Overexpression of PDGF-BB decreases colorectal and pancreatic cancer growth by increasing tumor pericyte content

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We hypothesized that overexpression of PDGF-BB in colorectal cancer (CRC) and pancreatic cancer cells would result in increased pericyte coverage of ECs in vivo, rendering the tumor vasculature more resistant to antiangiogenic therapy. We stably transfected the cDNA for the PDGF-B into HT-29 human CRC and FG human pancreatic cancer cells. Surprisingly, when HT-29 or FG parental and transfected cells were injected into mice (subcutaneously and orthotopically), we observed marked inhibition of tumor growth in the PDGF-B–overexpressing clones. In the PDGF-B–overexpressing tumors, we observed an increase in pericyte coverage of ECs. Treatment of PDGF-B–overexpressing tumors with imatinib mesylate (PDGFR inhibitor) resulted in increased growth and decreased total pericyte content compared with those in untreated PDGF-B–overexpressing tumors. In vitro studies demonstrated the ability of VSMCs to inhibit EC proliferation by approximately 50%. These data show that increasing the pericyte content of the tumor microenvironment inhibits the growth of angiogenesis-dependent tumors. Single-agent therapy targeting PDGF receptor must be used with caution in tumors when PDGFR is not the target on the tumor cell itself.

Introduction
Angiogenesis is essential for tumor growth. Angiogenesis is a complex process involving not only EC activation but also crosstalk among multiple cell types (1, 2). ECs are the central cells in angiogenesis, yet recent data suggest that pericytes, periendothelial smooth muscle cells that modulate EC function, are also critical for the development of a mature vascular network (3).

Pericytes serve several important functions. Pericytes regulate vascular function, including vessel diameter (and thus blood flow) and vascular permeability (reviewed in refs. 4, 5). Pericytes also provide mechanical support and stability to the vessel wall and maintain EC survival through direct cell-cell contact and paracrine circuits (6, 7). Another key function of pericytes is to regulate (inhibit) EC growth in order to establish a stable and mature vascular network (8). Abnormalities in pericyte coverage of ECs have been implicated in various diseases. For example, diabetic retinopathy is characterized by detachment of pericytes from the ECs, leading to the development of microaneurysms and subsequent blindness (9).

The role of pericytes within the tumor vasculature is currently an area of intense study. The degree of pericyte coverage of ECs in human tumors is controversial. Benjamin et al. demonstrated that 38% of the vessels within human prostate cancer tumors were associated with pericytes compared with almost 75% in normal tissue (10). Similarly, in glioblastoma multiforme, only 19% of the tumor vessels had associated pericytes (10). Eberhard et al. found that the percentage of ECs associated with pericytes varied according to tumor type; there was 65% pericyte coverage in colon cancer and only 13% in glioblastoma (11). Conversely, Morikawa et al. found that pericytes are present on more than 97% of the vessels within tumors, possibly because of differences in the number of markers used and the thickness of tissue sections (12). Importantly, within many tumor types, pericytes show distinct abnormalities, such as decreased numbers associated with ECs and variable adherence to ECs.

Since pericytes play a role in mediating EC survival (13), they have recently emerged as an important therapeutic target for antiangiogenic therapy (14). Studies of antiangiogenic agents targeting EC survival have demonstrated that these agents result in increased apoptosis in ECs that are not associated with pericytes, leading to a relative increase in the proportion of vessels with pericyte coverage (10, 15). These data have led to the hypothesis that pericytes mediate resistance to antiangiogenic therapy. If this hypothesis is correct, targeting both ECs and pericytes would increase the efficacy of antiangiogenic therapy, and in fact, this hypothesis is supported by preclinical studies (14, 16).

The exact molecular mechanism mediating pericyte coverage and its biologic relevance in tumors are currently being investigated. In normal tissue, multiple mediators of pericyte proliferation, migration, and function have been described (reviewed in ref. 17). One important family of growth factors implicated in enhanced proliferation and migration of pericytes is the PDGF family (reviewed in ref. 18). There are 4 known PDGF polypeptides — PDGF-A, PDGF-B, PDGF-C, and PDGF-D — that can form disulfide-linked homodimers or heterodimers. These dimers are expressed by a multitude of different cell types, including activated platelets, macrophages, ECs, osteoclasts, and tumor cells. PDGFs interact with 2 tyrosine kinase receptors, PDGFRα and PDGFRβ. PDGFRβ, the receptor that is highly expressed on pericytes and VSMCs, is the primary receptor for the PDGF-BB homodimer. The interaction between PDGF-BB and PDGFRβ induces receptor activation, downstream signaling, VSMC and pericyte proliferation, and enhanced migration in vitro. Importantly, PDGF-B– and PDGFRβ-knockout mice show a similar phenotype characterized by embryonic-lethal defects such as a lack of pericytes or VSMCs.
leading to widespread hemorrhages and microaneurysms (19, 20). These data suggest the importance of PDGF-BB and PDGFRβ in mediating pericyte coverage in normal tissues during development. However, the effect of PDGF-BB on the vasculature, and specifically pericytes, in tumors is not as well understood.

Our initial hypothesis was that pericytes serve as barriers to antiangiogenic therapy that targets ECs. To test this hypothesis, we transfected HT-29 human colorectal cancer (CRC) and FG pancreatic cancer cells with a plasmid encoding the full-length gene for PDGF-B in an effort to increase pericyte coverage of ECs in tumors. However, in the course of this study, we observed some unexpected findings. Surprisingly, PDGF-BB overexpression significantly inhibited in vivo tumor growth both ectopically and orthotopically. Further analysis revealed that PDGF-BB–overexpressing tumors contained an increased proportion of cells that expressed pericyte markers. Confocal microscopy confirmed a high degree of pericyte coverage of ECs in PDGF-BB–overexpressing tumors. Our results suggest that pericytes, through regulation of EC proliferation, may inhibit the angiogenic process in vivo and thus inhibit tumor growth.

**Results**

Expression of PDGF ligands in control and transfected human cancer cells. Conditioned medium collected from human cancer cell lines (HT-29, KM12L4, KM12SM, GEO, RKO, and FG) after 72 hours of culture was evaluated for PDGF-AA, PDGF-AB, and PDGF-BB ligand expression levels by ELISA. There was no detectable PDGF-AA, PDGF-AB, or PDGF-BB in the conditioned medium from any of the cell lines listed above.

To study the effects of forced expression of PDGF-BB in cancer cells, HT-29 and FG cells were transfected with a plasmid encoding the full-length PDGFB gene. Sense clones with various increased levels of PDGF-BB were chosen for further study. More than 25 clones were isolated and evaluated for PDGF-BB expression by ELISA. Two clones of intermediate- and high-expression levels were chosen for each cell line (colon cancer: HT-29 S2 and S4; and pancreatic cancer: FG S1 and S11). HT-29 S2 produced 11 pg of PDGF-BB per 1 × 10^6 cells per 24 hours, while HT-29 S4 produced 66 pg of PDGF-BB per 1 × 10^6 cells per 24 hours. HT-29 cells transfected with vector alone (Neo pool cells), similar to HT-29 parental cells, did not produce detectable levels of PDGF-BB. FG S1 produced 2,899 pg of PDGF-BB per 1 × 10^6 cells per 24 hours, while FG S11 produced 676 pg of PDGF-BB per 1 × 10^6 cells per 24 hours. FG Neo pool cells, like the FG parental cells, did not produce detectable levels of PDGF-BB. Since PDGF-B transfection can hypothetically lead to an increase in PDGF-AB, an ELISA was performed for PDGF-AB protein levels. The results demonstrated that none of the cell lines (either parental or transfected clones) produced detectable levels of PDGF-AB or PDGF-AA.

To determine whether PDGF-BB can induce autocrine effects in HT-29 cells, the expression of PDGFRα and PDGFRβ was determined by reverse transcription–polymerase chain reaction and Western blotting. HT-29 cells did not express detectable levels of PDGFRα mRNA but did express very low levels of PDGFRβ mRNA and protein (data not shown). This finding is consistent with observations of Kitadai et al. (21), who found that tumor cell PDGFRβ is rarely expressed in human specimens and, when present, is expressed at exceedingly low levels.

Cell proliferation assays were performed to determine whether overexpression of PDGF-BB or addition of exogenous PDGF-BB stimulated HT-29 cell proliferation. There was no significant difference between the doubling times of the HT-29 S2 and HT-29 S4 clones (32 hours and 36 hours, respectively) and HT-29 parental and HT-29 Neo pool cells (27 hours and 31 hours, respectively). There was also no significant difference between the doubling times of the FG S1 clone (40 hours) and the FG parental and FG Neo pool cells (32 and 34 hours, respectively). The addition of exogenous PDGF-BB also did not cause enhanced proliferation (data not shown). FACS analysis confirmed that there was no difference in cell cycle parameters among HT-29 parental, HT-29 Neo pool, and the PDGF-BB–overexpressing HT-29 clones (data not shown).

**Effect of PDGF-BB overexpression on HT-29 and FG tumor growth in vivo.** To determine the effects of PDGF-BB overexpression on HT-29 cells, we injected cell suspensions into the subcutaneous tissues of nude mice. On day 27, several mice in the control group (parental and Neo pool) became lethargic; the experiment was terminated and all mice were sacrificed. The mean volume...
Therefore, we stained for numerous pericyte markers, including α-SMA, NG2, desmin, and PDGFRβ. The data suggested that there was a significant increase in the number of pericytes and pericyte precursor cells within the PDGF-BB–overexpressing tumors (Figure 2 and Supplemental Video 1; available online with this article; doi:10.1172/JCI31334DS1). We quantified the amount of pericyte coverage using 2 markers (desmin and NG2) in combination with CD31 to stain for ECs and demonstrated that there is increased pericyte coverage in the PDGF-BB–overexpressing tumors (Table 1).

PDGFRβ has also been shown to be expressed in cells within the stromal compartment of tumors. To determine whether the PDGF-BB–overexpressing cells led to an increase in stromal tumor content, Gomori trichrome staining was performed on tumors derived from the HT-29 parental, Neo pool, S2, and S4 cell lines. There was no significant difference in the amount of stromal tissue content within tumor as determined by a gastrointestinal pathologist in a blinded fashion (data not shown).

Effect of PDGF-BB overexpression on tumor growth in the liver. Since tumor growth and angiogenesis are influenced by the organ micro-

### Table 1

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<tr>
<td>Tumor cell proliferation, no. of cells/HPF</td>
<td>13.3 ± 2.9</td>
<td>17.4 ± 3.2</td>
<td>7.1 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.2 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Tumor cell apoptosis, no. of cells/HPF</td>
<td>1.6 ± 0.7</td>
<td>3.2 ± 2.0</td>
<td>6.4 ± 1.9</td>
<td>3.0 ± 1.1</td>
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<td>EC proliferation, % CD31+/BrdU- cells/HPF</td>
<td>3.8 ± 0.7</td>
<td>5.3 ± 0.8</td>
<td>2.7 ± 0.7</td>
<td>3.0 ± 0.4</td>
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<td>% ECs covered by pericytes&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.6 ± 1.7</td>
<td>36.7 ± 4.8</td>
<td>45.8 ± 4.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>48.8 ± 4.6&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>% Area occupied by pericytes/HPF&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.0 ± 0.8</td>
<td>6.0 ± 0.8</td>
<td>10.8 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.4 ± 1.6&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup>P < 0.005 by Student’s t test versus HT-29 Neo pool.  
<sup>b</sup>P < 0.04 by Student’s t test versus HT-29 Neo pool.  
<sup>c</sup>Determined using pericyte marker α-SMA.  
<sup>d</sup>P < 0.02 by Student’s t test versus HT-29 P.  
<sup>e</sup>Determined using pericyte marker NG2.

### Figure 2

Effect of PDGF-BB overexpression on pericyte coverage. HT-29 tumor sections from mice injected with HT-29 control (Neo pool) or PDGF-BB–overexpressing (S2) cells were double stained for ECs (CD31; red) and pericyte markers (NG2 and α-SMA; green). There was an increased influx of pericytes or pericyte-like cells within the PDGF-BB–overexpressing tumors. Representative photomicrographs are shown for the HT-29 Neo pool and the HT-29 S2 groups. Scale bar: 50 μm.

**PDGFRβ, NG2, desmin, and α-SMA.**
To observe any growth inhibition of CRC cells by pericyte-like cells when cells were grown so as to allow cell-cell contact; this effect was not observed when cells were cultured in Boyden chambers (data not shown). These data suggested that pericyte-like cells are capable of inhibiting EC proliferation and that this inhibition of EC proliferation by pericytes requires cell-cell contact. In similar studies done with cocultures of 10T 1/2 and CRC cells, we did not observe any growth inhibition of CRC cells by pericyte-like cells (data not shown).

Figure 3

Effect of inhibition of PDGFR activity on tumor growth in the liver. HT-29 control (Neo pool) and PDGF-BB–overexpressing (S2 and S4) cells (1 × 10^6) were injected directly into the livers of nude mice. Mice were sacrificed, and the tumors were measured and weighed. There was a significant reduction in tumor mass in the PDGF-BB–overexpressing clones (P < 0.001) (Table 2 and Figure 3). Tumor volume was also significantly decreased (~86% to 91%) in the mice injected with PDGF-B–transfected clones compared with the control mice (P < 0.05) (Table 2 and Figure 3). Tumor volume was also significantly decreased (~86% to 91%) in the mice injected with PDGF-B–transfected clones (P < 0.05) (Table 2).

Effect of PDGF-BB overexpression on pericyte-like cell function. To determine the in vitro effect of PDGF-BB–overexpressing tumor cells on pericytes, a variety of cell proliferation, migration, and coculture assays were performed utilizing VSMCs, as these are believed to be the in vivo precursors to pericytes. As shown in Figure 4A, conditioned medium from the PDGF-BB–overexpressing clones significantly increased proliferation of human VSMCs by almost 2-fold (P < 0.01). Conditioned medium from the control or vector-transfected cells did not increase VSMC proliferation. These results were confirmed in an experiment using 10T 1/2 murine mural/pericyte-like cells (data not shown). In a cell migration assay in which HT-29 cells were placed in the lower chamber of a modified Boyden chamber, migration of VSMCs from the upper to the lower chamber was significantly greater in the presence of the PDGF-BD-expressing clones (Figure 4B; P < 0.05).

Table 2

| Effect of PDGF-BB overexpression on tumor growth and weight in the orthotopic environment |
|---------------------------------|-----------------|-----------------|-----------------|
| Liver                           | HT–29 Neo pool  | HT–29 S2         | HT–29 S4         |
| Tumor incidence, no. of mice    | 9/10 (90%)      | 4/10 (40%)       | 3/10 (30%)^A     |
| Tumor volume, mm^3 (mean ± SEM) | 423 ± 120       | 45 ± 29B         | 13 ± 11C         |
| Tumor mass, g (mean ± SEM)      | 0.25 ± 0.06     | 0.06 ± 0.03C     | 0.02 ± 0.01C     |

Pancreas

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<th>FG Neo pool</th>
<th>FG–S1</th>
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<tr>
<td>Tumor incidence, no. of mice</td>
<td>14/14 (100%)</td>
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<tr>
<td>Tumor volume, mm^3 (mean ± SEM)</td>
<td>3,349 ± 1,400</td>
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<tr>
<td>Tumor weight, g (mean ± SEM)</td>
<td>2.0 ± 0.8</td>
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^A P < 0.03 by χ^2 test versus Neo pool. ^B P < 0.05 by Student’s t test versus Neo pool. ^C P < 0.007 by Student’s t test versus Neo pool.

The total number of cells per well remained the same. After 48 hours, the cells were sorted and quantified by FACS. The number of HUVECs in cocultures with pericyte-like cells was decreased by 47% compared with the number of HUVECs cultured with HUVECs only (Figure 5). This effect was only observed when cells were grown so as to allow cell-cell contact; this effect was not observed when cells were cultured in Boyden chambers (data not shown). These data suggested that pericyte-like cells are capable of inhibiting EC proliferation and that this inhibition of EC proliferation by pericytes requires cell-cell contact. In similar studies done with cocultures of 10T 1/2 and CRC cells, we did not observe any growth inhibition of CRC cells by pericyte-like cells (data not shown).

Effect of inhibition of PDGFR activity on tumor growth and pericyte content in vivo and tumor cell number in vitro. To determine whether tumor growth inhibition due to PDGF-BB overexpression was mediated by activation of PDGFR, imatinib mesylate was utilized to block PDGFR activity in vivo. Control vector transfecteds (HT–29 Neo pool) and PDGF–BB–overexpressing cells (HT–29 S2) were injected subcutaneously into nude mice, and mice were treated with either imatinib mesylate or solvent (water; control). Similar to what was observed in the above studies, overexpression of PDGF-BB led to a marked inhibition of tumor growth (Figure 6).
In this study, we demonstrated that overexpression of PDGF-BB by human CRC and pancreatic cancer cells inhibited tumor growth in vivo. Neither the HT-29 human CRC cells nor the FG human pancreatic cancer cells expressed detectable levels of PDGF-BB or showed altered proliferation with the addition of PDGF-BB. Transfection of the PDGFβ gene into HT-29 or FG cells resulted in selective overexpression of PDGF-BB but not of the heterodimer PDGF-AB. There was a significant increase in the number of pericytes in the PDGF-BB-overexpressing tumors concomitant with a decrease in the microvessel counts of these tumors compared with control tumors. In vitro studies demonstrated that pericyte-like cells are able to inhibit EC proliferation. Treatment with imatinib mesylate, a tyrosine kinase inhibitor of PDGFRβ, resulted in a partial reversal of the tumor growth inhibition in mice injected with HT-29 PDGF-BB–overexpressing transfecteds. The imatinib mesylate–treated tumors also demonstrated a decreased number of pericytes, again suggesting that pericytes mediate tumor growth inhibition, possibly through their inhibition of EC proliferation.

The PDGF/PDGFR system has previously been shown to be directly involved in oncogenic transformation (25, 26). PDGF-B was the first growth factor shown to be a viral oncogene (PDGF-B/v-sis) (25). Many of the studies demonstrating the oncogenic role of PDGF-B were initially done in murine cell lines (such as NIH3T3) (25, 26). In human cell lines, the oncogenic properties of PDG-B are more difficult to demonstrate (27). There is extensive evidence for the overexpression of PDGF-B/PDGFRβ in tumors of neuroectodermal origin leading to enhanced tumor growth and progression (28). There is also evidence implicating the growth-promoting effects of PDGF in other tumor types, including leukemia (29, 30), melanoma (31), mesothelioma (32), osteosarcoma (33, 34), breast cancer (35), and lung cancer (36).

Despite the wealth of literature on PDGF and PDGFR in other tumor types, there are comparatively few published data about the effects of PDGF-B/PDGFRβ in colorectal or pancreatic cancers. Elucidating the role of PDGF in these tumor types is important, since in our studies, there was no effect of PDGF-BB on either colon or pancreatic cancer cell proliferation in vitro. An initial study of PDGF/PDGFR in human colon cancer cell lines revealed that 67% expressed PDGF-B, but none expressed PDGFR at the mRNA level (37). Another study determined that only 33% of human colon adenocarcinoma cell lines expressed detectable levels of PDGF protein (38). Yet another study analyzing full-thickness...
angiogenesis and tumor growth in the

overexpression with an increase in pericytes that results in

Importantly, although previous studies have also reported an

might be due to pericyte-mediated inhibition of EC proliferation. Pericytes and VSMCs express high levels

nism by which this occurred was reversal of pericyte-mediated inhi-

We hypothesize that the mecha-

Data from the current study demonstrate that pericyte-like cells

grown in vitro are able to inhibit EC proliferation. Thus, this study

confirms previous work suggesting that one function of pericytes is to inhibit the growth of ECs (8, 24). The mechanism of action for this inhibition has been reported to be mediated both by direct cell-cell contact and through the production of soluble mediators such as TGF-β1 (8, 44).

In the current study, treatment of HT-29 tumors with imatinib mesylate, a known inhibitor of PDGFR-β (as well as c-Kit and Abl), resulted in different effects on tumor growth in control and PDGF-BB-overexpressing tumors. As in previously published studies in which this compound was tested against epithelial tumors (45), imatinib mesylate had no effect on the growth of HT-29 control tumors. Other studies have demonstrated that imatinib mesylate has little or no effect on tumor growth in vivo even though this agent demonstra-

confirms significant antiproliferative effects in vitro (45). Strikingly, however, in our studies, treatment of the PDGF-BB-overexpressing tumors with imatinib mesylate partially reversed the PDGF-BB-mediated growth-inhibitory effects. We hypothesize that the mecha-

inhibitory effects of PDGF-BB in vivo is most likely due to an alteration in cells in the host tumor microenvironment. These data indicate the importance of understanding cell-specific expression of PDGF-B and PDGFRβ in tumors treated with inhibitors of this pathway.

In human tumors, pericytes are localized to the periendothelial cell location, since vessel maturation depends on not only PDGFR ligands but other EC-derived angiogenic mediators such as angiopoietin-1. Although aberrations in pericyte recruitment in tumors
overexpressing PDGF-BB may occur, this has not been analyzed in detail in human tumors due to the difficulty in studying numerous pericyte markers simultaneously. However, we recognize that forced expression of PDGF-BB to the level observed in our studies may not reflect the level observed in the majority of tumor types in patients. In conclusion, our data demonstrate that overexpression of PDGF-BB in human CRC and pancreatic cancer cells results in decreased tumor growth. PDGF-BB–overexpressing tumors are characterized by an increase in the relative proportions of pericyte (and possibly pericyte precursor) cells. In vitro studies confirmed that pericytes have the ability to decrease EC proliferation, suggesting that one function of pericytes is to regulate EC growth. Additional studies utilizing imatinib mesylate in vivo demonstrated decreased pericyte coverage and adherence in the treated tumors and thus confirmed the role of PDGF-BB in increasing pericyte coverage within tumors. Also, imatinib mesylate partially reversed the growth inhibition in PDGF-BB transfectants, supporting the hypothesis that pericytes tightly control EC proliferation and angiogenesis within tumors.

Methods

Cell culture and transfection

Human cancer cell lines were either obtained from ATCC (HT-29, GEO, and RKO) or were the generous gifts of I.J. Fidler (The University of Texas MD Anderson Cancer Center) (KM12L4, KM12SM, and FG). All cell lines were cultured and maintained in modified Eagle medium supplemented with 10% FBS as described previously (22). HUVECs, VSMCs, and 10T 1/2 cells (murine mural/pericyte-like cells) were obtained from ATCC and cultured and maintained in modified Eagle medium supplemented with 10% FBS as described previously (22). HUVECs, VSMCs, and 10T 1/2 cells (green; 50,000 cells). After 48 hours of coculture, HUVECs (red; 50,000 cells) were then plated with an equal number of undyed cells or 10T 1/2 cells (green; 50,000 cells). After 48 hours of coculture, HUVECs were trypsinized and sorted using FACS analysis, and the total number of fluorescent red cells was determined.

**Figure 7**

Effect of imatinib mesylate on pericyte coverage of HT-29 tumors. HT-29 tumors were double-stained for ECs and pericyte markers. NG2 staining (red) was used as a marker of pericytes or pericyte-like cells, whereas CD31 (blue) was used to identify ECs. There was an increased influx of NG2-positive cells within the PDGF-BB–overexpressing tumors, similar to what was shown previously (Figure 2). This influx was reduced in the imatinib mesylate–treated group, in which pericyte coverage appeared similar to that in control tumors. Representative photomicrographs are shown for each group. Scale bar: 50 μm.

overexpressing PDGF-BB was subcloned from the pSM-1 plasmid, obtained from ATCC, by digesting a 2.0-kb BamHI fragment of the original plasmid and subcloning it into pcDNA3.1 (Invitrogen) containing a neomycin resistance gene. Vector alone or vector containing the PDGFB gene was transfected into HT-29 cells using FuGENE 6 (Roche Diagnostics) per the manufacturer’s protocol, and the cells were grown in selective medium (10% modified Eagle medium plus 400 μg/ml neomycin; G418; Life Technologies Inc.). For in vivo studies, HT-29 and FG cells transfected with PDGF-B and mock-transfected with pcDNA were harvested from subconfluent cultures. Briefly, cells were rinsed with PBS solution, trypsinized (0.25% trypsin, 0.1% EDTA), and resuspended in modified Eagle medium containing 10% FBS. Cells were counted, and cell viability was assessed using the trypan blue exclusion assay (>90% viability). Cells were resuspended in HBSS for tumor inoculation into nude mice.

**ELISA for PDGF-AA, PDGF-AB, and PDGF-BB**

HT-29 and FG cells were plated at 10^5 cells per 6-well plate and allowed to attach overnight. Fresh medium (1% modified Eagle medium) was added to the cultures, and cells were cultured for 72 hours. Medium was harvested, spun at 210 g, filtered using a 0.22-μm filter, and frozen at −20°C until needed. Cells were counted using the trypan blue exclusion assay, and ELISAs were performed per the manufacturer’s instructions (R&D Systems).

**Cell proliferation and cell-cycle analysis of transfected cells**

**MTT assay.** A stock solution was prepared by dissolving 5 mg of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; M2128; Sigma-Aldrich) in 1 ml of PBS and filtering the solution to remove particulates. The solution was protected from light, stored at 4°C, and used within 1 month. Cells were seeded into 96-well plates in triplicate and allowed to adhere overnight. The cultures were then washed, and medium was changed, exposing cells to PDGF-BB (10 ng/ml) or control. After 24, 48, and 72 hours, absorbance was determined by MTT assay. After 2 hours of incubation in medium containing 0.42 mg/ml of MTT, the cells were lysed in dimethyl sulfoxide. The conversion of MTT to formazan by metabolically viable cells was monitored by an MR-5000 96-well microtiter plate reader at 570 nm (Dynatech Inc.).

**FACS analysis.** FACS analysis was performed to detect potential changes in cell cycle in transfected cell lines. Cells were grown to approximately 50% confluence, washed in PBS, and fixed in 70% ethanol overnight at 4°C. Propidium iodide supplemented with RNAse was added, and 30 minutes later, FACS analysis was performed using an EPICS XL-MCL flow cytometer (Beckman Coulter) with a 488-nm argon ion laser. Green fluorescence was detected at 520 nm, and red fluorescence was detected at 575 nm.

To determine whether EC proliferation is inhibited by the presence of pericyte-like cells, HUVECs and 10T 1/2 cells were dyed fluorescent red (HUVEC) or fluorescent green (10T 1/2) according to the manufacturer’s protocol (PKH67/PKH26 Fluorescent Cell Linker Kit; Sigma-Aldrich). HUVECs (red; 50,000 cells) were then plated with an equal number of undyed cells or 10T 1/2 cells (green; 50,000 cells). After 48 hours of coculture, HUVECs were trypsinized and sorted using FACS analysis, and the total number of fluorescent red cells was determined.
Cell migration assay
The effects of tumor cell–secreted PDGF-BB on VSMC migration in vitro were investigated using a modified Boyden chamber (BD Biosciences) with uncoated inserts (8.0-μm pores). VSMCs or 10T 1/2 cells were plated into migration inserts (50,000 cells per insert in 1% medium), and HT-29 parental, HT-29 Neo pool, HT-29 S2, or HT-29 S4 cells (50,000 cells in 1% medium) were plated into the bottom wells. Inserts were plated in triplicate for each cell line and incubated for 24 hours at 37°C. Migrating cells were stained (DiffQuick; Dade Behring), and 5 random fields were counted per insert at ×50 magnification.

Animal studies
Eight-week-old male athymic nude mice (obtained from the National Cancer Institute Animal Production Area) were acclimated for 1 week and caged in groups of 5. All animal studies were approved by the Animal Care and Use Committee of the MD Anderson Cancer Center.

Subcutaneous studies. HT-29 or FG cells (10⁵) in HBSS were injected into the subcutaneous space on the flanks of nude mice. Tumor growth was monitored and measured with calipers 3 times per week. Approximately 1 hour prior to sacrifice, mice were injected intraperitoneally with 1 mg of BrdU. When mice became moribund, they were sacrificed by CO₂ asphyxiation. Mice were weighed, and tumors were excised and weighed. Tumor tissue was harvested and either placed in 10% buffered formalin for paraffin fixation or frozen in OCT compound (Miles Inc.) in liquid nitrogen for subsequent immunohistochemical analysis.

Direct liver injection. The preferential metastatic site for CRC is the liver. Therefore, we have developed a technique for directly injecting tumor cells into the liver (22). Mice were anesthetized using methoxyflurane (Medical Developments International) and, under sterile conditions, subjected to an upper midline laparotomy. HT-29 cells (10⁵) were injected under the capsule into the lateral lobe of the liver. Tumors were allowed to grow until mice became moribund. Mice were sacrificed via CO₂ asphyxiation and weighed. Tumor tissue was harvested and either placed in 10% buffered formalin for paraffin fixation or frozen in OCT compound (Miles Inc.) in liquid nitrogen for subsequent immunohistochemical analysis.

Intrapancreatic injection. For intrapancreatic injections, mice were anesthetized using methoxyflurane (Medical Developments International) and, under sterile conditions, subjected to a left flank incision, as previously described (46). FG cells (10⁵) were injected into the tail of the pancreas. Tumors were allowed to grow until mice became moribund. Mice were sacrificed via CO₂ asphyxiation and weighed, and the pancreas was excised. Total pancreatic weight (including pancreatic tumors) was determined, and tumors were carefully dissected free of surrounding fat, measured, and weighed. Tumor tissue was harvested and processed as described above.

Analyses of immunostained tissue sections
Immunostained tissue sections were examined using a Zeiss photomicroscope equipped with a 3-chip charge-coupled device color camera (DXC-960 MD; Sony Corp.). The images were analyzed using Optimas image analysis software (version 5.2). Positive cells were counted using Scion software based on the NIH image program for Macintosh (Scion Corp.). The number of positive cells was expressed as the average of the number of cells in 0.05-μm² high-power fields at ×200. Five fields per tumor section from either 8-μm-thick sections (BrdU, TUNEL) or 20-μm-thick sections (CD31, desmin, α-SMA, NG2) were chosen randomly, and 5 or more specimens per group were analyzed. Areas of obvious necrosis (as determined by either H&E or Hoechst counterstain) were excluded from analysis. To determine the percentage of vessel area, sections were analyzed using Optimas software. The cross-sectional area of CD31-positive structures (i.e., vessel area) was also determined in the same 4 quadrants by capturing images, converting them to grayscale, and analyzing the NG2/CD31-stained areas with NIH ImageJ software (version 1.62; http://rsb.info.nih.gov/ij/) by setting a consistent threshold for all slides so that only CD31-stained cells were apparent. The NG2/CD31-positive area was then expressed as the number of pixels squared per high-power field.

Statistics
Statistical differences among groups were examined using the 2-tailed Student’s t test, the χ² test, or, for analysis of nonparametric data, the Mann-Whitney U test with InStat Statistical Software (GraphPad Software). The results of the in vivo experiments were tested for outliers using Grubb’s test (www.graphpad.com). A P value of less than 0.05 was considered statistically significant.

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