lines and induction of vascular endothelial growth factor (VEGF) in endothelial progenitor cells (EPC) by cancer cell-derived SHH. (a) Relative SHH mRNA expression in various pancreatic cancer cells and gastric (MNK28), colonic (SW480), hepatic (HepG2), and lung (NCI-H1975) cancer cell was evaluated by quantitative reverse transcription–polymerase chain reaction (RT-PCR). In KP-1 N, Panc-1, PK-8, BxPc3, and MIA Paca2, SHH mRNA was detected. Western blotting shows strong SHH protein expression in KP-1 N, PK-8, and Panc-1 (upper panel). Secreted SHH within culture supernatant was also identified (lower panel). (b) Quantitative RT-PCR analysis of proangiogenic factors, VEGF, SDF-1, and Ang-1 in EPC incubated with concentrated supernatant from KP-1 N ($CM^{KP\text{-}1N}$). Up-regulation of VEGF mRNA was observed when EPC were cultured with $CM^{KP\text{-}1N}$, and the induction was significantly higher than that of supernatant from PK-1, which faintly expresses SHH. Blocking SHH pathway by MAB4641 or cyclopamine significantly attenuated the induction of VEGF by $CM^{KP\text{-}1N}$. Values are shown as the average of three independent experiments.

We next examined whether SHH may enhance the production of angiogenic cytokines in EPC. In addition to the PTCH1 up-regulation, CM^{SHH} strongly induced VEGF mRNA. The induction was first detected at 12 h (2.7- ± 1.3-fold) and continued to increase up to 48 h (10.3- ± 1.8-fold) (Fig. 3d). A similar time-course has been observed in SHH-stimulated fibroblasts.[11] Other potent pro-angiogenic factors such as SDF-1 and Ang-1, but not HGF, were also up-regulated in the presence of CM^{SHH} (Fig. 3d). Again, no such induction was observed in HUVEC and HMVEC. These results indicated that EPC can respond to SHH, resulting in an enhancement of their expression of angiogenic cytokines.

Induction of VEGF in EPC by pancreatic cancer cell-derived SHH. In order to evaluate the interaction between cancer cells and endothelial cells, we next examined the effect of conditioned media from pancreatic cancer cells on EC. SHH mRNA and protein expression was observed in the pancreatic cancer cell lines KP-1 N, PK-8, and Panc-1, and the SHH mRNA levels are higher than those in cell lines from other common cancers such as stomach, colon, liver, and lung cancer. (Fig. 4a). A strong induction of VEGF mRNA was observed when EPC were cultured with concentrated supernatant from KP-1 N ($CM^{KP\text{-}1N}$) (Fig. 4b), Panc-1, and PK-8 (data not shown) pancreatic cancer cells highly expressing SHH. Supernatant from PK-1, which faintly expresses SHH, also induced VEGF, albeit at low levels, but the induction was significantly lower than $CM^{KP\text{-}1N}$ (Fig. 4b). Blocking the SHH pathway either by the neutralizing antibody, MAB4641, or the specific inhibitor cyclopamine significantly attenuated the induction of VEGF by $CM^{KP\text{-}1N}$ (from 10.5- ± 2.8-fold to 6.2- ± 1.2-fold; $P < 0.05$) (Fig. 4b). In addition to VEGF, $CM^{KP\text{-}1N}$ also induced Ang-1 (8.7- ± 1.7-fold) and SDF-1 (2.5- ± 1.3-fold) in EPC, and the induction was attenuated when SHH was selectively blocked (data not shown). These data indicate that secreted factors from pancreatic cancer cells can enhance the production of angiogenic factors including VEGF in EPC, and SHH at least partially mediates this effect.

SHH derived from pancreatic cancer cells enhanced the ability of EPC to stimulate tube formation by HUVEC. In order to examine whether EPC can promote angiogenesis in a paracrine manner, a tube formation assay was performed by coculturing EPC with HUVEC on matrigel. In the absence of growth factors including VEGF and at low serum condition (2% FBS in EBM2), HUVEC alone hardly formed tube structures. However, when HUVEC were cocultured with EPCs at 5:1 ratio, capillary morphogenesis was clearly induced (Fig. 5a). This was more prominently observed when EPC were preconditioned with $CM^{KP\text{-}1N}$, although the number of EPC associated with tubes was not altered (2.2 ± 1.57 *versus* 3.7 ± 1.49 cells/mm tube in non-stimulated and $CM^{KP\text{-}1N}$-stimulated EPCs; $P = 0.31$; Fig. 5b). Inhibition of SHH by cyclopamine and MAB4641 during preconditioning inhibited the number of branch points and tube length (Fig. 5c). These results suggest that SHH derived from pancreatic cancer cells enhances the ability of EPC to promote capillary morphogenesis of mature EC in a paracrine manner.

Discussion

Aberrant activation of the SHH pathway has been shown to occur in the majority of human pancreatic cancers,[18,33] and can be seen in early benign lesions.[19] A marked reduction in tumor size following systemic administration of cyclopamine, a specific hedgehog inhibitor,[15] supports its crucial role for maintaining the malignant biological behavior of pancreatic cancer.[18] The significance of SHH in the proliferation of cancer

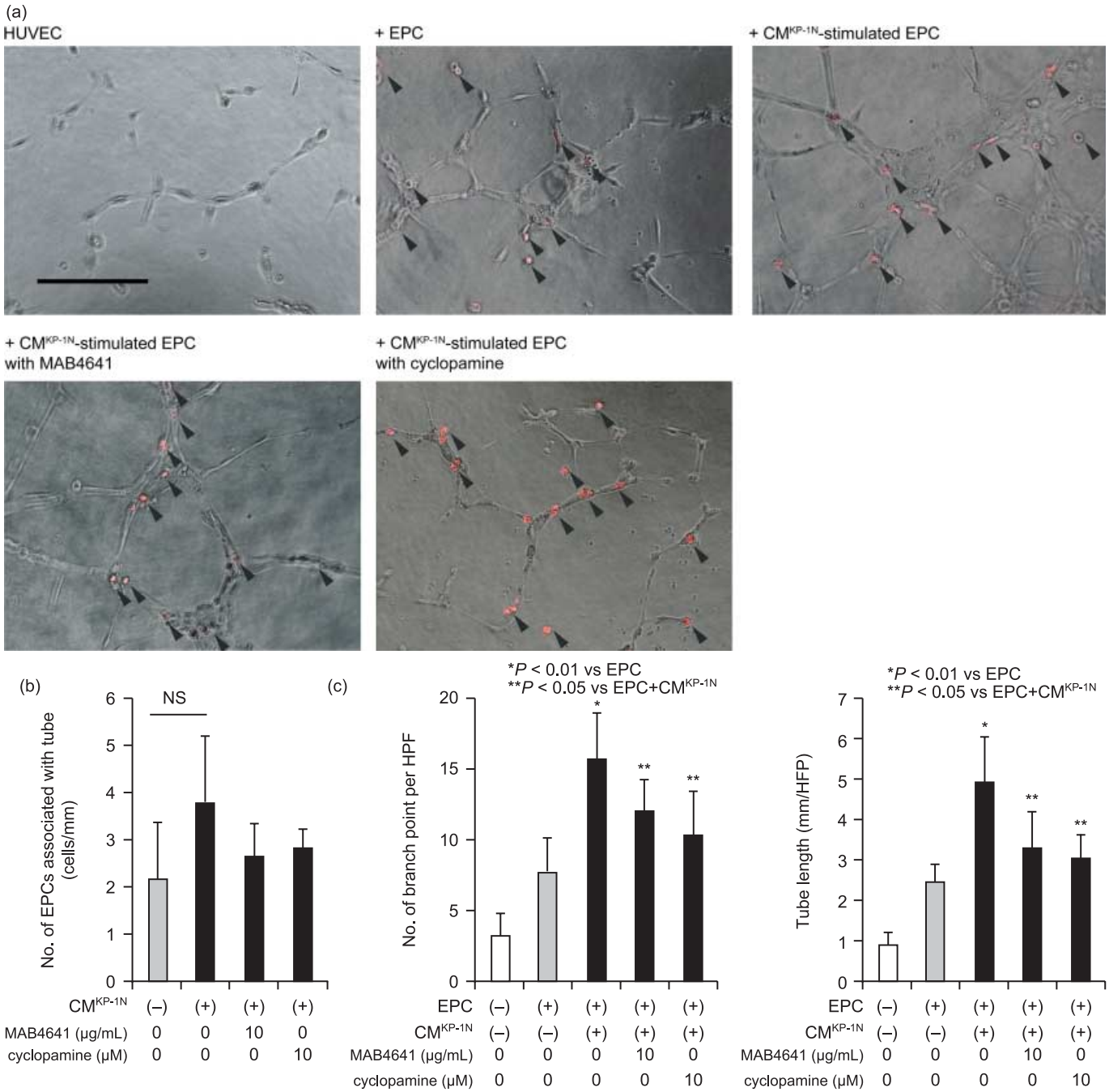

Fig. 5. Sonic hedgehog (SHH) enhanced the ability of endothelial progenitor cells (EPC) to stimulate tube formation by HUVEC in a paracrine manner. (a) HUVEC alone hardly formed tube structures, but capillary morphogenesis was clearly observed when human umbilical vein endothelial cell (HUVEC) were cocultured with EPC. The tube formation was significantly augmented by preconditioning EPC with $CM^{KP\text{-}1N}$, which was partially blocked by cyclopamine and MAB4641. Arrow heads indicate DiI-acLDL-labeled EPCs. Scale bar, 300 μm. (b) Number of EPC associated with tube was not altered by SHH inhibition. (c) Number of branch points and tube length was quantified. Inhibition of SHH during preconditioning of EPCs by $CM^{KP\text{-}1N}$ significantly attenuated the tube formation and number of branch points.

cells has been recognized, but its role during tumor-associated angiogenesis has not been clarified. Here, we demonstrated a novel role for SHH in the development of the tumor vasculature.

Recent advances in our understanding of the role of SHH in vascular development during embryogenesis,[7,10] as well as postnatal vasculogenesis in response to ischemic events in the cardiovascular system,[11,13] and to tissue injury,[12] has led us to speculate on the involvement of SHH in tumor-associated angiogenesis. Since immunostaining for PTCH1, a marker of SHH activation, in reactive stromal cells in human pancreatic cancer has been demonstrated,[18] we were then particularly curious whether tumor-associated EC may be a predominant source of stromal expression of PTCH1. For the first time, we confirmed the expression of PTCH1 in tumor EC within human pancreatic cancer tissues, but not in the normal pancreas, indicating an alternative role for the hedgehog pathway in pancreatic cancer. Our results imply that SHH secreted from cancer cells facilitates tumor growth not only by stimulating proliferation of cancer

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cells in an autocrine manner but also by promoting angiogenesis through EPC activation in a paracrine manner. Other stromal components such as fibroblasts may also be involved in the SHH-mediated angiogenic process, because previous studies have identified fibroblasts as a direct target of SHH.

In the setting of ischemic heart disease, *Shh* can stimulate angiogenesis primarily by acting on fibroblasts through an induction of VEGF and Ang-1 but not directly on EC.[11] However, Asai *et al*. recently described the direct action of *Shh* on murine BM-derived EPC during postnatal vasculogenesis utilizing a wound healing model.[12] We first demonstrated that SHH secreted from pancreatic cancer cells can promote tumor angiogenesis primarily through activation of human EPC, but not mature EC. These data indicate that within the tumor microenvironment, EPC derived from BM have a distinct response when compared to resident EC.[34] In human pancreatic cancer tissue, only a small fraction of tumor-associated EC expresses PTCH1 (6.8%). Although it is difficult to discriminate BM-derived EC from resident (mature) EC in human tissues, a recent study analyzing tumor-associated EC in individuals who develop cancer after BMT with donor cells from an individual of the opposite sex demonstrated that BM-derived EPC indeed contributed to the tumor endothelium, albeit at low levels (averaging at 4.9% of the total).[35] We identified strong PTCH1 protein expression in cultured EPC but not in HUVEC and HMVEC. Thus, a small fraction of PTCH1 positive EC in cancer tissue may reflect the small contribution of BM-EPC to the development of the tumor neovasculature.

Previous reports have identified PTCH1 expression HUVEC *in vitro*,[32] and its expression can be further induced during tube formation in matrigel.[28] In our study, PTCH1 mRNA levels in EPC was significantly higher than those in HUVEC and HMVEC, and the expressions were down-regulated in long-term culture, indicating that EC may lose their responsiveness to *Shh* during maturation. The naturally occurring hedgehog pathway antagonist HIP can bind to all three hedgehog proteins with an affinity equal to that of PTCH1.[6] Olsen *et al*. showed that overexpression of HIP inhibits the ability of these cells to respond to exogenous SHH, confirming that HIP can block hedgehog signaling when overexpressed.[28] HIP is predominantly expressed in vascular EC of normal tissues, while HIP mRNA levels are decreased or even absent in several types of human tumor-associated EC. In pancreatic neoplasms, HIP is epigenetically silenced by promoter methylation, and its restoration by 5-Aza-2′-deoxycytidine down-regulated hedgehog signaling.[36] We demonstrated that mature EC such as HUVEC and HMVEC expressed HIP mRNA, whereas the expression was totally absent in EPC. Taken together, the pattern of expression of PTCH1 and HIP may explain the distinct responses of EPC to SHH compared to mature EC. The precise mechanisms delineating how EC lose their response to SHH during maturation remains to be determined, but the sensitivity to SHH is likely to be a specific property to immature EPC.

Induction of angiogenic factors such as VEGF, Ang-1, and SDF-1 by *Shh* in murine fibroblasts and BM-EPC has been demonstrated by some authors.[11,12,37] We confirmed the similar induction by cell-secreted SHH in human peripheral blood EPC. The precise mechanisms how SHH induces various angiogenic cytokines remains to be determined in EC and fibroblasts. Many hedgehog-inducible genes are regulated by the transcriptional factor Gli; however, no Gli-response elements are present in the VEGF or Ang-1 promoter regions.[11] Alternatively, a Gli-independent pathway through COUP-TFII that can be induced by SHH[38] may also exert an effect on the regulation of angiogenic factors. However, we and others have identified that strong induction was observed 24 h after stimulation by SHH, suggesting that transcriptional up-regulation is unlikely. Thus, the hedgehog pathway may mediate its effect on angiogenesis by interacting with other signals. Recently, the role of the PI3K/Akt pathway was identified as a potential downstream signaling pathway of SHH; inhibition of PI3K almost completely blocked the induction of VEGF by *Shh* in BM-EPC,[37] although the link between SHH and PI3K is still unknown. Further studies are required to fully understand the molecular events during SHH-mediated angiogenesis.

Activation of the SHH signaling pathway is crucial not only for aberrant proliferation of cancer cells but also for the angiogenic response. We have demonstrated that SHH derived from pancreatic cancer cells can promote the development of the tumor vasculature in a paracrine manner, and this is mediated by an effect on EPC specifically. Several compounds targeting SHH have been tested *in vivo* to block tumor growth but these reagents would alternatively be a promising approach to control EPC-mediated tumor angiogenesis.

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Supplementary Material

The following supplementary material is available for this article:

1 Characterization of cultured endothelial progenitor cells derived from peripheral blood. (a) Morphology of cultured human endothelial progenitor cells (EPC). Spindle-shaped attached cells were observed by culturing peripheral blood–mononuclear cells (peripheral blood-MNC) for 4–7 days in a human fibronectin-coated dish. Cluster formation similar to a blood island was occasionally seen. (b) More than 95% attached cells are positive for both DiI-acLDL uptake (red) and fluorescein-isothiocyanate-Ulex-lectin binding (green). (c) Flow cytometric analysis of attached cells (EPC). Representative histograms from six independent experiments are shown. Attached cells were positive for CD31 ($55.7 \pm 7.7\%$), CD105 ($90.4 \pm 3.1\%$), and CD34 ($19.8\% \pm 5.2\%$) but not for c-kit ($2.3 \pm 2.1\%$) shown as filled histograms (open histograms; isotype control).

2 Aberrant expression of the patched homolog 1 (PTCH1) in tumor-associated endothelial cells. Co-localization of PTCH1 (green) and CD31 (red) immunostaining in human pancreatic cancer specimens was demonstrated by 2–3 μm thick confocal images (middle panel; magnified view dashed area is in right panel). No PTCH1 staining was observed in in normal pancreatic tissues (left panel). Arrow heads indicate PTCH1 staining in tumor associated endothelial cells. Scale bar, 20 μm.

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