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VEGF-null cells require PDGFR α signaling-mediated stromal fibroblast recruitment for tumorigenesis

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We generated VEGF-null fibrosarcomas from VEGF-loxP mouse embryonic fibroblasts to investigate the mechanisms of tumor escape after VEGF inactivation. These cells were found to be tumorigenic and angiogenic *in vivo* in spite of the absence of tumor-derived VEGF. However, VEGF derived from host stroma was readily detected in the tumor mass and treatment with a newly developed anti-VEGF monoclonal antibody substantially inhibited tumor growth. The functional significance of stroma-derived VEGF indicates that the recruitment of stromal cells is critical for the angiogenic and tumorigenic properties of these cells. Here we identified PDGF AA as the major stromal fibroblast chemotactic factor produced by tumor cells, and demonstrated that disrupting the paracrine PDGFR α signaling between tumor cells and stromal fibroblasts by soluble PDGFR α-IgG significantly reduced tumor growth. Thus, PDGFR α signaling is required for the recruitment of VEGF-producing stromal fibroblasts for tumor angiogenesis and growth. Our findings highlight a novel aspect of PDGFR α signaling in tumorigenesis.

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Introduction

Angiogenesis, the formation of new blood vessels, is crucial for tumor growth, as tumor cells require oxygen and nutrients for proliferation and survival (Folkman, 1995; Carmeliet and Jain, 2000). Tumor angiogenesis is a complex process regulated by both pro- and antiangiogenic factors produced by the tumor cells as well as the stromal cells in the tumor microenvironment (Hanahan and Folkman, 1996; Jung *et al*, 2002). One of the most important angiogenic factors is VEGF, which regulates endothelial cell survival, proliferation and migration (Carmeliet *et al*, 1996; Ferrara, 1999). The critical role of VEGF in tumor angiogenesis has been well documented in animal studies using anti-VEGF antibodies, or small molecules targeting VEGFR2 (Kim *et al*, 1993; Fong *et al*, 1999), and has recently been validated by the success of VEGF-targeted cancer therapy in clinical trials (Fernando and Hurwitz, 2003).

Expression of VEGF has been detected in both tumor and stromal compartments, raising the question of the relative importance of each compartment for VEGF-mediated angiogenesis. Genetic inactivation of VEGF in tumor cells resulted in severe inhibition of tumor growth and angiogenesis in studies using wild-type embryonic stem (ES) cells (Ferrara *et al*, 1996), ras-transformed ES cells (Shi and Ferrara, 1999), embryonic fibroblasts (Grunstein *et al*, 1999) or endocrine pancreatic cells (Inoue *et al*, 2002). These gene-targeting studies demonstrate that tumor-derived VEGF is essential for tumor growth and neovascularization. Interestingly, appreciable VEGF expression was detected in tumor-associated fibroblasts (TAFs) and immune cells in many tumor specimens (Hlatky *et al*, 1994; Lewis *et al*, 2000; Pilch *et al*, 2001; Barbera-Guillem *et al*, 2002). In a human rhabdomyosarcoma xenograft model, it was shown that complete inhibition of tumor growth and angiogenesis required blockade of both tumor and host VEGF (Gerber *et al*, 2000). However, two transgenic mouse lines expressing green fluorescent protein (GFP) driven by different human VEGF promoter regions generated conflicting results regarding the extent to which the stromal compartment could constitute a source of VEGF for angiogenesis (Fukumura *et al*, 1998; Kishimoto *et al*, 2000). In addition, a recent study using ras-transformed VEGF-deficient adult dermal fibroblasts concluded that VEGF production by tumor stroma had a modest role in tumor angiogenesis (Viloria-Petit *et al*, 2003). Thus, the contribution of stromal-derived VEGF to tumor angiogenesis is unclear.

In the tumor stromal compartment, fibroblasts are the predominant cell type and potentially are a significant source of VEGF. These TAFs are phenotypically different from their normal counterparts and are active participants in tumor development (Kunz-Schughart and Knuechel, 2002a, b). However, very little is known about the mechanisms of stromal cell recruitment. Efforts to purify fibroblast migratory factor(s) from human lung fibroblasts and from a mouse colon carcinoma cell line identified fibronectin (FN) as a potential stromal fibroblast recruitment factor (Hu *et al*, 1997; Morimoto and Irimura, 2001), although the role of this protein in tumor stroma recruitment has yet to be directly

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addressed. The platelet-derived growth factor (PDGF) family members are the most extensively investigated regulators of mesenchymal cell proliferation and migration during development (Hoch and Soriano, 2003); they are also highly expressed in tumors and could play important roles in stromal fibroblast recruitment. In fact, pathological studies with human tumor samples localized PDGFR β expression in the peripheral stroma and PDGF-B expression in epithelial tumor cells (Coltrera *et al*, 1995; Bhardwaj *et al*, 1996; Kawai *et al*, 1997; Sundberg *et al*, 1997), suggesting that PDGF-B may be implicated in stroma recruitment. Furthermore, overexpression of PDGF-B stimulated development of vascular connective stroma in a human melanoma xenograft model and induced tumorigenic conversion of nontumorigenic human keratinocytes by stroma activation (Forsberg *et al*, 1993; Skobe and Fusenig, 1998), demonstrating that tumor cell-produced PDGF-B can facilitate tumor growth through its paracrine effects on stromal cells. In comparison, the role of PDGF-A in tumorigenesis has been much less appreciated, in part due to its weaker mitogenic and chemotactic activities (Beckmann *et al*, 1988; Siegbahn *et al*, 1990). PDGF-A has been documented to stimulate tumor growth in an autocrine fashion (Betsholtz *et al*, 1989; Harsh *et al*, 1990; Sulzbacher *et al*, 2000), whereas direct evidence for a role of this factor in tumor stroma activation is still lacking. Likewise, the two newly identified PDGF members (Heldin *et al*, 2002), PDGF-C and PDGF-D, have been implicated in autocrine stimulation of sarcoma and glioblastoma cells (Lokker *et al*, 2002; Zwerner and May, 2002), while any role as paracrine mediators remains to be established.

In this study, using fibrosarcomas generated from VEGF-null ras-transformed mouse embryonic fibroblasts (MEFs), we demonstrate that VEGF production in the stromal compartment plays a critical role in tumor angiogenesis. We further identified PDGF AA as the major factor produced by tumor cells to recruit tumor stromal fibroblasts and uncovered a key role for PDGFR α signaling in tumor angiogenesis and growth.

Results

Tumor formation by VEGF-null ras-transformed MEFs

We generated ras-transformed cells that are deficient in VEGF production using a strategy depicted in Figure 1A. Briefly, MEFs were derived from VEGF/*loxP*(+/+) mice (Gerber *et al*, 1999), immortalized with SV40 large T antigen, and then transformed with H-ras. Cre recombinase was introduced to generate VEGF-null cell lines. The loss of VEGF expression in the VEGF-null cell lines was verified by quantitative RT–PCR analysis of cellular RNA using primers and probes specific for VEGF exon 3 (Figure 1B). VEGF ELISA also demonstrated the absence of VEGF protein in the conditioned media (CM) from the VEGF-null cells (data not shown).

We next examined the tumorigenic ability of the $VEGF^{-/-}$ clones. In initial experiments, all seven clones examined were found to be tumorigenic *in vivo*, although to a reduced extent compared to the parental C2P cells. Figure 1C illustrates three representative VEGF-null clones, G5, F10 and F4. Tumors derived from these clones consistently weighed about half as those derived from C2P. This is in agreement with the notion that tumor-derived VEGF is significant for tumor growth. However, vascular structures were evident in the $VEGF^{-/-}$ tumors, and there was a slight decrease in vessel density in G5 tumors compared to C2P tumors (Figure 1D). This led us to speculate that angiogenic factors other than tumor cell-produced VEGF might contribute to tumor formation.

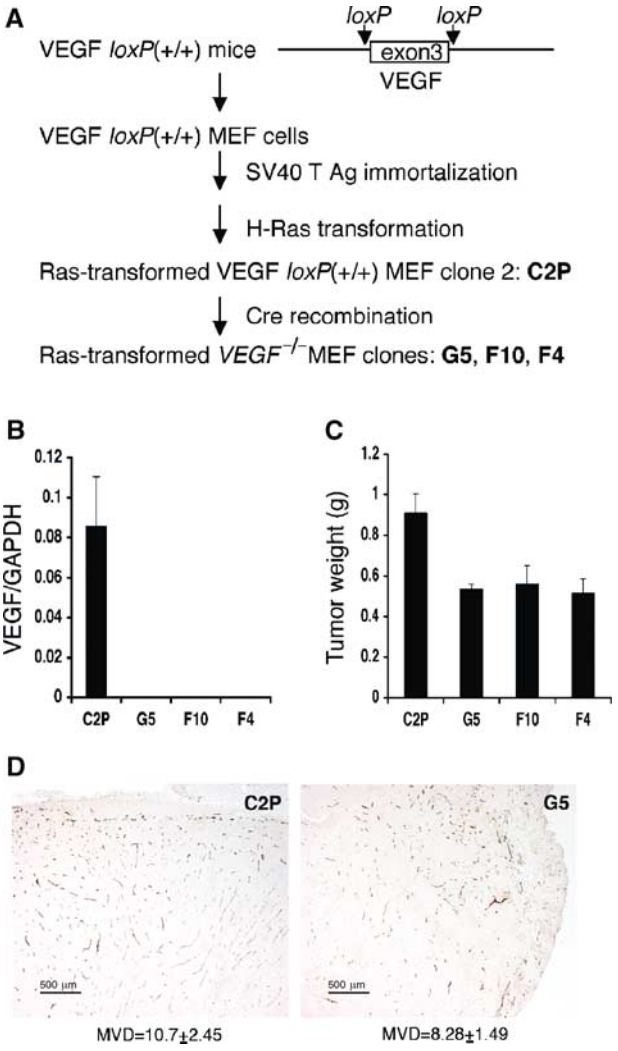

Figure 1 Generation of ras-transformed $VEGF^{-/-}$ MEF cell lines and fibrosarcomas. (**A**) A diagram of the strategy to derive ras-transformed $VEGF^{-/-}$ cells from the VEGF/*loxp*(+/+) mice, in which exon 3 of the VEGF gene is the target for deletion. (**B**) Quantitative RT–PCR analysis of VEGF RNA using primer/probe sets specific for exon 3, demonstrating the loss of VEGF exon 3 transcript in the $VEGF^{-/-}$ clones (G5, F10 and F4). (**C**) Fibrosarcoma formation by the parental $VEGF^{+/+}$ C2P clone and the $VEGF^{-/-}$ clones. Tumor weight was determined 3 weeks after tumor cell implantation. (**D**) Sections of tumors originated from $VEGF^{+/+}$ C2P and $VEGF^{-/-}$ G5 cells were stained for endothelial cell marker Flk-1. Vessel density in units/μm is indicated below the corresponding images.

Tumor stroma provides VEGF for angiogenesis and tumorigenesis

One potential mechanism by which ras-transformed $VEGF^{-/-}$ cells form tumors could be through recruitment of stromal cells, which in turn may produce VEGF. To determine whether VEGF was expressed within the tumor mass, quantitative RT–PCR specific for VEGF exon 3, the region deleted in $VEGF^{-/-}$ cells, was performed on RNA isolated from tumors. VEGF exon 3-specific RNA message was detected in

G5, F10 and F4 tumors, although at a markedly lower level compared to the parental C2P tumors (Supplementary Figure 1A). Also, VEGF protein was detected in F10, F4 and G5 tumor lysates, at concentrations ~10–20% of the C2P tumors (Supplementary Figure 1B). To examine the distribution of VEGF expression in these fibrosarcomas, *in situ* hybridization was performed with a VEGF exon 3-specific probe. In C2P tumors (Figure 2A–D), the VEGF signal was more evenly distributed in the tumor mass, predominantly arising from tumor cells. In the G5, F10 and F4 tumors (Figure 2E–P), the VEGF signal was noted in discontinuous clusters of cells near the necrotic zones, consistent with stromal patterns, while no signal was detected in tumor cells.

To test the possibility that angiogenesis in $VEGF^{-/-}$ tumors may be mediated by VEGF from recruited stromal cells, we examined the effect of mFlt(1-3)-IgG, a soluble VEGF receptor chimeric protein (Ferrara *et al*, 1998), on the growth of these tumors. We found that administration of mFlt(1-3)-IgG significantly reduced the growth rate of C2P tumors as well as those of G5 and F4 tumors (data not shown), suggesting that stroma-derived VEGF is important for tumor angiogenesis in these fibrosarcomas. However, because VEGFR1/Flt-1 can also bind to PlGF and VEGF-B in addition to VEGF (Ferrara *et al*, 2003b), the effect of mFlt(1-3)-IgG may not be solely through inhibiting VEGF-A. In order to more specifically assess the contribution of stromal VEGF-A to tumor growth, we treated tumor-bearing animals with a newly developed anti-VEGF antibody, G6-23 (Fuh *et al*, manuscript in preparation). As shown in Figure 3A–C, relative to an isotype-matched control antibody, G6-23 substantially inhibited the growth rate of not only C2P tumors but also G5 and F10 tumors. G6-23 treatment caused a significant decrease in the tumor weight in all three cases (Figure 3D, $P<0.05$). Interestingly, in agreement with the observation that G5 tumors express a higher level of VEGF than F10 tumors, G6-23 reduced the average tumor weight of G5 tumors by more than 62%, whereas the reduction of F10 tumor weight was smaller, by about 50%. These results demonstrate surprisingly that even the small amounts of VEGF from the stroma play an important role in tumor angiogenesis.

Tumor cells secrete factors with fibroblast chemotactic and mitogenic activity

Stromal fibroblasts represent a major component of the tumor stroma and are known to produce VEGF (Hlatky *et al*, 1994; Pilch *et al*, 2001). To examine the mechanism by which our VEGF-null clones recruit stromal cells, we sought to test CM from these cells for chemotactic and proliferative activity on cultured fibroblasts. Initial experiments demonstrated that 3T3 fibroblasts respond to a variety of proliferative and chemotactic stimuli similar to primary stromal fibroblasts isolated from xenografted tumors. Therefore, we used 3T3 cells as a stromal fibroblast cell model system in our study. We found that the CM from tumor cells strongly stimulated migration as well as proliferation of 3T3 fibroblasts (Figure 4A and B). The data indicate that these ras-transformed MEF

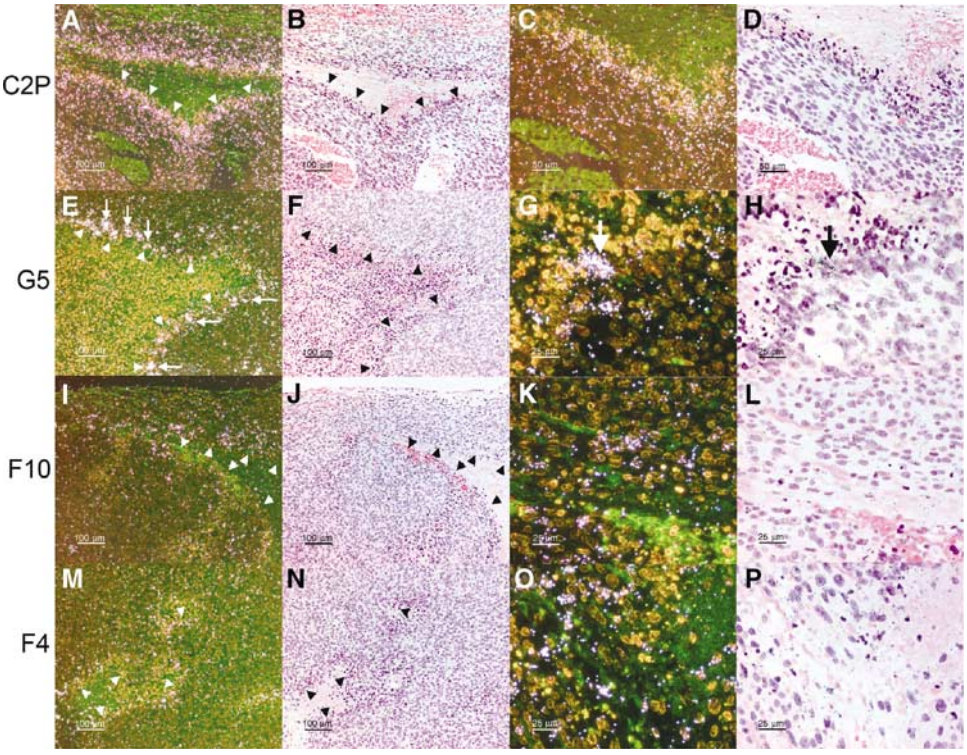

Figure 2 Examination of VEGF RNA expression in tumors by *in situ* hybridization. Paraffin sections of tumors grown from $VEGF^{+/+}$ C2P or $VEGF^{-/-}$ G5, F10 and F4 cell lines were hybridized with a ^{33}P-labeled antisense riboprobe specific for the deleted exon 3 of VEGF sequence. (**A–D**) In C2P tumors, positive signal arises predominantly from tumor cells. The arrowheads indicate a continuous rim of hypoxic tumor and host stromal cells expressing increased levels of VEGF surrounding a necrotic area. (**E–H**) In G5 tumors, increased VEGF signal is noted in the hypoxic tumor zone (arrowheads); VEGF signal here is discontinuous, associated with stromal cells (arrows). (**I–P**) In F10 and F4 tumors, punctuate VEGF signal is noted at the boundary between necrotic tumor and viable tissue (arrowheads); the signal occurs in discrete regions consistent with origin in host stroma. Parallel images were taken with dark-field (A, C, E, G, I, K, M, O) or bright-field (B, D, F, H, J, L, N, P) illumination. Scale bars are 100μm (A, B, E, F, I, J, M, N) or 25μm (C, D, G, H, K, L, O, P).

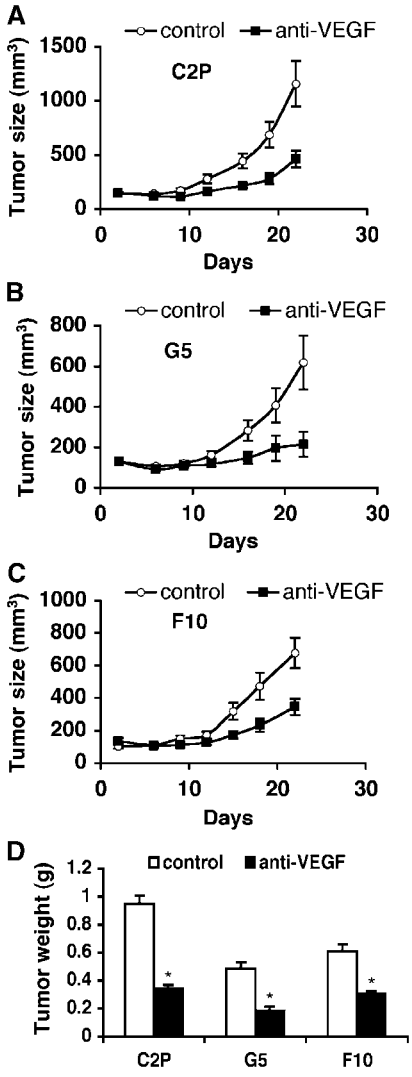

Figure 3 Inhibition of C2P, G5 and F10 tumor growth by anti-VEGF treatment. Treatments were started 2 days post tumor cell inoculation by intraperitoneal administration of the anti-VEGF G6-23 or a control antibody anti-ragweed at 10 mg/kg, twice weekly. (**A–C**) Tumor growth was monitored by measurement with a vernier caliper. (**D**) Tumor weight was determined 3 weeks post tumor cell implantation. Statistical analyses were performed with Student's *t*-test comparing the anti-VEGF treatment groups with the control groups; $^{*}P<0.05$.

cells produce secreted factors able to recruit host stromal fibroblasts.

Identification of PDGF AA as the major fibroblast recruitment factor produced by tumor cells

We sought to identify the mediators of stromal recruitment in our fibrosarcoma model. Because G5 tumors had the highest VEGF levels among VEGF-null clones, G5 cells are potentially a good model system to investigate fibroblast recruitment. We partially purified stromal fibroblast recruitment factor candidates from G5 CM, sequentially using cation exchange, size-exclusion and reversed-phase chromatography. The bioactivity of the eluted fractions was monitored by fibroblast migration and proliferation assays. Most of the activity of G5 CM was retained by a HiTrap S cation exchange column and was recovered in the 1 M NaCl factions (data not shown), suggesting that it is due to basic protein(s). The subsequent

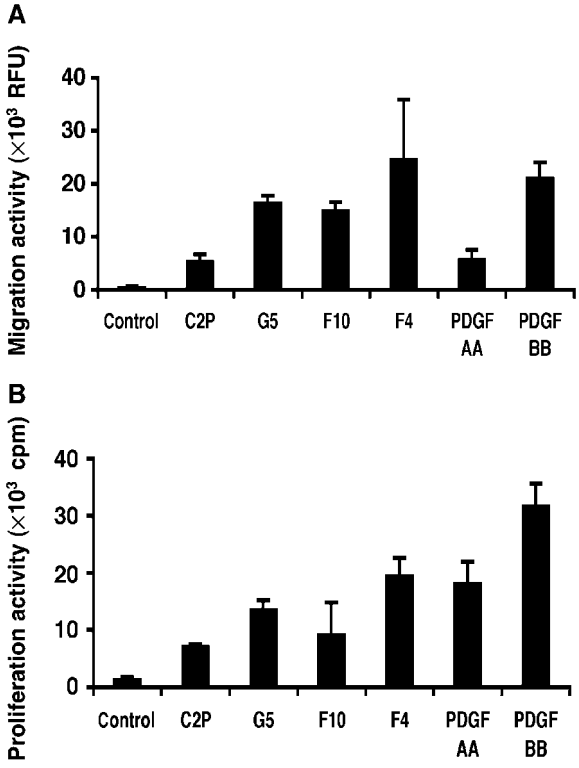

Figure 4 Simulation of 3T3 fibroblast migration and proliferation by CM from ras-transformed MEF cells. (**A**) Relative fluorescence unit (RFU) indicates the relative number of chemotactic cells. (**B**) Proliferation activity was represented by [^{3}H]thymidine incorporation as quantified by scintillation counting.

fractionation by size-exclusion chromatography revealed a major peak of activity with apparent molecular mass between 20 and 50 kDa (fractions 28–31) (Figure 5A). FN, the fibroblast migratory factor previously identified from human fibroblasts and mouse colon cancer cells (Hu *et al*, 1997; Morimoto and Irimura, 2001), was indeed detected in fractions 15–17, with an apparent molecular mass of >500 kDa (data not shown). Since these fractions showed little activity in our assays, we ruled out FN as a major contributor to the activity of G5 CM. Another potential stromal recruitment factor, PDGF BB, is within the estimated molecular weight range of the major bioactivity peak. Using ELISA kits specific for the different members of the PDGF family, to our surprise, we did not detect any immunoreactive PDGF BB. However, PDGF AA was identified in these active fractions at concentrations in excess of 100 ng/ml. Further purification by reversed-phase chromatography demonstrated that the PDGF AA-containing fractions overlapped with the activity peak (Figure 5B). To test the hypothesis that PDGF AA contributes to the fibroblast chemotactic and mitogenic activity in G5 CM, we employed neutralizing, soluble PDGFR IgGs in our activity assays. Since PDGF AA binds only PDGFR α, its activity can be blocked by soluble PDGFR α but not by soluble PDGFR β. We found that soluble PDGFR α-IgG inhibited 70–80% of the activity of G5 CM in a dose-dependent fashion, with a maximal effect at 30 ng/ml (Figure 6). In contrast, PDGFR β-IgG had no effect at all concentrations tested (Figure 6).

In addition to PDGF AA, a newly identified member of the PDGF family, PDGF CC, also preferentially signals through

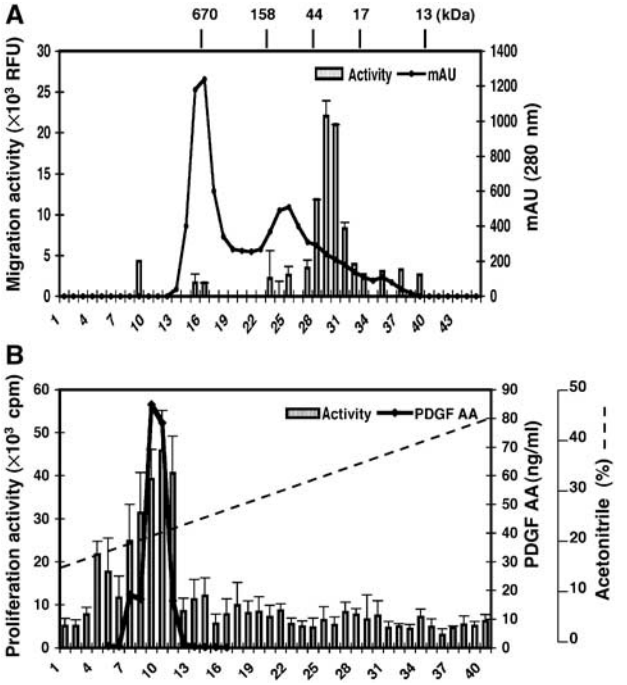

Figure 5 Partial purification of fibroblast chemotactic and mitogenic factors from G5 CM. (**A**) Fibroblast migration activity profile of fractions from the TSK size-exclusion column, which had been calibrated with known protein markers. The bioactive TSK fractions (28–31) were pooled and applied to the C4 Sepharose reversed-phase column. (**B**) Detection of PDGF AA in the active fractions from reversed-phase chromatography. The C4 column was eluted with a 15–50% gradient of acetonitrile. The collected fractions were tested for fibroblast proliferation activity and assayed for the presence of PDGF AA by ELISA.

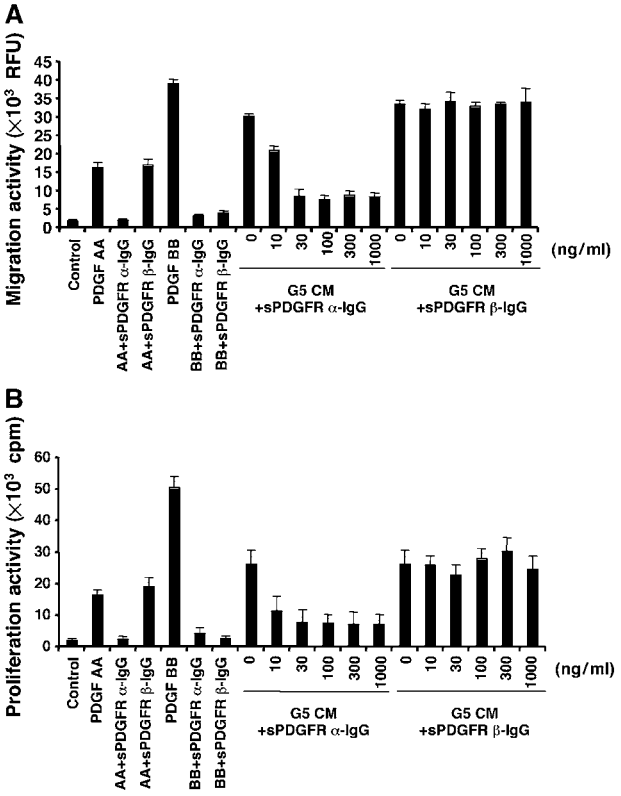

Figure 6 Inhibition of G5 CM-induced fibroblast migration and proliferation by soluble PDGFR α-IgG. Migration (**A**) and proliferation (**B**) assays were conducted with G5 CM in the presence of different concentrations of either soluble PDGFR α-IgG or PDGFR β-IgG. Recombinant human PDGF AA and PDGF BB were included as controls.

PDGFR α (Li *et al*, 2000; Gilbertson *et al*, 2001). Using quantitative RT–PCR, we detected PDGF-C RNA in tumor cells, but we were unable to demonstrate the protein in G5 CM using a commercially available antibody (data not shown). Thus, it is unclear whether PDGF CC contributes to the chemotactic activity of the CM. Also, very little PDGF-B and essentially no PDGF-D RNA was expressed by these tumor cells (data not shown).

PDGF ligands and PDGF receptors are differentially expressed in tumor and stromal cells in vivo

To further dissect the mechanisms of stromal recruitment, we performed *in situ* hybridization studies to examine the expression patterns of PDGF-A, -B, -C and the two PDGF receptors in tumors. PDGF-A signal was particularly intense throughout the tumor mass (Figure 7A–D), while PDGF-C signal was moderate and diffuse (Figure 7I–L). The localizations of PDGF-A and PDGF-C signals are consistent with tumor source, in agreement with the *in vitro* data showing that tumor cells strongly express PDGF-A and to a lesser extent PDGF-C. Distinctly, PDGF-B expression was found to be associated with vascular endothelial cells in the surrounding normal tissues and in discrete clusters, consistent with vascular endothelial origin in the tumors (Figure 7E–H). Whereas PDGFR α expression showed a punctuate pattern consistent with normal stromal fibroblasts (Figure 7M–P), PDGFR β expression was strongly associated with tumor stromal vessels (Figure 7Q–T). It is noteworthy that there was no PDGFR α signal associated with normal vessels, where the appositional expression of PDGF-B and PDGFR β was evident (Figure 7E, M and Q). The expression patterns are consistent with paracrine signaling between PDGF-A (and perhaps PDGF-C) produced by tumor cells and PDGFR α expressed on stromal cells. The blood vessel-associated expression of PDGF-B and PDGFR β is consistent with their role in pericyte recruitment and vascular maturation (Abramsson *et al*, 2003; Bergers *et al*, 2003; Lindblom *et al*, 2003).

The differential expression profile of PDGF family members, combined with the distinct effects of soluble PDGFR α- and β-IgGs on the fibroblast chemotactic activity in the tumor cell CM, suggests that PDGFR α signaling is an important mechanism by which tumor cells recruit stromal fibroblasts.

Soluble PDGFR α and PDGFR β inhibit tumor growth

Since VEGF-null tumor cells are largely dependent on stroma-derived VEGF for angiogenesis, we suspected that PDGFR signaling may play an important role in their angiogenesis and tumorigenesis. Tumor-bearing animals were treated with antagonistic, soluble PDGFR α-IgG or PDGFR β-IgG, which were delivered through adenoviral expression vectors directly into the tumor mass. Figure 8A illustrates a representative experiment, while similar results were obtained in three additional independent experiments. Relative to the control Av-LacZ, Av-PDGFR α-IgG significantly inhibited G5 tumor growth by 50% while Av-PDGFR β-IgG inhibited G5 tumor

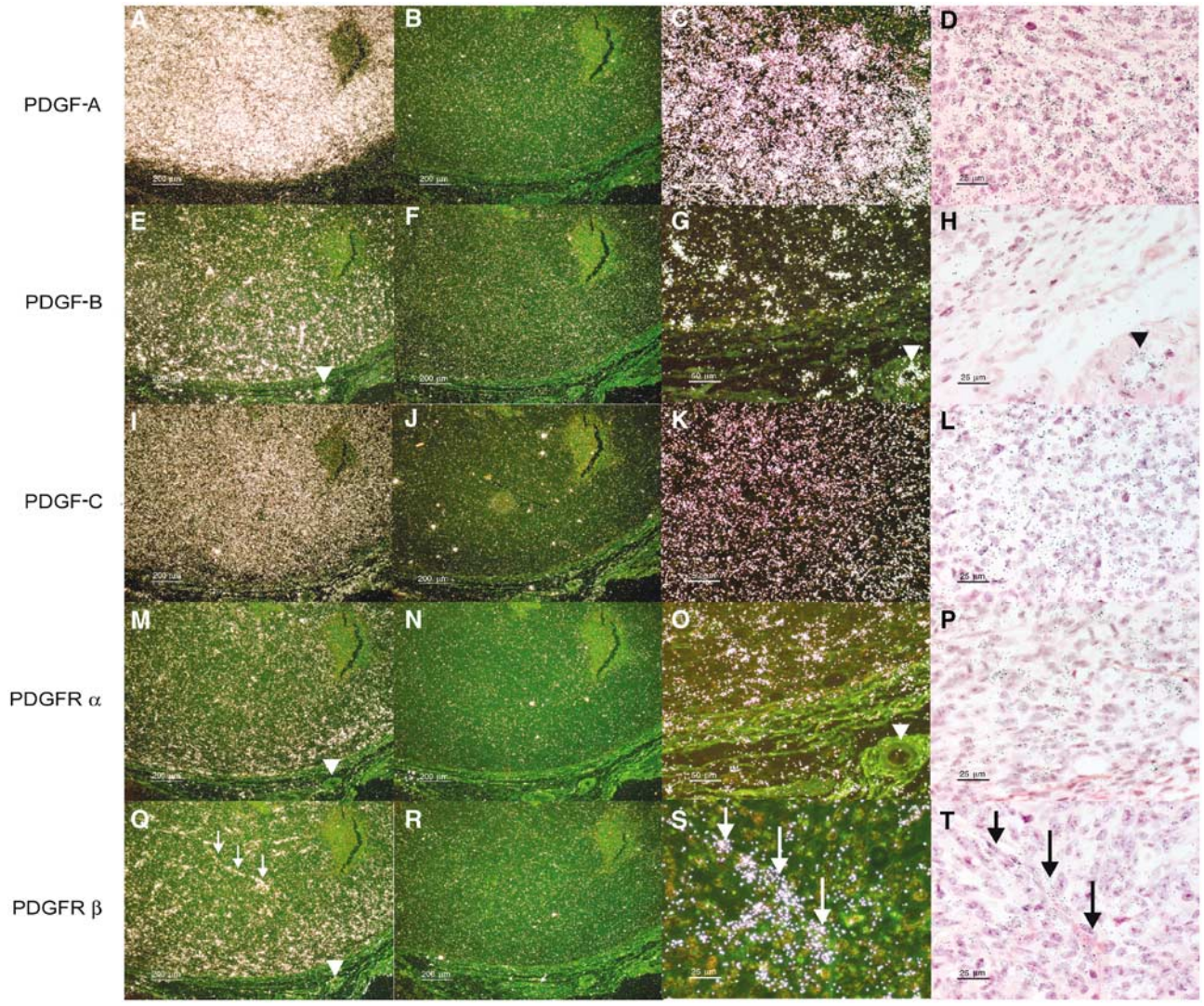

Figure 7 Analysis of PDGF-A, PDGF-B, PDGF-C, PDGFR α and PDGFR β expression in G5 tumors by *in situ* hybridization. Paraffin sections of G5 tumors were hybridized with ^{33}P-labeled riboprobes specific for PDGF-A, PDGF-B, PDGF-C, PDGFR α or PDGFR β as indicated. For each gene, antisense (columns 1, 3, 4) and control sense riboprobes (column 2) were applied to parallel sections. (**A–D**) PDGF-A expression is strong and uniform in the tumor mass. **(E–H)** PDGF-B expression occurs in discrete cell clusters consistent with vascular endothelial origin in tumors and is associated with vascular endothelial cells in the surrounding normal tissue (arrowheads at small arteriole in E, G, H). (**I–L**) PDFG-C signal is diffuse in tumors, and less strong than PDGF-A. (**M–P)** PDGFR α expression is associated with punctuate cell clusters consistent with stromal fibroblasts; no signal is associated with normal vessels in the surrounding tissue (arrowheads in M, O). (**Q–T)** PDGFR β expression is associated with stromal vessels (arrows in Q, S, T); positive signal is present in vascular smooth muscle in normal arterioles (arrowhead in Q). Parallel images were taken with bright-field (D, H, L, P, T) or dark-field (all others) illumination. Scale bars are 200 μm (A, B, E, F, I, J, M, N, Q, R) or 25 μm (C, D, G, H, K, L, O, P, S, T).

growth by 38%, indicating that both PDGFR α and PDGFR β signaling are important in the tumorigenic process. Furthermore, combination of Av-PDGFR α-IgG and Av-PDGFR β-IgG induced an additive inhibitory effect on G5 tumor growth compared to either one alone (Supplementary Figure 2). Av-PDGFR IgGs or soluble PDGFR IgGs had no direct inhibitory effect on tumor cell growth as they had no effect on G5 cell proliferation in culture (Figure 8B and Supplementary Figure 3). Thus, soluble PDGFR β-IgG inhibited G5 tumor growth most likely by interfering with the recruitment of pericytes, which provide VEGF and other factors for vessel survival (Darland *et al*, 2003); in contrast, soluble PDGFR α-IgG would disrupt the paracrine PDGFR α signaling between tumor cells and stromal fibroblasts, the major source of stromal VEGF. Consistently, we observed a significant decrease in the VEGF protein level in tumors treated with Av-PDGFR α-IgG and to a lesser degree in tumors treated with Av-PDGFR β-IgG (Figure 8C), suggesting that recruitment of VEGF-producing host stromal cells was reduced in the treated tumors. However, both Av-PDGFR α-IgG and Av-PDGFR β-IgG had a smaller (~18%) inhibitory effect on the growth of parental C2P tumors, which did not achieve statistical significance (Figure 8D). That tumor growth inhibition by Av-PDGFR IgGs was more effective on VEGF-null tumors than on parental C2P tumors is consistent with our hypothesis that VEGF-null cells are more dependent on PDGFR signaling-mediated recruitment of an angiogenic, VEGF-producing stroma.

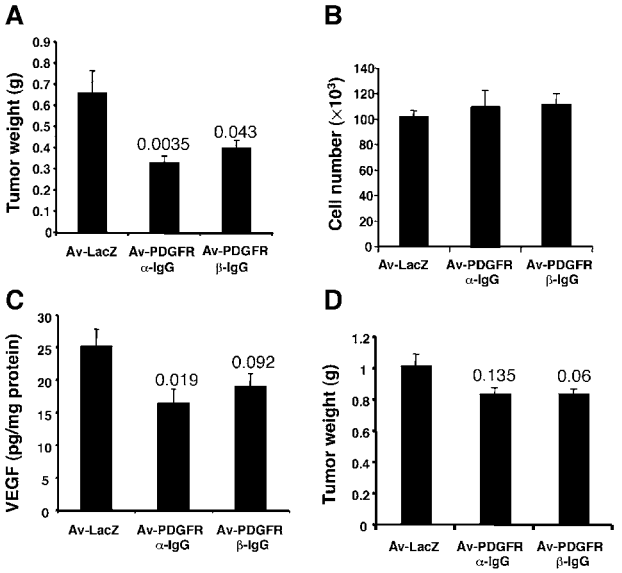

Figure 8 Inhibition of tumor growth by soluble PDGFR α-IgG and PDGFR β-IgG. (**A**) G5 tumor-bearing animals were treated with Av-LacZ, Av-PDGFR α-IgG or Av-PDGFR β-IgG once weekly. Tumor weight was determined 3 weeks later. (**B**) G5 cells in culture were infected with the indicated adenoviruses, and counted 5 days later. (**C**) ELISA was performed to measure VEGF protein concentrations in tumor lysates derived from different treatment groups. (**D**) C2P tumor-bearing animals were treated as described in (A). Student's *t*-test comparing the Av-PDGFR IgG treatment groups with the Av-LacZ group was performed to assess significance. $P<0.05$ was considered significant.

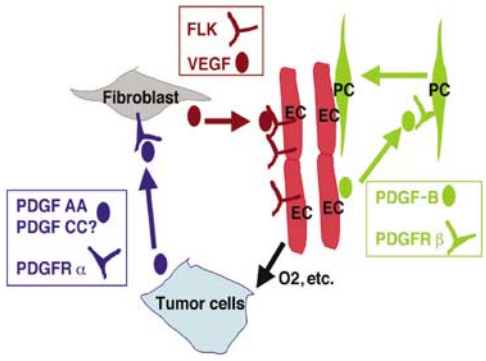

Figure 9 Multiple pathways involved in tumorigenesis by VEGF-null tumor cells. VEGF-null tumor cells secrete PDGF AA (and perhaps CC) to recruit host stromal fibroblasts through PDGFR α signaling pathway. The recruited host stromal fibroblasts in turn provide VEGF to simulate endothelial cell (EC) and to initiate angiogenesis. The stability of tumor vessels is dependent on the interaction between pericytes (PC) and endothelial cells (EC) via the PDGFR β signaling pathway.

Discussion

The importance of VEGF produced by the tumor cells in tumor angiogenesis has been well documented in various studies, while the contribution of stromal-derived VEGF is unclear (Grunstein *et al*, 1999; Shi and Ferrara, 1999; Gerber *et al*, 2000; Viloria-Petit *et al*, 2003). Using ras-transformed $VEGF^{-/-}$ MEFs, we demonstrated that tumor stroma is an important source of VEGF for tumor angiogenesis. Furthermore, we identified PDGFR α signaling as a mechanism utilized by the VEGF-null tumor cells to recruit VEGF-producing stromal fibroblasts (Figure 9). This role of PDGFR α signaling in stromal fibroblast recruitment and tumor angiogenesis is distinct from the specific role of PDGFR β signaling in pericyte recruitment. Since recruitment of host stromal fibroblasts is important for tumor angiogenesis and growth when tumor cells are deficient in VEGF production, the PDGFR α signaling pathway is likely to be an additional target for antiangiogenesis cancer therapy.

Our study demonstrated that stroma-produced VEGF played a significant role in tumor growth and neovascularization since anti-VEGF treatment significantly inhibited the growth of tumors grown from ras-transformed VEGF-null MEFs. This finding is at variance with a recent study using ras-transformed VEGF-null adult dermal fibroblasts (Viloria-Petit *et al*, 2003), which showed that even though appreciable levels of VEGF expression were detected in stromal cells, VEGF production appeared to have a modest role in oncogene-driven tumor angiogenesis. It is noteworthy that we used a newly developed potent anti-VEGF antibody (G6-23) in our study, while an antibody (DC101) against VEGFR-2/flk-1 was used in the study employing adult dermal fibroblasts. DC101 might not be able to block VEGF activity completely, as increasing evidence indicates that VEGFR-1/flt-1 may be a positive regulator in tumor angiogenesis (Hiratsuka *et al*, 2001, 2002; Luttun *et al*, 2002), and has specific roles in the recruitment of endothelial progenitor cells and monocytes (Hattori *et al*, 2002; Pipp *et al*, 2003), both of which could promote vessel growth. Thus, the seemingly conflicting results regarding stromal VEGF contribution to tumor angiogenesis between the two studies could be due, at least in part, to the difference in the efficacy of the VEGF inhibitors employed. In addition, differences in genetic compositions in the two cell sources and in the expression levels of VEGF or ras in the derivative tumors could also render variations in sensitivity to VEGF inhibition. Nevertheless, our findings are in agreement with one significant conclusion of the study by Viloria-Petit *et al* (2003) that at least some of the angiogenesis associated with fibrosarcoma growth is VEGF-independent. Although in the present study VEGF was found to be important for tumor angiogenesis in the fibrosarcomas originated from the ras-transformed MEFs, neither the anti-VEGF Mab G6-23 nor mFlt(1-3)-IgG treatment was able to block tumor growth completely. That such escape from VEGF inhibition was not due to incomplete VEGF blockade is strongly suggested by the finding that, in parallel experiments, the same inhibitors could completely block the *in vivo* growth of the A673 rhabdomyosarcoma cell line (data not shown). In the VEGF-null adult dermal fibroblast tumor model, ras-mediated downregulation of antiangiogenic factor thrombospondin-1 (TSP-1) was shown to be the driving force for tumor angiogenesis. In our system, TSP-1 expression in ras-transformed MEF cells was also found to be significantly lower compared to nontransformed cells, while there was little difference in TSP-1 expression between $VEGF^{-/-}$ and $VEGF^{+/+}$ ras-transformed tumor cells. Therefore, downregulation of TSP-1 by ras may have contributed to the partial tumor escape from VEGF dependence in our model as well. As oncogenic ras is known for both upregulating proangiogenic VEGF and downregulating antiangiogenic factor TSP-1

(Rak and Kerbel, 2001; Watnick *et al*, 2003), it may be necessary to target both factors to disrupt more efficiently oncogene-driven tumor angiogenesis.

Expression of PDGF gene family members has been shown in a number of solid tumors, ranging from gliomas to prostate cancers, and the role of PDGF signaling in these tumors can vary from autocrine stimulation of tumor cell growth to paracrine interaction between tumor cells and stromal cells (George, 2003; Pietras *et al*, 2003). In our fibrosarcoma model, PDGF autocrine signaling was not a significant factor in tumor growth because the tumor cells express little receptors and they were transformed with SV40 large T antigen and ras, both of which have been shown to downregulate PDGF signaling (Zhan and Goldfarb, 1986; Cook *et al*, 1993; Paasinen-Sohns and Holtta, 1997). Instead, we found that PDGFR α-mediated paracrine signaling between tumor cells and stromal fibroblasts was a principal mechanism for stroma recruitment, and demonstrated that this pathway was critical for tumor growth. Our finding underlines the importance of paracrine PDGFR α signaling in tumorigenesis, which has received very little attention so far. Moreover, we found very little PDGF-B expression in these ras-transformed tumor cells, while PDGF-B along with PDGFR β was detected mostly on stromal vessels within the tumor mass. The observation that soluble PDGFR β-IgG inhibited tumor growth is consistent with previous reports that PDGFR β signaling is essential for pericyte recruitment and tumor vessel stabilization (Abramsson *et al*, 2003; Bergers *et al*, 2003). It is also possible that PDGFR β may play a minor role in stromal fibroblast recruitment by interacting with tumor cell-produced PDGF-C and endothelial-derived PDGF-B. Our study implicates that the two PDGF signaling pathways mediated by PDGFR α and PDGFR β play distinct and complementary roles in tumor angiogenesis. These two pathways are conceivably more important for the tumorigenic properties of the VEGF-deficient tumor cells, as these cells rely on the recruitment of an angiogenic stroma for tumor angiogenesis.

Besides PDGF AA, other factors expressed by ras-transformed MEF cells might also play a minor role in stromal fibroblast recruitment, as the fibroblast stimulatory activity in tumor cell CM was substantially but not completely inhibited by soluble PDGFR α-IgG. We did detect some low level of transforming growth factor-β1 (TGF-β1) in our tumor cell CM, and it is possible that TGF-β1 expressed by tumor cells could also contribute to tumor angiogenesis by increasing VEGF production in stromal cells (Berking *et al*, 2001; Tuxhorn *et al*, 2002). Recruited stromal cells can potentially produce other angiogenic factors in addition to VEGF; thus blocking stromal fibroblast recruitment could have impacts on tumor angiogenesis and growth beyond just inhibiting VEGF. Although the focus of this current study was on stromal fibroblasts, we expect that the inflammatory cells in the tumor stromal compartment could also be a significant player in tumor angiogenesis and are currently investigating this possibility.

We have shown here that VEGF-mediated tumor angiogenesis is a complex process that both tumor cells and stromal cells contribute to, and that tumor cells can indirectly facilitate new blood vessel formation via their stimulatory effects on stromal cells. Furthermore, we have identified PDGFR α signaling pathway as an important mechanism for stromal fibroblast recruitment, which is an essential route for tumor angiogenesis when tumor cells are deficient in VEGF production. In agreement with others, we have also found that PDGFR β signaling localized on tumor vessels exerts an important function in tumor growth. The distinct roles of these signaling pathways in tumor angiogenesis warrant combination therapy targeting multiple pathways in order to reduce more efficiently the blood supply for tumors and thus to make better approaches for cancer therapy.

Materials and methods

Generation of VEGF-null ras-transformed mouse embryonic fibroblastic cell lines

MEFs were isolated from VEGF/*loxP*(+/+) mice, in which exon 3 of the VEGF gene was flanked by *loxP* sites (Gerber *et al*, 1999). The transgenic MEFs were immortalized by stable transfection with SV40 large T antigen and then transformed with a vector expressing oncogenic mutant H-Ras (Val-12). Subsequently, the VEGF/*loxp*(+/+) ras-transformed MEFs were infected with an adenovirus expressing cre recombinase to delete exon 3 of the VEGF gene. Several $VEGF^{-/-}$ subclones were isolated from the infected pool through limiting dilution. Cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 HAM (DMEM/F12, 50:50 mixture), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/ml penicillin and 100 μg/ml streptomycin.

Real-time quantitative RT–PCR analysis

RNA was isolated from culture cells or frozen tumor tissues using the Qiagen RNeasy kit, and RT–PCR reactions were run in a Model 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA) and the results were normalized to GAPDH levels. The sequences of primers and probes are presented in Supplementary material.

NIH 3T3 fibroblast migration

Migration assay was performed using 24 transwell FluoroBlok plates (BD Biosciences, Bedford, MA). A 500 μl portion of serum-free assay medium containing testing samples, recombinant human PDGF AA (30 ng/ml) or PDGF BB (10 ng/ml) (R&D systems, Minneapolis, MN) was added to the bottom chamber, and then 2×10^4 3T3 cells (ATCC, Manassas, VA) in 100 μl assay medium were plated into the top reservoir of the transwell. The assay was conducted for approximately 15 h at 37°C. The transmigrated cells on the bottom side of the membrane were stained with 10 μM YoPro dye (Molecular Probes, Eugene, OR). Images were taken under a Nikon fluorescence microscope using OpenLab software (Improvision, Lexington, MA), and the relative fluorescence unit (RFU), indicative of the number of cells migrated, was quantified using ImagePro Plus software (Media Cybernetics, Silver Spring, MD).

NIH 3T3 fibroblast proliferation

Cells were seeded into 96-well plates at a density of 3000 cells/well, and grown in complete culture medium (DMEM/F12 50:50, 10% FBS) for 1 day. Cells were then serum-starved for another day before being treated with testing samples, recombinant human PDGF AA (30 ng/ml) or PDGF BB (10 ng/ml) for approximately 24 h. [^{3}H]thymidine (1 μCi/well) was added to the culture for the last 6 h of treatment. Cells were harvested on Unifilter 96-well white microplate (GF-C) using a Packard Filtermate Harvester and were counted with a Packard luminescence counter (Packard, Meriden, CT).

Partial purification of stroma recruitment factors from CM

VEGF-null G5 cells were incubated in serum-free DMEM/F12 for 3 days. A 2 L volume of G5 CM was collected as initial starting material for purification. The CM was diluted in 25 mM sodium phosphate, pH 6.0, and then loaded to a 5 ml HiTrap S-Sepharose cation exchange column (Amersham Pharmacia, Sweden). The bound proteins were eluted stepwise from the column with buffers containing 0.2 M and 1 M NaCl. The bioactive fractions were pooled and applied to a TSK size-exclusion column (21.5 × 30 cm) (TOSOH Biosep LLC, Japan) equilibrated with 20 mM Tris buffer, pH 7.2, containing 2 M NaCl and 0.02% Tween 20. The subsequent

bioactive fractions were pooled, adjusted to 0.1% trifluoroacetic acid and applied to a reversed-phase C4 HPLC column (100×4.6 mm) (Eichrom Technologies, Darien, IL). Proteins were eluted with a linear gradient of 15–50% acetonitrile. Aliquots of each fraction were diluted and assayed for fibroblast migration and proliferation activity.

Adenovirus generation

Adenoviral vectors expressing chimeric human soluble PDGF receptors, PDGFR α-IgG or PDGFR β-IgG were generated using the AdEasy adenoviral vector system (BD Biosciences, Bedford, MA) according to the manufacturer's manuals. PDGFR α-IgG and PDGFR β-IgG consist of the extracellular domains of human PDGFR α and β respectively fused to the Fc region of human IgG1.

Fibrosarcoma formation and tumor growth inhibition studies

Five million cells in 0.1 ml of growth factor-reduced Matrigel (BD Biosciences, Bedford, MA) were injected subcutaneously into the dorsal flank region of 6- to 8-week-old beige nude mice from Harlan Sprague Dawley. At 2 days after tumor cell inoculation, mice were injected intraperitoneally with anti-VEGF G6-23 or an isotype control antibody anti-ragweed at 10 mg/kg body weight, twice weekly. There were 5–10 animals per treatment group. Tumor growth was monitored twice weekly by measurement of length (L) and width (W) with a vernier caliper and tumor volume was calculated using the formula $V = LW^2/2$ (Blaskovich *et al*, 2000). After 3 weeks, tumors were harvested, weighed and processed for histological analysis.

To assess the antitumor activity of the soluble PDGFR IgGs, adenoviruses expressing human chimeric PDGFR α-IgG (Av-PDGFR α-IgG), PDGFR β-IgG (Av-PDGFR β-IgG) or a control LacZ gene (Av-LacZ) were injected directly into tumor mass. Different cell lines and treatment conditions were tested in groups of 5–8 mice. The tumor-bearing mice were dosed at 1×10^9 PFU weekly, starting 1 day after tumor cell inoculation. After 3 weeks, tumors were weighed and harvested for pathology and histologic analyses.

Tumor cell proliferation

Cells were plated in 12-well plates at 2×10^4 cells/well and infected with Av-LacZ, Av-PDGFR α-IgG or Av-PDGFR β-IgG at 200 PFU/cell. Cells were cultured in growth medium containing 1% serum for 5 days and cell numbers were determined using a coulter counter.

In situ hybridization and immunohistochemistry

Tumors were fixed in 10% neutral buffered formalin for approximately 16 h prior to paraffin embedding. Sections 5 μm thick were processed for *in situ* hybridization (Ferrara *et al*, 2003a). ^{33}P-UTP-labeled sense and antisense probes were PCR amplified using primers described in Supplementary material. Immunohistochemical staining for VEGFR-2/flk-1 was performed as described (Gerber *et al*, 1999). Vessel surface density was measured using Photoshop software and reported in units/μm.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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