PDGF-BB modulates hematopoiesis and tumor angiogenesis by inducing erythropoietin production in stromal cells

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The platelet-derived growth factor (PDGF) signaling system contributes to tumor angiogenesis and vascular remodeling. Here we show in mouse tumor models that PDGF-BB induces erythropoietin (EPO) mRNA and protein expression by targeting stromal and perivascular cells that express PDGF receptor-β (PDGFR-β). Tumor-derived PDGF-BB promoted tumor growth, angiogenesis and extramedullary hematopoiesis at least in part through modulation of EPO expression. Moreover, adenoviral delivery of PDGF-BB to tumor-free mice increased both EPO production and erythropoiesis, as well as protecting from irradiation-induced anemia. At the molecular level, we show that the PDGF-BB–PDGFR-β signaling system activates the EPO promoter, acting in part through transcriptional regulation by the transcription factor Atf3, possibly through its association with two additional transcription factors, c-Jun and Sp1. Our findings suggest that PDGF-BB–induced EPO promotes tumor growth through two mechanisms: first, paracrine stimulation of tumor angiogenesis by direct induction of endothelial cell proliferation, migration, sprouting and function of tumor blood vessels. Such vascular alterations can eventually lead to antiangiogenic drug resistance.¹,⁴,⁵

The stroma, which constitutes a large portion of various solid tissues and organs, consists of extracellular matrix, vascular cells and mesenchymal cells, including fibroblasts, myofibroblasts and inflammatory cells.¹⁵–¹⁸ The tumor stromal compartment contributes to tumor growth by stimulating neovascularization.¹⁹ PDGF-BB (a dimer of the PDGF-B chain), a multifunctional member of the PDGF family, signals through the receptors PDGFR-α and PDGFR-β.¹⁰ PDGF-BB stimulates angiogenesis in vivo and in tumors.¹¹–¹³ PDGFR-β is a tyrosine kinase receptor localized to the plasma membrane that is abundantly expressed in stromal and mural cells, including fibroblasts, myofibroblasts, pericytes and VSMCs.¹³,²⁴ In addition to affecting angiogenesis, PDGF-BB also has marked effects on vascular remodeling, maturation and stability by recruiting pericytes and VSMCs to newly formed angiogenic vessels. Genetic deficiency of PDGF-BB or PDGFR-β leads to early embryonic lethality caused by depletion of pericytes and vascular leakage.²⁵,²⁶

EPO, a hormone that stimulates erythropoiesis, is produced in fetal liver and in the kidneys of adults.²⁷ EPO expression is elevated during tissue hypoxia through activation of the hypoxia-inducible factor-1α (HIF-1α) transcription factor pathway.²⁸ In addition to stimulation of erythropoiesis, EPO has been reported to promote angiogenesis, vascular stability and endothelial cell survival.²⁹ Extramedullary hematopoiesis associated with hepatosplenomegaly is often seen in

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individuals with various types of cancer^{10,31}, and high expression of EPO and its receptor is associated with poor survival in these individuals^{32}. In this study, we show that PDGF-BB modulates extramedullary hematopoiesis and angiogenesis through activation of EPO production in the stromal compartment.

RESULTS
PDGF-BB in stromal expansion, angiogenesis and tumor growth

We evaluated the ability of PDGF-BB to promote tumor growth, stromal expansion and angiogenesis by overexpressing PDGF-BB in subcutaneous tumors using mouse T241 fibrosarcoma and Lewis lung carcinoma (LLC) cell lines. In T241 tumors, PDGF-BB expression significantly accelerated the tumor growth rate compared to tumors transfected with control vector (hereafter called vector tumors) (Fig. 1a).

A histological analysis showed that T241 tumors overexpressing PDGF-BB (hereafter called PDGF-BB tumors) contained a high proportion of stroma, which infiltrated the area between tumor cells and cells expressing PDGFR-β (Fig. 1b, arrows). We estimated that >50% of the T241 PDGF-BB tumor tissue consisted of stroma. In contrast, vector tumors contained only a modest amount of stromal tissue (Fig. 1b and Supplementary Fig. 1e). Additionally, T241 PDGF-BB tumors also had a high density of α-smooth muscle actin (α-SMA)-positive structures, which probably correspond to myofibroblasts distributed among the tumor cells (Fig. 1b).

We co-stained cells expressing PDGFR-β with several cell-type-specific markers; platelet/endothelial cell adhesion molecule (CD31), isoelectinB4 or perfused dextran, marking vascular endothelial cells; chondroitin sulfate proteoglycan 4 (NG2), marking pericytes; and α-SMA, marking VSMCs and myofibroblasts. In addition to the observed stromal tissue expansion in T241 PDGF-BB tumors, the density of the microvessels was significantly greater in PDGF-BB compared to vector tumors, suggesting that PDGF-BB contributes substantially to tumor angiogenesis (Fig. 1c,d). PDGFR-β was expressed in stromal cells, myofibroblasts and subpopulations of pericytes and VSMCs but was not localized in T241 tumor cells (Fig. 1c).

A quantitative analysis confirmed that T241 PDGF-BB tumors contained a significantly higher density of PDGFR-β+ cells than did vector tumors (Fig. 1e).

Tumor-derived PDGF-BB induces extramedullary hematopoiesis

Necropsy revealed that mice with T241 PDGF-BB tumors showed marked splenomegaly and hepatomegaly compared to mice with vector tumors (Supplementary Fig. 1a,c), suggesting that tumor-derived PDGF-BB enters the circulation to induce a systemic effect. Circulating amounts of PDGF-BB were significantly higher in mice with T241 PDGF-BB compared to vector tumors (Fig. 1f). Notably, a histological analysis showed that hematopoietic foci were often present in the livers of mice with PDGF-BB tumors (Fig. 1g) along with a marked expansion of red pulp in the spleen, which eventually became indistinguishable from white pulp (Fig. 1h). Similarly, implantation of LLC PDGF-BB tumors resulted in hepatosplenomegaly and increased extramedullary hematopoiesis in the liver and spleen (Supplementary Fig. 1f). However, the liver and spleen returned to the sizes observed in healthy mice after surgical removal of established T241 PDGF-BB tumors (Supplementary Fig. 1c), showing that the presence of tumors is required to induce hepatosplenomegaly. Similarly, the number of
Extramedullary hematopoiesis and expression of PDGFRs in the stromal compartment. (a–c) Single-cell suspensions from liver (a), spleen (b) and bone marrow (c) of mice with PDGF-BB or vector tumors were double stained with PE-Ter119 and DRAQ5, followed by FACS analysis. The cell population with high Ter119 and DRAQ5 staining represents erythroblasts, the cell population with low Ter119+ and DRAQ5+ staining represents reticulocytes, and the cell population with high Ter119 and no DRAQ5 staining represents erythrocytes. A representative FACS plot from each group and a quantification of the number of erythroblasts compared to the total number of Ter119+ cells is shown (n = 4 per group). The numbers in red indicate the percent of the total cell population. (d–f) Single-cell suspensions from liver (d), spleen (e) and bone marrow (f) from mice with PDGF-BB or vector tumors were cultured with colony-forming assay media to detect BFU-Es, mature BFU-Es, CFU-Es and CFU-GMs. Quantification of the number of colonies is shown (n = 3 per group). (g,h) Liver and spleen tissue sections from mice with PDGF-BB or vector tumors were double stained with antibody specific to PDGFR-β (g, red) or antibody specific to PDGFR-α (h, red) and DAPI (blue). (i) Quantification of PDGFR-β+ signals in the liver (n = 8 per group) (×20 magnification). Data are means ± s.e.m.

Figure 2 Extramedullary hematopoiesis and expression of PDGFRs in the stromal compartment. (a–c) Single-cell suspensions from liver (a), spleen (b) and bone marrow (c) of mice with PDGF-BB or vector tumors were double stained with PE-Ter119 and DRAQ5, followed by FACS analysis. The cell population with high Ter119 and DRAQ5 staining represents erythroblasts, the cell population with low Ter119+ and DRAQ5+ staining represents reticulocytes, and the cell population with high Ter119 and no DRAQ5 staining represents erythrocytes. A representative FACS plot from each group and a quantification of the number of erythroblasts compared to the total number of Ter119+ cells is shown (n = 4 per group). The numbers in red indicate the percent of the total cell population. (d–f) Single-cell suspensions from liver (d), spleen (e) and bone marrow (f) from mice with PDGF-BB or vector tumors were cultured with colony-forming assay media to detect BFU-Es, mature BFU-Es, CFU-Es and CFU-GMs. Quantification of the number of colonies is shown (n = 3 per group). (g,h) Liver and spleen tissue sections from mice with PDGF-BB or vector tumors were double stained with antibody specific to PDGFR-β (g, red) or antibody specific to PDGFR-α (h, red) and DAPI (blue). (i) Quantification of PDGFR-β+ signals in the liver (n = 8 per group) (×20 magnification). Data are means ± s.e.m.

hematopoietic foci in the liver and the separation of white pulp and red pulp in the spleen were also normalized after T241 PDGF-BB tumor removal (Supplementary Fig. 1b–d). In addition, treatment with imatinib (STI571), a tyrosine kinase inhibitor of PDGFR-β, reversed the hematopoietic phenotype induced by T241 PDGF-BB tumors (Supplementary Fig. 1b–d).

We next asked whether erythroblasts in hematopoietic foci were actively proliferating. We double immunostained tissue sections from liver and spleen with Ter119 and Ki67 to define the proliferating erythroblast population. Indeed, we found a significantly higher number of Ki67+ proliferating erythroblasts in the hematopoietic foci of livers from mice with T241 PDGF-BB tumors compared to mice with vector tumors (Fig. 1g,i). We also observed a significant increase of Ter119+Ki67+ hematopoietic populations in the spleens of mice with PDGF-BB tumors (Fig. 1h,j), indicating that tumor-derived PDGF-BB induces active extramedullary hematopoiesis.

We next carried out a fluorescence-activated cell sorting (FACS) analysis on liver, spleen and bone marrow to further characterize the red blood cell (RBC) precursors present in tumor-bearing mice. FACS analysis showed that the number of DRAQ5+Ter119+ RBC precursors in spleen and liver was significantly increased in mice with T241 PDGF-BB tumors compared to those with vector tumors (Fig. 2a,b).

In contrast, we found no significant difference in the numbers of RBC precursors in bone marrow (Fig. 2c).

An immunohistochemical analysis of liver tissue showed that the number of CD45+ myeloid cells (where CD45 is a specific marker...
of myeloid cells) was significantly \( P < 0.01 \) increased in mice with
t241 PDGF-BB tumors compared to those with vector tumors
(Supplementary Fig. 2a,b), suggesting that PDGF-BB–induced
hematopoiesis is not limited to erythropoiesis. In support of this view,
we detected a significantly elevated number of erythroid burst
forming units (BFU-Es), mature BFU-Es, erythroid colony forming units
(CFU-Es) and granulocyte-macrophage colony-forming units (CFU-
GMs) in the liver and spleen of mice with t241 PDGF-BB tumors
compared to those with vector tumors (Fig. 2d,e). In contrast to the
liver and spleen, numbers of BFU-Es, mature BFU-Es, CFU-Es and
CFU-GMs were unchanged in the bone marrow of mice with t241
PDGF-BB tumors compared to those with vector tumors (Fig. 2f),
suggesting that PDGF-BB preferentially stimulates extramedullary
hematopoiesis rather than bone marrow hematopoiesis.

**PDGF-BB protects against tumor-induced anemia**

To study the functional consequences of PDGF-BB–induced
extramedullary hematopoiesis, we measured RBC numbers, hemoglobin

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**Figure 3** Elevation of plasma EPO concentrations and transcriptional regulation of EPO expression by PDGF-BB. (a) Morphology of S17 cells stimulated or not with PDGF-BB that were double stained with DAPI and antibody specific to PDGFR-a or antibody specific to PDGFR-b. (b) Plasma concentrations of EPO in tumor-free mice or mice with t241 PDGF-BB, PDGF-BB LLC, or control vector tumors \( n = 6–8 \) per group. (c) Detection by immunoblotting of EPO protein in S17 cells treated with PDGF-BB or not treated. (d) Relative fold induction of EPO promoter activity in S17 cells by PDGF-BB treatment in the presence or absence of an antibody specific to PDGFR-b (Anti-R-b) or ST1571 (left); PDGF-AA or PDGF-BB (center); or siRNAs targeting Pdgfra (siPdgfra) or Pdgfb (siPdgfb) (right) \( n = 4 \) per group. EPOp-Luc, plasmid construct. (e,f) Gene array experiments were performed on S17 cells treated with PDGF-BB or buffer (n = 3 per group, and expression scale bars are shown on the top). (g) Data for the top ten most upregulated and downregulated transcription factor mRNAs. (g) Expression of Atf3, Klf5, Jun and Sp1 mRNAs in S17 cells treated with PDGF-BB or buffer quantified by qRT-PCR \( n = 3 \) per group. (h) EMSA analysis showing a lack of direct binding of Atf3 to the Epo promoter. A known Atf3-bound promoter (Ssf) served as a positive control. NS, nonspecific fragment. (i) Left, relative fold induction of Epo promoter activity by PDGF-BB in the presence or absence of siRNAs targeting Atf3 (siAtf3), Klf5 (siKlf5), Jun (siJun) or Sp1 (siSp1). Middle, detection of the Epo promoter binding activity of Sp1, c-Jun and Atf3 transcription factors by ChIP assay. Control, scramble DNA (a nucleotide of the same length with a random sequence). Right, relative fold induction of Epo promoter activity by PDGF-BB or by Atf3, and the effects of the indicated siRNAs on Atf3-mediated induction \( n = 3 \) per group. pAtf3, plasmid construct. (j) A diagram showing possible molecular mechanisms by which PDGF-BB induces EPO expression. See text for details. Data are means ± s.e.m.
concentration and hematocrits in the peripheral blood of tumor-bearing mice. Mice with T241 vector tumors had an anemic phenotype, with significant (P < 0.05) decreases in the average value of RBCs, average concentrations of hemoglobin and hematocrit compared to mice with PDGF-BB tumors (Supplementary Table 1). Notably, mice with T241 PDGF-BB tumors showed significant (P < 0.05) improvement in these parameters compared to mice with vector tumors (Supplementary Table 1). Moreover, the concentration of white blood cells was increased in mice with T241 PDGF-BB tumors compared to mice with vector tumors. However, the concentration of platelets was not significantly altered in mice with T241 PDGF-BB tumors compared to mice with vector tumors (Supplementary Table 1). These findings show that tumor-derived PDGF-BB substantially increases hematocrit, leading to an improvement of tumor-associated anemia.

We next tested whether PDGF-BB–induced erythropoiesis involves the BMP4-mediated stress erythroid response. BMP4 expression was significantly (P < 0.05) increased in the liver and spleen of mice with T241 PDGF-BB tumors compared to those with vector tumors (Supplementary Fig. 2c,d). Additionally, treatment of cell suspensions derived from spleen and liver of mice bearing T241 PDGF-BB tumors with BMP4 and Epo significantly (P < 0.05) increased the number of BFU-E colonies compared to treatment with Epo alone, whereas BMP4 did not have this effect on cell suspensions from spleen and liver of mice bearing vector tumors (Supplementary Fig. 2e,f). These data suggest that BMP4 may, in part, be involved in mediating the PDGF-BB–induced erythroid response.

Expression of PDGFRs in the liver and spleen

In the liver and spleen of mice bearing T241 PDGF-BB tumors, PDGFR-α and PDGFR-β were not expressed in hepatocytes or splenocytes, but were primarily expressed in the stromal compartment (Fig. 2g–i). In the liver, PDGFR-β* signals did not co-localize with albumin, a parenchymal marker expressed by hepatocytes (Supplementary Fig. 3a). Rather, PDGFR-β* signals were co-localized with α-SMA* (marking VSMCs and myofibroblasts) and NG2* cells (marking pericytes; Supplementary Fig. 3b,c). Although the expression of PDGFR-α in the liver did not differ markedly between mice with PDGF-BB tumors and those with vector tumors (Fig. 2g and data not shown), the amount of stromal PDGFR-β staining was more than ninefold higher in the liver of mice with PDGF-BB tumors compared to those with vector tumors (Fig. 2i). In the spleen, we observed PDGFR-β* signals exclusively in the red pulp area (Fig. 2g), consistent with the idea that PDGFR-β is actively involved in regulating extramedullary hematopoiensis.

We further validated that PDGFR-α and PDGFR-β are expressed in stromal cells using two stromal fibroblast cell lines, MS-5 and S17 (ref. 34). Addition of PDGF-BB to both types of cells resulted in marked morphological changes: the cells became elongated and had spindle-like fibers (Fig. 3a and Supplementary Fig. 3d). Consistent with the in vivo findings, these cells had high levels of PDGFR-α and PDGFR-β expression (Fig. 3a and Supplementary Fig. 3d).

PDGFR-β–dependent EPO promoter activity in stromal cells

To study the mechanisms underlying PDGF-BB–induced extramedullary hematopoiensis, we measured circulating EPO concentrations. EPO concentrations were increased more than threefold in the plasma...
of mice with T24I or LLC PDGF-BB tumors compared to mice with the corresponding vector tumors (Fig. 3b), suggesting that PDGF-BB upregulates EPO expression. Because PDGFRs are expressed in the stromal compartment and in perivascular cells such as pericytes and VSMCs in both the liver and spleen (Fig. 2g.h and Supplementary Fig. 3b,c), it is unlikely that PDGF-BB acts directly on hepatocytes or splenocytes to induce EPO expression. PDGF-BB markedly increased the amount of EPO protein in S17 stromal cells, as assessed by immunoblotting using an antibody specific to mouse EPO (Fig. 3c).

We next studied the molecular mechanisms by which PDGF-BB induces EPO expression in the stromal compartment. We constructed a full-length mouse EPO promoter upstream of the firefly luciferase reporter gene and transfected this reporter gene construct into S17 stromal cells. Stimulation of the transfected cells with PDGF-BB resulted in a nearly threefold increase of luciferase activity compared to buffer-treated controls (Fig. 3d), suggesting that PDGF-BB acts on target cells to activate EPO gene transcription. Treatment with STI571 or a PDGFR-β–specific antibody significantly attenuated transcriptional activation of the EPO promoter (Fig. 3d). Stimulation with PDGF-AA, which binds specifically to PDGFR-α, did not significantly induce EPO promoter activity (Fig. 3d). To further elucidate the roles of PDGFR-α and PDGFR-β in the transcriptional activation of EPO, we used siRNAs specific to each of the two receptors. Consistent with the data obtained using PDGF-AA, incubation of S17 cells with siRNA targeting PDGFR-β, but not siRNA targeting PDGFR-α, significantly inhibited PDGF-BB–induced EPO promoter activity (Fig. 3d and Supplementary Fig. 3e). Taken together, these results show that PDGF-BB transcriptionally induces EPO promoter activity through PDGFR-β but not PDGFR-α in stromal cells.

**Atf3 mediates PDGF-BB–induced EPO expression**

To determine the signaling pathways involved in the regulation of EPO expression by PDGF-BB, we performed an Affymetrix gene array analysis. Gene cluster and principal component analyses showed that the gene expression profiles were highly reproducible in three independent buffer-treated and three independent PDGF-BB–treated samples. We observed both genes that were upregulated and downregulated by PDGF-BB (Fig. 3e,f). We focused on transcription factors that were upregulated by PDGF-BB to study their potential role in activating EPO promoter activity. Because we identified Klf5 (encoding the transcription factor Klf5) and Atf3 (encoding the transcription factor Atf3) as highly upregulated (Fig. 3e), we further measured their expression by quantitative real-time PCR (qRT–PCR), which showed an approximately 70-fold increase of Atf3 and an eightfold increase of Klf5 in PDGF-BB–stimulated compared to buffer-treated stromal cells (Fig. 3g).

To study the effects of Atf3 and Klf5 on the regulation of EPO expression, we designed specific siRNA probes. In stromal cells transfected with the EPO promoter–luciferase reporter construct, Atf3 siRNA treatment significantly attenuated PDGF-BB–induced reporter activity (Fig. 3i), showing that Atf3 mediates the transcriptional upregulation of PDGF-BB–stimulated EPO expression.

**Figure 5** Anti-tumor and antiangiogenic activity, systemic impact of EPO or PDGFR antagonism and the direct effects of EPO on endothelial cells. (a) Growth rate of PDGF-BB tumors treated with either buffer, STI571, an antibody specific to PDGFR-β or an antibody specific to PDGFR-α (n = 8 per group). (b) Plasma EPO concentrations (left) and the weights of liver (center) and spleen (right) from mice with PDGF-BB tumors treated with the indicated agents (n = 8 per group). Anti-Rα, antibody specific to PDGFR-α; Anti-Rβ, antibodies specific to PDGFR-β. (c) Growth rate of PDGF-BB tumors treated with buffer or an antibody specific to EPO (n = 8 per group). (d) Images and weights of tumors, livers and spleens treated with buffer or an antibody specific to EPO (n = 8 per group).

(e) Left, tumor microvessels in mice with PDGF-BB tumors treated with an antibody specific to EPO or with buffer as visualized using CD31 + endothelial cell signals (red) and EGFP + tumor cells (green). Right, quantification of tumor blood vessel area (×20 magnification, n = 8 per group). (f) Left, tumor microvessels in mice with vector tumors treated with an antibody specific to EPO or with buffer as visualized using CD31 + endothelial cell signals (red) and EGFP + tumor cells (green). Top right, growth rate of vector tumors treated with an antibody specific to EPO or with buffer. Bottom right, quantification of tumor microvessel area (×20 magnification, n = 8 per group). (g,h) Proliferation (g) and migration (h) of mouse brain endothelial cells (EC) induced by EPO protein treatment (n = 6–8 per group). IU, international unit; A490, absorption at 490 nm. (i) Stimulation of endothelial cell tube formation by EPO. Quantification of the vessel-like tube area formed by endothelial cells per field (n = 8 per group) (×20 magnification). (j) Stimulation of vessel sprouting from mouse aortas by EPO. Quantification of the number of endothelial cell sprouts (n = 6 per group). Data are means ± s.e.m.
Conversely, treatment with Kif5 siRNA did not affect the promoter activity of EPO (Fig. 3i and Supplementary Fig. 3e). Sequence analysis of the EPO promoter region did not reveal any Atf3 consensus-binding sites, suggesting that Atf3 may not act directly on the EPO promoter. Indeed, an electrophoretic mobility shift assay (EMSA) did not provide evidence for direct binding of recombinant Atf3 to total erythrocytes (g) and RBC concentrations, hemoglobin concentrations and hematocrits (h) of sublethally irradiated tumor-free SCID mice that received AdPDGF-BB or control vehicle. The mice were analyzed on days 3, 5, 8 and 18 after irradiation (n = 6 per group). NI, non irradiated. Data are means ± s.e.m. (i) Mechanisms of tumor-derived PDGF-BB–induced hematopoiesis, tumor growth and angiogenesis. Tumor-derived PDGF-BB enters the circulation (endocrine) to target PDGFR-β and angiogenesis. PDGF-BB induces stromal EPO expression and activates extramedullary hematopoiesis. Elevated numbers of RBCs may promote tumor growth by decreasing hypoxia in tumors. EPO also protects the host against tumor-associated anemia. In addition to stimulation of hematopoiesis, EPO directly induces tumor angiogenesis. Enhanced hematopoiesis could potentially supply circulating endothelial progenitor cells to the growing tumor vasculature. Hyper-\( \text{O}_2 \) blood, blood with an increase in oxygen level.

**PDGF-BB induces stromal EPO expression in vivo**

Because EPO is almost exclusively produced in the kidneys of adults, we first evaluated EPO mRNA expression in kidneys by in situ hybridization. We used two probes, one directed against exon 3 and the other against exon 5 of the mouse Epo gene. Kidney EPO mRNA levels were increased only slightly in mice with T241 PDGF-BB tumors compared to those with vector tumors (Fig. 4a–c). In contrast, in situ detection showed that the expression levels of Epo mRNA were significantly elevated in both liver and spleen from mice with T241 PDGF-BB tumors compared to those with vector tumors (Fig. 4a–c). These findings show that upregulation of EPO production
in tumor-bearing animals occurs primarily in extramedullary sites, including the liver and spleen. Consistent with these results, qRT-PCR analysis showed that EPO mRNA levels in the liver and spleen from mice with T241 PDGF-BB tumors were significantly elevated compared to those with vector tumors (Fig. 4d). In the kidneys, there was a slight increase in EPO expression, which was not statistically significant.

As shown by immunohistochemistry, EPO+ cells were present in PDGFR-β+ stromal tissue of tumors, spleen and liver from mice with T241 PDGF-BB tumors (Fig. 4). To further define the cellular source of EPO, we used FACS to sort cells from liver and spleen into PDGFR-β+ (mice with T241 PDGF-BB tumors) and PDGFR-β− (mice without tumors) fractions. As expected, the amount of EPO mRNA in the PDGFR-β+ cell fraction in both liver and spleen was significantly higher than that in the corresponding PDGFR-β− cell fraction (Fig. 4e). In the kidney, EPO-producing cells appeared to correspond to peritubular interstitial cells (PICs), which are known to have high expression of EPO (Fig. 4a). In a further immunohistochemical analysis, EPO-producing cells in both tumor and non-tumor tissue of mice with T241 PDGF-BB tumors co-localized with α-SMA, vimentin, CD73, endomucin and desmin (Supplementary Fig. 4). In the spleen and liver, co-localization of a subset of EPO+ signals with α-SMA suggested that VSMCs or myofibroblasts might be involved in EPO production (Supplementary Fig. 4a,b). In tumor tissue, however, EPO-producing cells were not generally associated with α-SMA or desmin expression (Supplementary Fig. 4d). Notably, we detected CD73+ signals (a marker of PICs) in the kidney but not in other tissues (Supplementary Fig. 4), indicating that EPO-producing cells in non-kidney tissues do not correspond to PICs.

Taken together, these results further confirm that EPO expression is confined to the stromal compartment in both malignant and non-malignant tissues, and that stromal cells, rather than hepatocytes or splenocytes, are the source of PDGF-BB–induced EPO production. Moreover, EPO-producing cells in various tissues may represent different cell types and may not be directly related to PICs, the cell type that produces EPO in the kidney.

PDGFR-β in EPO maintenance and the role of hypoxia
To study whether the PDGFR-β-mediated signaling system is required for the physiological maintenance of EPO production in various tissues, we carried out an in vivo loss-of-function experiment. Global deletion of Pdgfrb in adult mice was achieved by crossing mice containing a Pdgfrblox/lox allele with transgenic mice containing a tamoxifen-inducible Cre construct30. Treatment of the resulting mice when they were 4 weeks old with tamoxifen resulted in deletion of Pdgfrb30. Expression of PDGFR-β mRNA in the spleen, liver and kidney was significantly (P < 0.01) reduced in these tamoxifen-treated mice compared to tamoxifen-treated wild-type (WT) mice (Fig. 4g). Notably, these mice showed a significant (P < 0.05) decrease in EPO mRNA in spleen but not in liver or kidney (Fig. 4h), indicating that the PDGFR-β–mediated signaling system is required for the physiological maintenance of EPO expression in the spleen, but not in the liver or kidney. Circulating EPO concentrations in the PDGFR-β–deleted mice and tamoxifen-treated WT mice were similar (Supplementary Fig. 3f), supporting the conclusion that the spleen is not a major site for EPO production under physiological conditions.

As it has previously been shown that tissue hypoxia can upregulate EPO expression through activation of the HIF-1α transcription factor pathway37, we considered the possibility that hypoxia in various tissues and organs in mice with PDGF-BB tumors might contribute to the upregulation of EPO expression. We analyzed tumor and non-tumor tissue sections for tissue hypoxia using a standard hypoxia probe, but observed no clear differences in tumor, liver or spleen tissue between mice with T241 PDGF-BB tumors compared to those with vector tumors (Supplementary Fig. 5). This finding suggests that tissue hypoxia does not substantially contribute to PDGF-BB–mediated EPO upregulation, although the role of tissue hypoxia in this regulation warrants further investigation.

EPO in tumor growth, angiogenesis and hematopoiesis
The finding that PDGF-BB stimulates EPO production suggests that EPO might mediate some of the in vivo effects of PDGF-BB. A PDGFR-β–specific antibody, but not a PDGFR-α–specific antibody, significantly (P < 0.05) attenuated PDGF-BB–stimulated T241 tumor growth (Fig. 5a) and tumor neovascularization (Supplementary Fig. 6a,b). Treatment with STI571 or a PDGFR-β–specific antibody resulted in a reduction of plasma EPO concentrations compared to treatment with buffer (Fig. 5b), consistent with our previous results that activation of the PDGFR-β signaling pathway is essential for upregulation of EPO expression in stromal cells. Notably, the PDGFR-β–specific antibody had a more potent inhibitory effect than did STI571, which could be explained by the observation that STI571 had a tendency to increase circulating EPO concentrations in tumor-free mice, as we also saw using other antiangiogenic agents (Supplementary Fig. 6d). Because STI571 targets not only PDGFRs but also several other kinases, it is possible that other kinase-mediated pathways, including those mediated by the tyrosine kinase Abl, are involved in the regulation of EPO expression. In addition to inhibiting tumor growth, treatment with STI571 or with PDGFR-β–specific antibody normalized liver and spleen size and weight in mice with PDGF-BB tumors, but treatment with PDGFR-α–specific antibody did not (Fig. 5b). These findings show that PDGFR-β is responsible for the accelerated tumor growth rate and hepatosplenomegaly induced by PDGF-BB overexpression.

Similar to the effects of treatment with PDGFR-β–specific antibody, treatment with an antibody specific to mouse EPO38 also significantly inhibited PDGF-BB tumor growth (Fig. 5c,d) and angiogenesis (Fig. 5e) and prevented PDGF-BB–induced splenomegaly and hepatomegaly (Fig. 5d). However, treatment of mice with vector tumors with the EPO-specific antibody did not significantly inhibit tumor growth or angiogenesis (Fig. 5f), indicating that the suppression of tumor growth by EPO blockade does not result from global anemia. To further delineate the direct angiogenic activity of EPO, we tested the effects of EPO protein on primary endothelial cells. Treatment with EPO protein significantly stimulated endothelial cell proliferation, cell migration and tube formation in a concentration-dependent manner (Fig. 5g–i). Similarly, EPO significantly (P < 0.05) induced endothelial cell sprout formation in the aortic ring assay (Fig. 5j). These findings support the notion that EPO may act directly on endothelial cells to promote angiogenesis.

Adenoviral PDGF-BB increases EPO levels and hematopoiesis
To exclude the possibility that tumor-derived factors other than PDGF-BB are involved in EPO upregulation and in promoting hematopoiesis, we tested the effects of PDGF-BB delivery alone. We made use of an adenovirus vector expressing PDGF-BB (AdPDGF-BB)39, which we injected into severe combined immunodeficiency (SCID) mice. We intravenously injected approximately 1 × 108 particles of AdPDGF-BB or an adenovirus vector (AdVector) into each mouse. Because adenoviruses preferentially infect hepatocytes in vivo, effects on the liver might be difficult to interpret, and we therefore focused...
on splenomegaly as the phenotype of interest. Similar to tumor-derived PDGF-BB, delivery of AdPDGF-BB resulted in splenomegaly (Fig. 6a,b), a significant increase in the concentration of circulating EPO (Fig. 6c), increased concentrations of RBCs and hemoglobin, and an increase in hematocrit (Fig. 6d). Notably, administration of ST1571 significantly attenuated splenomegaly and reduced the concentrations of RBCs and hemoglobin (Fig. 6d), suggesting that PDGFR-mediated upregulation of EPO is responsible for these effects. Similarly, PDGFR-β-specific antibody (Fig. 6a–d), but not PDGFR-α-specific antibody (Supplementary Fig. 7a–c), markedly reduced splenomegaly, circulating EPO concentrations and the values of RBCs in AdPDGF-BB–treated mice. Circulating PDGF-BB concentrations in the adenovirus model is comparable to those seen in the tumor model (Supplementary Fig. 7d). However, treatment with an EPO-specific antibody resulted in only a slight decrease in the concentrations of RBCs and hemoglobin (Fig. 6d). These relatively small effects might be due to the long life span of RBCs (approximately 40 d) compared to the duration of treatment (14 d). In support of this view, treatment of WT mice with EPO-specific antibodies did not significantly affect circulating concentrations of RBCs or hemoglobin or hematocrits (Supplementary Fig. 7h). Analysis of DRAQ5+Ter119+ cell populations by FACS showed that the number of RBC precursors in the spleen was significantly (P < 0.05) increased at day 14 after delivery of AdPDGF-BB compared to control mice with AdVector (Supplementary Fig. 7f), indicating that AdPDGF-BB triggers activation of splenic erythropoiesis. Similar to the results seen in mice with PDGF-BB tumors, the numbers of splenic BFU-Es, mature BFU-Es, CFU-Es and CFU-GMs were significantly (P < 0.05) increased in AdPDGF-BB–treated mice compared to those treated with AdVector (Supplementary Fig. 7e).

To test whether delivery of AdPDGF-BB could protect against irradiation-induced hematopoietic suppression, we irradiated tumor-free SCID mice at a sublethal dose and then injected them with AdPDGF-BB (Fig. 6e–h). We used non-irradiated mice as controls. At day 3 after irradiation, we observed anemia in the irradiated mice: the concentrations of circulating RBCs and hemoglobin and the hematocrits in these mice were significantly decreased compared to those in non-irradiated mice (Fig. 6h). Irradiated mice that received AdPDGF-BB showed increased concentrations of circulating RBCs and hemoglobin and hematocrits compared to the control group (Fig. 6h). Consistent with the improved numbers of RBCs and hemoglobin concentration, the number of peripheral erythroblasts was also significantly increased in these mice (Fig. 6g). A time-course analysis showed that AdPDGF-BB–treated mice recovered faster from irradiation-induced anemia than did control mice (Fig. 6g,h). Notably, AdPDGF-BB significantly (P < 0.01) upregulated the expression of BMP4 in the spleen (Supplementary Fig. 7g), which is involved in erythroid radioprotection through a stress–triggered mechanism. These data suggest that BMP4-dependent stress erythropoiesis is required for the recovery of erythropoiesis induced by AdPDGF-BB in irradiated mice. Taken together, these results show that PDGF-BB administration can protect irradiated mice from anemia, consistent with its ability to stimulate extramedullary hematopoiesis.

**DISCUSSION**

In this study, we delineate two EPO-mediated mechanisms of PDGF-BB–induced tumor growth (Fig. 6i). In a paracrine mechanism, PDGF-BB acts on stromal cells, pericytes or VSMCs that express the PDGF-BB receptor PDGFR-β to expand the stromal compartment and activate EPO expression, leading to enhanced tumor angiogenesis. In an endocrine mechanism, tumor-derived PDGF-BB enters the circulation to induce extramedullary hematopoiesis, which accelerates tumor growth by improving the oxygen and nutrient supply. At the molecular level, we show that the PDGF-BB–PDGFR-β signaling system activates the EPO promoter through induction of the Atf3 transcription factor in association with c-Jun and Sp1. Although our results indicate that the level of tissue hypoxia in tumor, liver and spleen is not altered in mice with tumors overexpressing PDGF-BB compared to those with control tumors, a role for hypoxia in EPO induction cannot be ruled out completely, as hypoxia has previously been reported to regulate Atf3 levels.

The finding that stromal cells, pericytes and VSMCs in tumors and other tissues express EPO in response to PDGF-BB is unexpected to us because under physiological conditions, EPO in adults is primarily produced in the kidney. Based on our current work and other previously published findings, it seems that EPO might contribute to tumor angiogenesis through four possible mechanisms (Fig. 6i): first, by direct induction of endothelial cell proliferation and migration; second, by decreasing vessel stability to further support tumor growth; third, stroma-derived EPO may enter the circulation and stimulate extramedullary hematopoiesis, which would further support tumor growth by improving the nutrient and oxygen supply; and fourth, by stimulating proliferation, differentiation and mobilization of endothelial progenitor cells, which participate in neovascularization. Additionally, the EPO receptor is expressed in certain types of tumor cells and, thus, might act directly on tumor cells.

The circulating concentrations of PDGF-BB (approximately 1 ng ml−1) observed in our preclinical models are similar to those found in individuals with cancer and hence are clinically relevant. For example, in individuals with glioblastoma, the average concentration of plasma PDGF-BB is 880 pg ml−1 (ref. 46). Elevated concentrations of plasma PDGF-BB have also been found in individuals with a wide range of other cancers, including lymphoma, sarcomas and epithelial cancers. Our preclinical findings suggest that PDGF-BB might also cause systemic effects in individuals with cancer. Indeed, a considerable number of these individuals suffer from hepatomegaly and splenomegaly that have unknown causes. PDGF-BB–induced extramedullary hematopoiesis may have a systemic impact on hematocrit in individuals with cancer. A substantial number of individuals with cancer suffer from bone-marrow–associated anemia, as also seen in our mouse tumor model. We showed that PDGF-BB–induced extramedullary hematopoiesis protects the host against the tumor-associated anemic phenotype. Considering that PDGF-induced elevation of hematocrit may improve the nutrient and oxygen supply in the tumor environment, leading to accelerated tumor growth, the possibility that high hematocrit levels are associated with accelerated tumor growth rates in individuals with cancer warrants further investigation.

Despite the clinical success of targeting the PDGF signaling system in cancer therapy, the underlying mechanisms of such therapy are not well understood. Activation of the PDGF signaling system often occurs as a compensatory switch to alternative angiogenic pathways that lead to resistance to current antiangiogenic therapy. For example, the PDGF signaling pathway may contribute to the acquisition of resistance to VEGF antibody therapy by activating tumor stromal cells. However, treatment with a combination of VEGF and PDGF-specific antibodies produced only modest antitumor activity. Similarly, deletion of host-derived PDGF-BB using a genetic approach did not increase the antitumor activity of a VEGF-specific
antibody.\textsuperscript{49,50} Thus, the concept of combination therapy targeting both VEGF and PDGF warrants further preclinical validation.

Our results demonstrate the complex interplay between angiogenic factors in the tumor environment. In light of the protumorigenic effects of EPO, we suggest that elevated EPO levels might confer resistance to the anti-tumor effects of angiogenic therapy, such as agents that target PDGF or VEGF pathways.\textsuperscript{51} EPO expression can be induced not only by PDGF but also by other factors or mechanisms involving PDGFR-β auto-activation. We anticipate that combinations of PDGF-specific and EPO-specific neutralizing agents would produce greater therapeutic benefits than would single-agent therapy and might be able to overcome the drug resistance associated with therapy using VEGF-specific antibodies. Taken together, our data define a previously unidentified mechanism of PDGF-BB–induced tumor angiogenesis and provide compelling evidence for the key role of EPO in mediating PDGF-BB–induced angiogenesis, tumor growth and hematopoiesis. Although we studied only PDGF-BB, similar regulatory mechanisms might also apply to other members of the PDGF family.

\section*{METHODS}

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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\section*{AUTHOR CONTRIBUTIONS}

Y.C. designed the study and wrote the manuscript. Y.X., S.L., K.H., Z.W., L.D.E.J., R.C. and E.-M.H. performed mouse experiments, as well as histological and immunohistological analyses. Y.X. and S.L. measured the plasma EPO concentrations by ELISA, measured the luciferase activity, performed hematological analyses and performed adenoviral analyses. S.L. performed radiation experiments. Y.X., Z.W. and S.L. cultured stromal cells in vitro assays. Y.Y. performed qRT-PCR, EMSA and ChIP assays. K.H. and Y.Y. performed FACS hybridization, and D.G. performed assays. S.L. cultured stromal cells for radiation experiments. Y.X., Z.W. and S.L. cultured stromal cells for ELISA, measured the luciferase activity, performed qRT-PCR, EMSA and ChIP assays. K.H. and Y.Y. performed FACS hybridization, and D.G. performed assays. Y.X. and O.L. analyzed the microarray data. M.S. provided the PDGFR-β knockout mice for this study.

\section*{COMPETING FINANCIAL INTERESTS}

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Female 6- to 8-week-old C57Bl/6 or SCID mice were bred, acclimated and caged in groups of six or fewer mice at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institute. C57Bl/6 mice were used in all tumor-related experiments, and SCID mice were used for the adenoivirus experiments. Mice were anesthetized by injection of a 1:1:2 mixture of hypnorm (Vetapharma), dormicium (Roche) and distilled water before all procedures and killed by a lethal dose of CO2, which was followed by cervical dislocation. PDGFR-β was depleted in C57 mice by estrogen receptor transgenic mice (University of Toyama, Japan) by oral administration of tamoxifen (Sigma-Aldrich; 9 mg per 40 g of body weight for 5 d consecutively) at 4 weeks of age. Pdgfrβfl/fl mice were treated identically and used as controls. After another 4 weeks, the mice were used for experimentation. The mouse studies were approved by the Northern Stockholm Experimental Animal Ethical Committee.

Affymetrix gene array analysis. S17 stromal cells were treated with or without 100 ng ml−1 PDGF-BB for 72 h, following which total RNA was extracted and fixed cultured cells on cover slips were incubated with specific antibodies to mouse EPO (anti-mEPO, R&D Systems) and a monoclonal antibody specific to mouse EPO (Anti-mEPO, R&D Systems Inc.) and reagents used. Immunofluorescent staining. Cryostat tissue sections (9 μm thick) and fixed cultured cells on cover slips were incubated with specific antibodies against CD31, Ki67, Ter119, ERα, PDGF-α or PDGF-β using standard immunohistochemical procedures. In the co-localization experiments, several combinations of antibodies, including those to CD31, Ki67, Ter119, EPO, PDGF-α or PDGF-β, were simultaneously incubated with the same tissue sample. See Supplementary Methods for details about the antibodies and reagents used.

Mouse tumor experimentation and treatment. Mouse fibrosarcoma T241 or LLC cell lines were transfected to overexpress enhanced green fluorescent protein (EGFP) and PDGF-BB or were transfected with empty vector only carrying EGFP, as previously described. Approximately 2 × 106 tumor cells transduced with vector or PDGF-BB were subcutaneously implanted on the dorsal back of each 6- to 8-week-old female C57Bl/6 mouse (n = 8 per group), and tumor sizes were measured as previously reported. In the PDGF-BB blockade experiments, an antibody specific to PDGFR-α (H3H) or an antibody specific to PDGFR-β (2CS) (ImmClone) was injected intraperitoneally (i.p.) (0.8 mg per mouse three times per week) into each tumor-bearing mouse (n = 8 per group). Antibody or drug administration began 5 d after tumor implantation. PBS was administered on the same schedule as a control. In some experiments, primary tumors were surgically removed when they reached ~1 cm3 in size, and the experiments were then continued for an additional 14 d. An antibody specific to EPO (EPO neutralizing antibody, R&D Systems; see Supplementary Methods) was i.p. administered to healthy mice or to mice with PDGF-BB or vector tumors (n = 8 per group) at a dose of 0.2 mg per kg of body weight three times weekly, with the first administration at day 5 after tumor implantation. In non-tumor bearing mice, ST1571 at a dose of 1 mg per mouse was i.p. administered daily for 10 d (n = 5). Blood and tissue samples were collected at the end of the experiments.