

PDGF-C Mediates the Angiogenic and Tumorigenic Properties of Fibroblasts Associated with Tumors Refractory to Anti-VEGF Treatment

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SUMMARY

Tumor- or cancer-associated fibroblasts (TAFs or CAFs) from different tumors exhibit distinct angiogenic and tumorigenic properties. Unlike normal skin fibroblasts or TAFs from TIB6 tumors that are sensitive to anti-VEGF treatment (TAF-TIB6), TAFs from resistant EL4 tumors (TAF-EL4) can stimulate TIB6 tumor growth even when VEGF is inhibited. We show that platelet-derived growth factor C (PDGF-C) is upregulated in TAFs from resistant tumors. PDGF-C-neutralizing antibodies blocked the angiogenesis induced by such TAFs in vivo, slowed the growth of EL4 and admixture (TAF-EL4 + TIB6) tumors, and exhibited additive effects with anti-VEGF-A antibodies. Hence, our data reveal an additional mechanism for TAF-mediated tumorigenesis and suggest that some tumors may overcome inhibition of VEGF-mediated angiogenesis through upregulation of PDGF-C.

INTRODUCTION

A variety of genetic/epigenetic changes within epithelial cells that initiate neoplastic transformation and promote invasive behavior and angiogenesis have been identified. In addition, signaling resulting from interactions between epithelial cells and stroma also contributes to tumor development (Bhowmick and Moses, 2005; Bissell and Radisky, 2001; Kalluri and Zeisberg, 2006; Red-Horse et al., 2007). Alterations in the stroma can promote epithelial tumorigenesis (Barcellos-Hoff and Ravani, 2000; Bhowmick et al., 2004; Maffini et al., 2004).

The stroma consists of extracellular matrix and mesenchymal cell types, which are fibroblasts and myofibroblasts, various inflammatory cells (Shojaei et al., 2008), endothelial cells, and pericytes (Orimo and Weinberg, 2006). Similar to transformed epithelial cells, tumor stroma may also exhibit drastic changes at the transcriptional level (Allinen et al., 2004). Adding tumor-associated fibroblasts (TAFs) to “initiated” but nontumorigenic or malignant epithelial cells stimulates tumor growth and angiogenesis in vivo (Olumi et al., 1999; Orimo et al., 2005). Orimo et al. (2005) demonstrated that SDF-1, which is upregulated in tumor

fibroblasts, stimulates tumor cell proliferation and angiogenesis. TGF- β 1 and HGF have been also implicated in the tumor-promoting effects of TAFs (Bhowmick et al., 2004; Kuperwasser et al., 2004). Moreover, a recent study has indicated that gene expression signatures within the stroma can be useful prognostic predictors of breast cancer progression (Finak et al., 2008).

Fibroblasts are thought to promote tumor growth in part through stimulation of tumor angiogenesis. One key mediator of angiogenesis is vascular endothelial growth factor A (VEGF-A) (Ferrara, 2004). VEGF-A has been also reported to promote recruitment of bone marrow-derived cells (Grunewald et al., 2006). Targeting VEGF-A, in combination with chemotherapy, is efficacious in treating several human tumors (Ellis and Hicklin, 2008; Ferrara and Kerbel, 2005; Kerbel, 2008).

Platelet-derived growth factor C (PDGF-C) (Li et al., 2000) is a member of the PDGF family (Aase et al., 2002; Andrae et al., 2008; Ding et al., 2000; Fredriksson et al., 2004). PDGF-C signaling through PDGF receptor (PDGFR) α and β homo- or heterodimers is important for the development of connective tissues and for wound healing (Andrae et al., 2008; Li et al.,

SIGNIFICANCE

Tumor-associated fibroblasts (TAFs) are active players in tumorigenesis. Here we show that TAFs may mediate resistance to antiangiogenic therapy with VEGF inhibitors. We identified platelet-derived growth factor C (PDGF-C) as a key mediator of TAF-induced angiogenesis. Blocking PDGF-C using specific antibodies reduces the growth of tumors that are resistant to anti-VEGF treatment and exhibits additive effects with anti-VEGF therapies. Hence, our data indicate that targeting stroma-derived mediators such as PDGF-C can be complementary to anti-VEGF strategies.

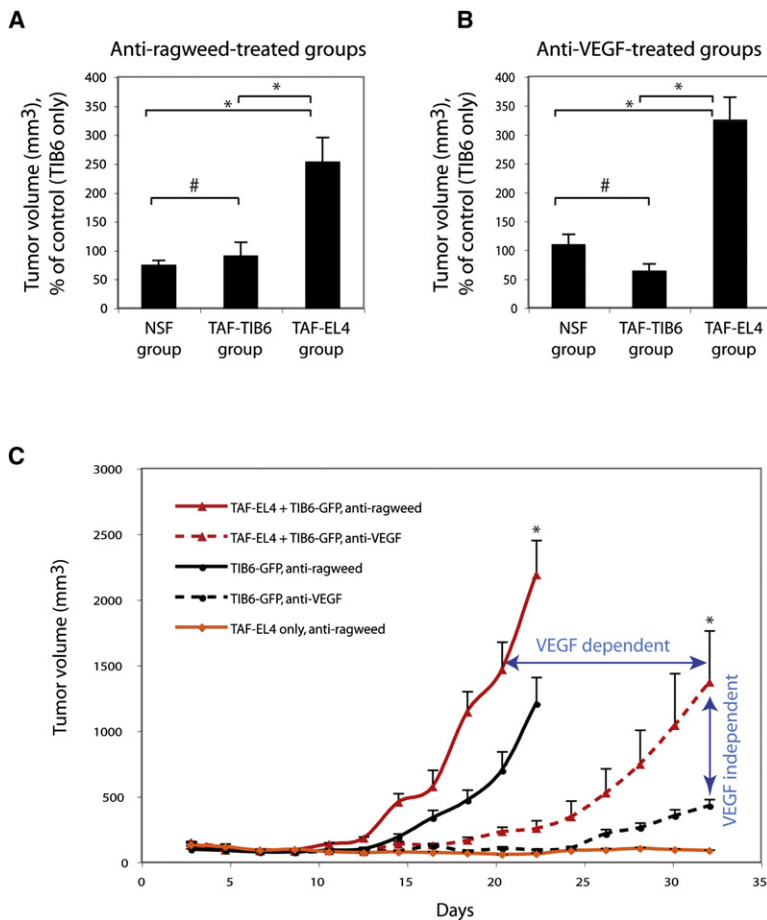


Figure 1. TAF-EL4 Promote TIB6 Tumor Growth in the Absence or Presence of VEGF Inhibition

(A) Addition of TAF-EL4, but not normal skin fibroblasts (NSFs) or TAF-TIB6, to TIB6 tumors promotes TIB6 tumor growth in anti-ragweed-treated animals ($*p < 0.0001$, TAF-EL4 group versus NSF group; $*p < 0.015$, TAF-EL4 group versus TAF-TIB6 group; $\#p = 0.23$, NSF group versus TAF-TIB6 group). Data are presented as % change in tumor volume relative to TIB6-only control across several independent experiments ($n = 3, 2$, and 5 , respectively).

(B) Addition of TAF-EL4, but not NSFs or TAF-TIB6, to TIB6 tumors results in increased TIB6 tumor growth in anti-VEGF mAb-treated animals ($*p < 0.00005$, TAF-EL4 group versus NSF group; $*p < 0.0018$, TAF-EL4 group versus TAF-TIB6 group; $\#p = 0.08$, NSF group versus TAF-TIB6 group). Data reflect several independent experiments ($n = 3, 2$, and 6 , respectively).

(C) TAF-EL4-mediated tumorigenesis contains VEGF-dependent and -independent mechanisms ($*p < 0.05$). Data are shown as means \pm SEM.

2000; Pietras et al., 2003). Recombinant PDGF-C stimulates angiogenesis in chick embryos and mouse corneas (Cao et al., 2002) and revascularizes ischemic tissues (Li et al., 2005). PDGF-C has also been implicated as an autocrine growth regulator of Ewing tumor cell lines in vitro (Zwerner and May, 2002).

Several studies have shown effects of TAFs on tumor growth and angiogenesis, but it is unclear whether there are any tumor-specific effects of TAFs. In this study, we examined TAFs isolated from two types of tumors, one of which is sensitive to anti-VEGF-A treatment and the other of which is refractory (Shojaei et al., 2007a). Unlike normal skin fibroblasts (NSFs) or TAFs from sensitive tumors, TAFs from refractory tumors can promote in vivo growth of VEGF-sensitive tumors, even in the presence of VEGF inhibitors. Our data indicate that PDGF-C is upregulated in TAFs derived from refractory tumors and that PDGF-C, together with VEGF-A, mediates the angiogenesis induced by such TAFs in vivo. This study also demonstrates that inhibition of PDGF-C may reduce tumor angiogenesis and growth in vivo.

RESULTS

Isolation and Culture of Primary Fibroblasts from Normal Skin and Subcutaneous Tumors

TAFs were isolated from EL4 (TAF-EL4) and TIB6 (TAF-TIB6) tumors of ~ 1000 mm³ in size. EL4 tumors are refractory to

anti-VEGF treatment, whereas TIB6 tumors are sensitive (Shojaei et al., 2007a) (see Figure S1 available online). All primary fibroblasts (NSFs, TAF-EL4, and TAF-TIB6) exhibited spindle morphology and expressed fibroblast surface protein (FSP) (Figures S2A and S2B). B16F1 cells, in contrast, did not express FSP. Primary fibroblasts were also positive for α -smooth muscle actin (α -SMA) (Figure S2C). Moreover, NSFs and TAFs (TAF-EL4 and TAF-TIB6) were largely negative for CD31 and CD45 expression (Figure S2D). Less than 0.3%, 0.3%, and 1% of cells were CD31 positive, CD45 positive, or double positive respectively for all isolated fibroblasts.

TAF-EL4 Promote TIB6 Tumor Growth Even in the Presence of an Anti-VEGF Antibody

Both TIB6 and TIB6-GFP were employed for recombination experiments. TIB6 and TIB6-GFP tumors exhibit similar responses to anti-VEGF treatment, although the growth rate of TIB6-GFP cells in mice is slower than that of TIB6 cells. Xenografts containing only TIB6 cells were used as a control and were implanted simultaneously with each admixture group. The addition of NSFs or TAF-TIB6 to TIB6 tumors did not alter TIB6 tumor growth (Figure 1A; Figures S3Aa and S3Ab). In contrast, the addition of TAF-EL4 significantly enhanced TIB6 tumor growth (Figures 1A and 1C; Figure S3Ac). The increase in tumor mass was not due to EL4 tumor cell contamination in the fibroblast preparation, since implants containing only TAF-EL4 fibroblasts did not develop any tumors (Figure 1C; Figure S3Ac). Furthermore, histological analysis revealed only a minimal number of fibroblasts (Figure S3Ad), indicating that increased tumor mass reflects the growth of TIB6 tumor cells rather than TAF-EL4. Tumors from all groups displayed histology similar to control TIB6 tumors (Figure S3Ad).

Similar in vivo recombination experiments were performed in the presence of anti-VEGF monoclonal antibody (mAb). Neither

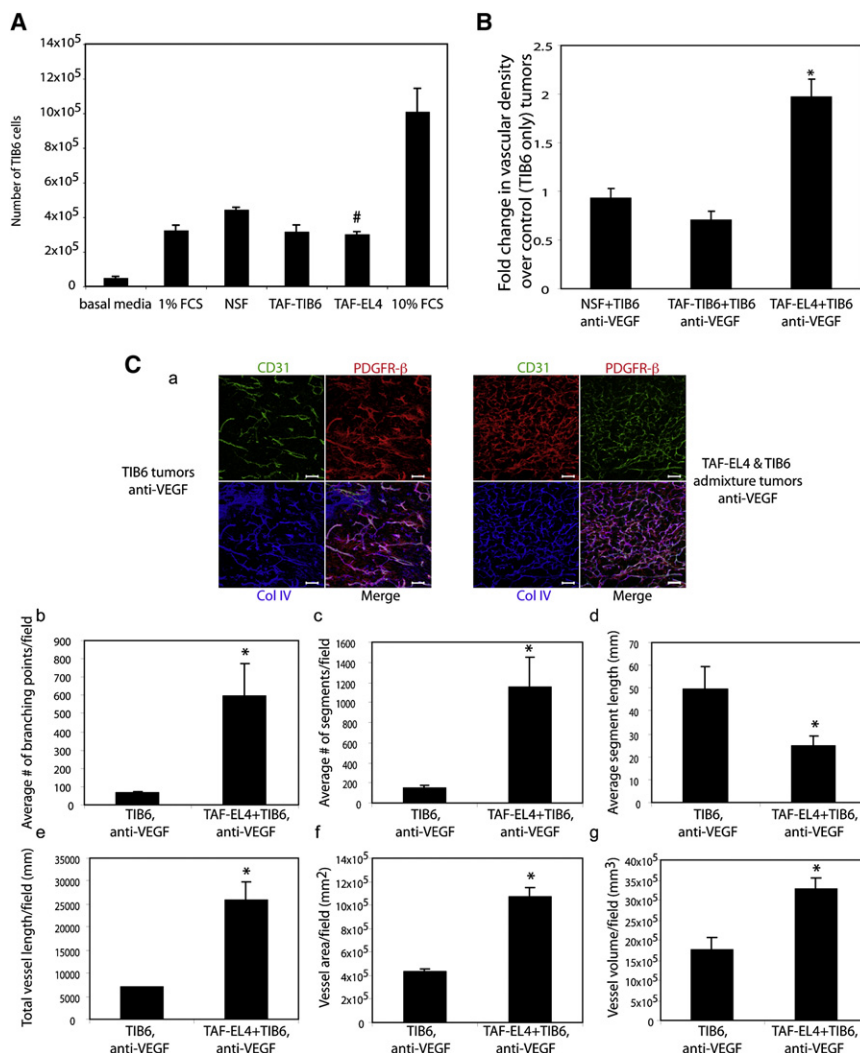


Figure 2. TAF-EL4 Stimulate Tumor Angiogenesis In Vivo

(A) TAF-EL4 are comparable to TAF-TIB6 in stimulating TIB6 cell growth in vitro (#p = 0.58, TAF-EL4 versus TAF-TIB6; n = 4).

(B) Addition of TAF-EL4 to TIB6 tumors stimulates tumor angiogenesis. Vascular density for each admixture tumor was normalized to the simultaneous TIB6 control tumors (*p < 0.0001, TAF-EL4 group versus NSF group and TAF-TIB6 group). Eight to fourteen fields were analyzed for each group of tumors harvested at day 26.

(C) TAF-EL4 and TIB6 admixture tumors exhibit denser vascular networks than TIB6 control tumors at day 26.

(Ca) Confocal images of TIB6 tumors and TAF-EL4 and TIB6 admixture tumors. Scale bars = 100 μ m.

(Cb-Cg) Vasculature quantifications for control TIB6 tumors and admixture tumors containing TAF-EL4 and TIB6 cells. Entire TIB6 tumor images and three random fields of the admixture tumors were used for quantification.

(Cb) Number of branching points was determined by counting the intersection points (*p < 0.05).

(Cc) Average number of vessel segments per field (*p < 0.05).

(Cd) Average segmental length per field (*p < 0.04).

(Ce) Average total vessel length per field (*p < 0.02).

(Cf) Average total vessel area per field (*p < 0.005).

(Cg) Average total vessel volume per field (*p < 0.02). Data shown are means \pm SEM.

NSFs nor TAF-TIB6 promoted TIB6 tumor growth when VEGF was inhibited (Figure 1B; Figures S3Ba and S3Bb). In contrast, addition of TAF-EL4 induced TIB6 tumor growth in the presence of VEGF blockade (Figures 1B and 1C; Figure S3Bc). Admixture of TIB6 and TAF-EL4 cells generated larger tumors (Figures S3Bc and S3Bd) compared to TIB6 controls. Again, few fibroblasts were observed in the admixture tumors (Figure S3Be). We also used TIB6-GFP tumor cells in recombination experiments. GFP-labeled TIB6 cells were the predominant cell type within the admixture tumors (Figure S3Bf). TAF-EL4-only implants did not grow over the duration of the experiment (Figure S3Bc). These findings argue against the possibility that contaminating EL4 (GFP-negative) tumor cells contributed to the growth of admixture tumors. VEGF-dependent mechanisms are involved in TAF-EL4's ability to promote TIB6 tumor growth, since the growth of admixture tumors containing TAF-EL4 and TIB6 cells was reduced by anti-VEGF antibodies (Figure 1C).

TAF-EL4 Are Not Different from TAF-TIB6 in Stimulating TIB6 Cell Growth In Vitro

We cocultured TIB6 cells with NSFs, TAF-TIB6, or TAF-EL4 over a period of 3 days. TAF-EL4 did not perform better

than NSFs or TAF-TIB6 in stimulating TIB6 proliferation in vitro (Figure 2A). Similarly, no difference was observed among various fibroblasts (NSFs or TAFs) in stimulating EL4 proliferation (Figure S4).

TAF-EL4 Promote Tumor Angiogenesis in the Presence of VEGF Blockade

We performed CD31 immunostaining in multiple sections from tumors with the largest and median sizes in all groups. Admixture tumors containing TAF-EL4 and TIB6 cells exhibited extensive vascular networks (Figures 2B and 2C; Figure S5). The microvascular density in TIB6 and TAF-EL4 admixture tumors was 2-fold higher than that in TIB6 control tumors (Figure 2B). In contrast, the vascular networks in admixture tumors containing NSFs or TAF-TIB6 and TIB6 cells were similar to those of the simultaneously harvested TIB6 control tumors.

We analyzed 80 μ m tumor sections by confocal microscopy and reconstructed 3D images. We measured the number of vessel branching points, number of vessel segments, segment length, total vessel length, vessel area, and vessel volume for TAF-EL4 and TIB6 admixture tumors and control TIB6 tumors. Addition of TAF-EL4 fibroblasts to TIB6 tumors led to a marked increase (~6-fold) in the number of vessel branching points (Figure 2Cb) and segmental vessels (~6 fold) (Figure 2Cc) and

reduced average segmental vessel length (~ 2 -fold) (Figure 2Cd) but increased total vessel length (>3 -fold) (Figure 2Ce), vessel area (~ 2.5 -fold) (Figure 2Cf), and vessel volume (~ 2.5 -fold) (Figure 2Cg) per field. We did not observe any difference in percent vessel coverage by PDGFR β^+ cells between TAF-EL4 and TIB6 admixture tumors and control TIB6 tumors ($72.6\% \pm 1.95$ and $74.5\% \pm 2.15$, respectively). We observed, however, that many PDGFR β^+ cells were also dispersed throughout TIB6 tumors, whereas the majority of PDGFR β^+ cells within the admixture tumors were associated with vessels (Figure 2C).

TAFs from Anti-VEGF-Refractory Tumors Induce Angiogenesis In Vivo

We injected fibroblasts in growth factor-deprived Matrigel subcutaneously into mice and allowed vessels to develop for 10 days. All primary fibroblasts (NSFs, TAF-TIB6, and TAF-EL4) expressed PDGFR β as assessed by flow cytometry (data not shown). Matrigel plugs without fibroblasts did not contain any CD31- or PDGFR β -positive cells (Figure 3A), suggesting little or no vessel formation and minimal invasion by host fibroblasts or pericytes. Very few vessels were observed in Matrigel plugs containing NSFs or TAF-TIB6. However, PDGFR β -positive cells were present within the implants, suggesting the survival of the implanted fibroblasts. In contrast, TAF-EL4 implants contained a well-developed microvasculature that was supported by PDGFR β -positive cells (Figures 3A and 3C). CD31-positive areas within Matrigel only, NSFs, TAF-TIB6, and TAF-EL4 implants amounted to 0.004%, 0.13%, 0.35%, and 10%, respectively (Figure 3B).

TAFs from two additional tumors refractory to anti-VEGF treatment, TIB42 and TIB48 (Figure S1), were able to induce angiogenesis in Matrigel plug assays (Figures 3C and 3D). We also isolated TAFs from GFP-positive EL4 and TIB6 tumors. However, only TAF-EL4 were able to induce angiogenesis in vivo (Figure 3E). No GFP-positive tumor cells were present in the implants, whereas such cells were readily observed in the positive controls. Similar experiments were also carried out using TAF-TIB48 from TIB48-GFP tumors. Again, TAF-TIB48 alone induced angiogenesis in vivo without contaminating TIB48-GFP cells (Figure 3D, left panel). These data indicate that the angiogenesis we observed in TAF-EL4 or TAF-TIB48 implants was due to signals from TAF-EL4 or TAF-TIB48 cells, not from contaminating tumor cells.

The onset of angiogenesis involves invasion of tip cells, followed by stalk endothelial cells, to populate avascular areas (Davis and Senger, 2005; Gerhardt and Betsholtz, 2005). Close examination of vessels within TAF-EL4 implants at high magnification ($40\times$) revealed that many CD31-positive endothelial cells possessed tip-cell-like phenotypes (i.e., multiple cytoplasmic extensions) (Figure 3F).

TAF-EL4 Express Higher Levels of VEGF-A, but Not SDF-1, In Vivo

We measured VEGF concentrations in NSFs, TAF-TIB6, and TAF-EL4 both in vitro and in vivo. TAF-TIB6 and TAF-EL4 secreted higher levels of VEGF than NSFs in vitro (Figure 4A). However, VEGF levels in TAF-EL4 or TAF-TIB6 conditioned media were similar. We then measured VEGF from fibroblast implant lysates. Unlike the in vitro data, VEGF levels were highest

in the TAF-EL4 groups (Figure 4B). TAF-TIB6 in vivo implants contained the lowest amounts of VEGF (~ 50 pg/ml). These data suggest that VEGF might partially account for the ability of TAF-EL4 to induce angiogenesis. However, NSF implants, which exhibit only 2-fold lower VEGF production, did not induce any microvascular sprouting, suggesting that one or more additional factors produced by TAF-EL4 cooperate with VEGF to stimulate angiogenesis.

It has been reported that TAFs promote tumor growth via up-regulation of SDF-1 (Orimo et al., 2005). However, we found reduced levels of SDF-1 in TAF-EL4 and TAF-TIB6 implants compared to NSF implants (Figure 4C), suggesting that the angiogenesis observed in TAF-EL4 implants is not mediated by SDF-1.

PDGF-C Is Upregulated in TAFs from Refractory Tumors

We compared gene expression profiles of TAFs from resistant versus sensitive tumors by microarray analysis. Unsupervised clustering analysis of genes that exhibited statistically significant changes revealed a pattern that clearly separated TAFs of resistant tumors (TAF-EL4, TAF-TIB42, and TAF-TIB48) from NSFs and TAF-TIB6 (Figure 5A). Microarray analysis (and subsequent TaqMan analysis) revealed that *PDGF-C*, *Angptl2*, *HOX-A10* and *A7*, and *COX-2* were among the upregulated genes in TAFs from resistant tumors. The complete data set is available at the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) with the accession number GSE13549.

We focused our analysis on secreted or cell surface proteins. Among the genes that demonstrated reproducible changes across multiple probes included in the microarray chip, *PDGF-C* was consistently upregulated in TAFs from three different resistant tumors. While all TAFs exhibited higher PDGF-C expression than NSFs did, expression of PDGF-C was highest in TAFs from resistant tumors (Figure 5B). Surprisingly, we observed a downregulation of SDF-1 in TAFs from refractory tumors (Figure 5C).

We also dissociated TIB6 and EL4 tumors and flow-sorted cells into four different populations: tumor cells (GFP $^+$), enriched fibroblasts (GFP $^-$ CD31 $^-$ CD45 $^-$), endothelial cells (GFP $^-$ CD31 $^+$ CD45 $^-$), and bone marrow-derived cells (including resident macrophages) (GFP $^-$ CD31 $^-$ CD45 $^+$). TaqMan analysis on such freshly isolated cells indicated that all cell types within TIB6 tumors had minimal PDGF-C expression (Figure 5D). Cells from EL4 tumors exhibited elevated levels of PDGF-C compared to those in TIB6 tumors, the highest expression being observed in enriched fibroblasts.

VEGF and PDGF-C Are Required for TAF-Induced Angiogenesis In Vivo

To determine whether the angiogenesis induced by TAF-EL4 requires VEGF-A, we treated the animals harboring the implants with anti-VEGF mAb for 30 days. We observed some vessels in TAF-EL4 implants treated with anti-VEGF. However, when compared to the control antibody-treated group, vascular densities were markedly reduced (Figure 6A; Figure S6), indicating that VEGF is required for the full angiogenic response.

We next asked whether PDGF-C is required for TAF-EL4-induced angiogenesis. We used a neutralizing affinity-purified anti-mPDGF-C IgG. Western blot analysis demonstrated that

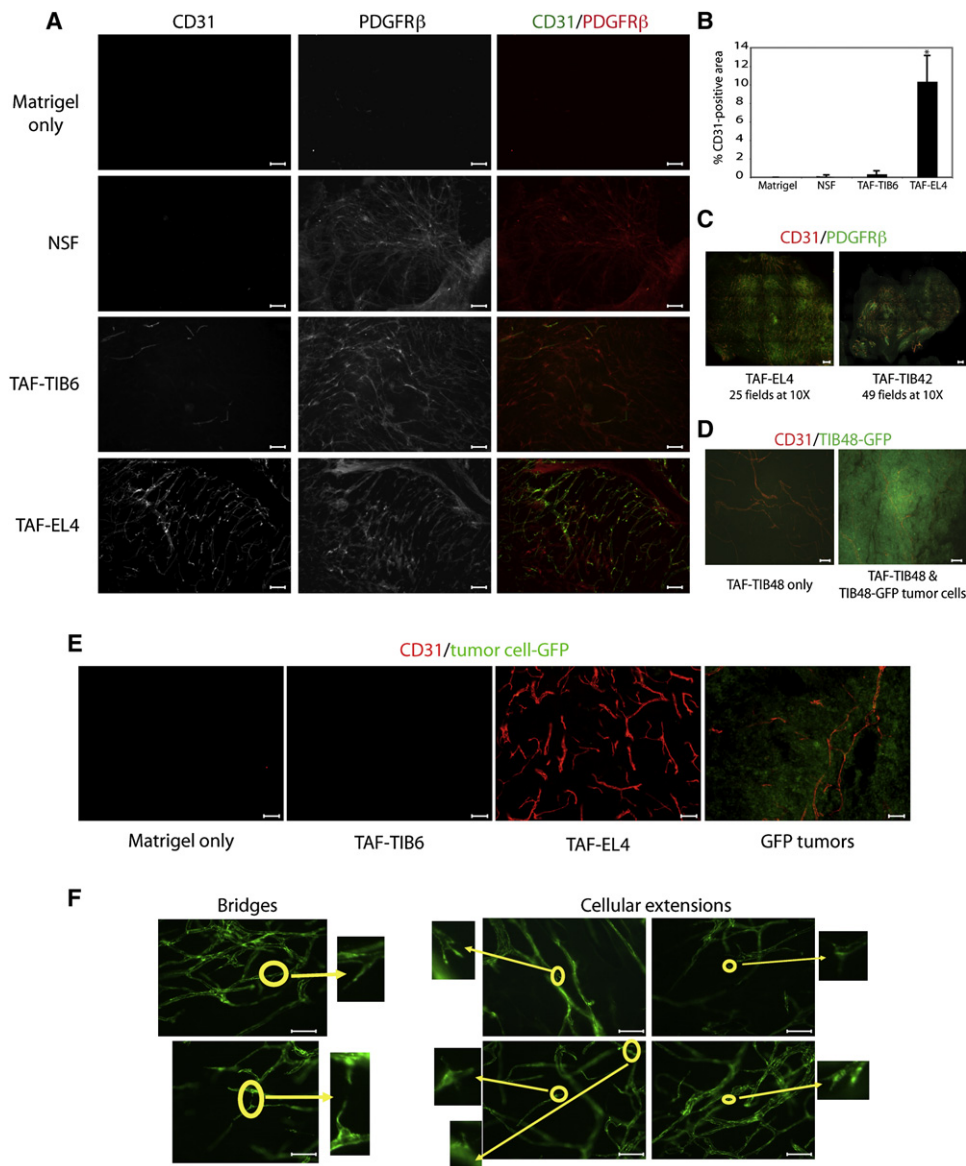


Figure 3. TAFs from Anti-VEGF-Refractory Tumors Induce In Vivo Angiogenesis in Matrigel Plug Assays

(A) TAF-EL4-induced angiogenesis in vivo. Scale bars = 100 μ m.

(B) CD31-positive areas in Matrigel implants (* $p < 0.01$, TAF-EL4 versus TAF-TIB6; $n = 5-6$). Data are shown as means \pm SEM.

(C) Overviews of TAF-EL4- and TAF-TIB42-induced angiogenesis in Matrigel plugs. Images shown here contain the entire Matrigel plugs and are tiled pictures of 25 or 49 images taken at 10 \times , respectively. Scale bars = 200 μ m.

(D) TAF-TIB48 are able to induce angiogenesis in vivo. As a positive control, TAF-TIB48 were mixed with TIB48-GFP tumor cells prior to implantation (right panel). Scale bars = 100 μ m.

(E) TAF-EL4-induced angiogenesis does not result from EL4 tumor cell contamination. TAF-TIB6 and TAF-EL4 fibroblasts were isolated from TIB6-GFP and EL4-GFP tumors, respectively. No GFP-positive tumor cells were observed in either TAF-TIB6- or TAF-EL4-only implants. GFP-positive tumor cells were used as positive control. Scale bars = 100 μ m.

(F) Cells with tip-cell-like phenotypes were observed in TAF-EL4-only implants. Scale bars = 50 μ m.

this antibody interacted strongly with mouse or human PDGF-C but had no detectable interaction with PDGF-A, PDGF-B, PDGF-D, or VEGF-A (Figure S7). In addition, the antibody specifically neutralized PDGF-C-induced endothelial cell migration (see below).

TAF-EL4 fibroblasts mixed with PBS, goat IgG, or anti-PDGF-C IgG were implanted subcutaneously. Addition of anti-PDGF-C

IgG to TAF-EL4 implants significantly delayed the angiogenic response (Figures 6B and 6C). There was >6-fold reduction in normalized vascular density in TAF-EL4 implants containing anti-PDGF-C antibody as compared to TAF-EL4 implants containing goat IgG (Figure 6C). The requirement for PDGF-C in TAF-induced angiogenesis was not limited to TAF-EL4, since similar results were obtained with TAF-TIB42 cells (data not

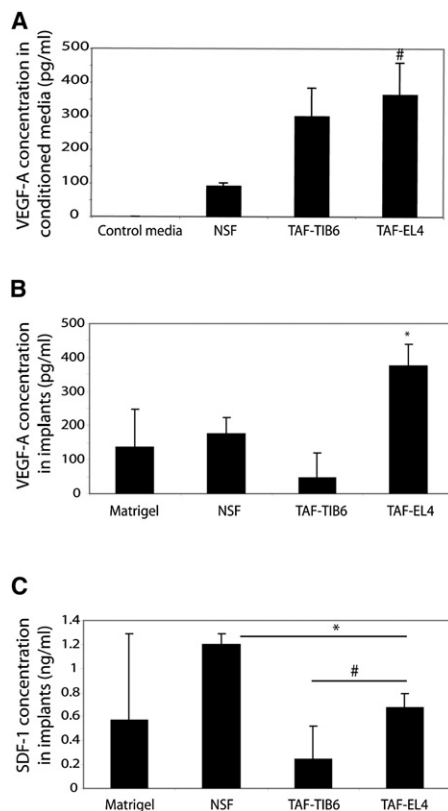


Figure 4. VEGF-A, but Not SDF-1, Is Upregulated in TAF-EL4 Implants

(A) VEGF-A concentrations in conditioned media ($\#p = 0.63$, TAF-EL4 versus TAF-TIB6; $n = 3$ independent experiments).

(B) VEGF concentrations in lysates from Matrigel implants ($*p < 0.05$, TAF-EL4 versus NSF implants; $n = 4$ each).

(C) SDF-1 concentrations in Matrigel lysates ($*p < 0.003$, TAF-EL4 versus NSF implants; $\#p = 0.38$, TAF-EL4 versus TAF-TIB6 implants; $n = 4$ each).

Data are shown as means \pm SEM.

shown). Incubation of TAF-EL4 or TAF-TIB42 with anti-PDGF-C did not affect TAF viability (Figure S8), indicating that the reduction in vascular density resulting from anti-PDGF-C was not due to impaired TAF viability.

Conversely, we added recombinant PDGF-C to nonangiogenic TAF-TIB6 in Matrigel plugs. While TAF-TIB6 alone were unable to induce angiogenesis, addition of PDGF-C ($\sim 3.3 \mu\text{g}$) led to an increase in vascular density compared to control (Figures 6D and 6E). SDF-1 ($\sim 3.3 \mu\text{g}$), a cytokine that has been shown to induce angiogenesis in other biological settings, did not induce such effects under the conditions tested (Figures 6D and 6E).

Anti-PDGF-C Antibodies Suppress the Growth of EL4 and TAF-EL4 Admixture Tumors and Are Additive with Anti-VEGF Antibodies

Addition of TAF-EL4 to TIB6-GFP tumor cells promoted TIB6 tumor growth in both anti-ragweed- and anti-VEGF-treated groups (Figure 7A). Single-agent treatment of admixture tumors with anti-PDGF-C IgG resulted in $\sim 63\%$ growth inhibition at day 20 relative to goat IgG (Figure 7A; Figure S9). Moreover, combination of anti-PDGF-C and anti-VEGF antibodies further reduced the

growth of admixture tumors compared to monotherapy with anti-VEGF mAb. By day 34, the combination resulted in $\sim 74\%$ growth inhibition compared to anti-VEGF alone. We next tested the efficacy of anti-PDGF-C antibodies on growth of EL4 tumors. Anti-PDGF-C IgG reduced the growth of EL4 tumors by $\sim 32\%$ (Figures 7Ba and 7Bd). A comparable degree of inhibition ($\sim 37\%$) was achieved using anti-VEGF mAb (Figures 7Bb and 7Bd). Greater inhibition ($\sim 62\%$) was observed in animals treated with both anti-PDGF-C and anti-VEGF antibodies (Figures 7Bc and 7Bd).

In contrast, anti-PDGF-C IgG had no effect on TIB6 tumor growth (Figure 7Ca), whereas anti-VEGF mAb resulted in a dramatic inhibition (Figures 7Cb and 7Cd). These findings are consistent with the minimal expression of PDGF-C in TAFs or other cell fractions from TIB6 tumors (Figure 5D).

PDGF-C Induces Migration of Endothelial Cells, but Not of CD11b⁺Gr1⁺ Cells

To identify the potential target (or targets) of PDGF-C, we analyzed the expression levels of PDGFR α and β in different cell populations within TIB6 and EL4 tumors. In addition to PDGF-C, PDGFR α and β were upregulated in enriched fibroblasts, endothelial cells, and bone marrow cells within EL4 tumors (Figure 8A). Since previous studies (Shojaei et al., 2007a) demonstrated that tumor infiltration by CD11b⁺Gr1⁺ myeloid cells, which also express CD45, can facilitate tumor growth in the presence of anti-VEGF antibodies, we tested whether PDGF-C might recruit CD11b⁺Gr1⁺ myeloid cells. CD11b⁺Gr1⁺ cells isolated from bone marrow of TIB6 or EL4 tumor-bearing mice expressed very low levels of PDGF-C receptors (Figure 8A). Accordingly, PDGF-C did not induce the migration of myeloid cells (Figure 8B). However, SDF-1 strongly induced migration of these cells. Moreover, PDGF-C did not upregulate any proangiogenic genes tested (VEGF-A and B, PLGF, HGF, FGF2, PDGF-A, -B, and -C, Angpt2, Angptl1 and 2, MMP9, and MMP13) in CD11b⁺Gr1⁺ cells (data not shown).

While mesenchymal cells are PDGF-C's primary target, PDGF-C can also induce migration of mouse endothelial progenitors and human endothelial cells (Li et al., 2005). Endothelial cells within EL4 tumors expressed PDGFRs, raising the possibility that the PDGF-C-mediated angiogenesis we observed could be explained at least in part by its direct effect on endothelial cells. We isolated and cultured primary mouse endothelial cells (mECs) from normal lungs. mECs expressed mRNAs for PDGFR α and β (Figure 8A), VEGFRs, and other endothelial cell specific markers (Figures S10 and S11). VEGF-A induced mEC migration in vitro (Figure 8Ca). PDGF-C induced migration of mECs comparable to VEGF (Figure 8Cb). Anti-PDGF-C IgG specifically blocked mEC migration induced by PDGF-C, whereas goat IgG had no inhibitory effects.

DISCUSSION

We chose murine lymphoma models for our study because tumor-free TAFs could be easily isolated from these tumors, providing a suitable and reproducible experimental system. TAF-EL4, but not TAF-TIB6, were able to stimulate the in vivo growth of the anti-VEGF-sensitive TIB6 tumor, suggesting that signals from tumor cells can regulate TAF tumorigenic properties. Furthermore, TAF-EL4 can also stimulate TIB6 tumor

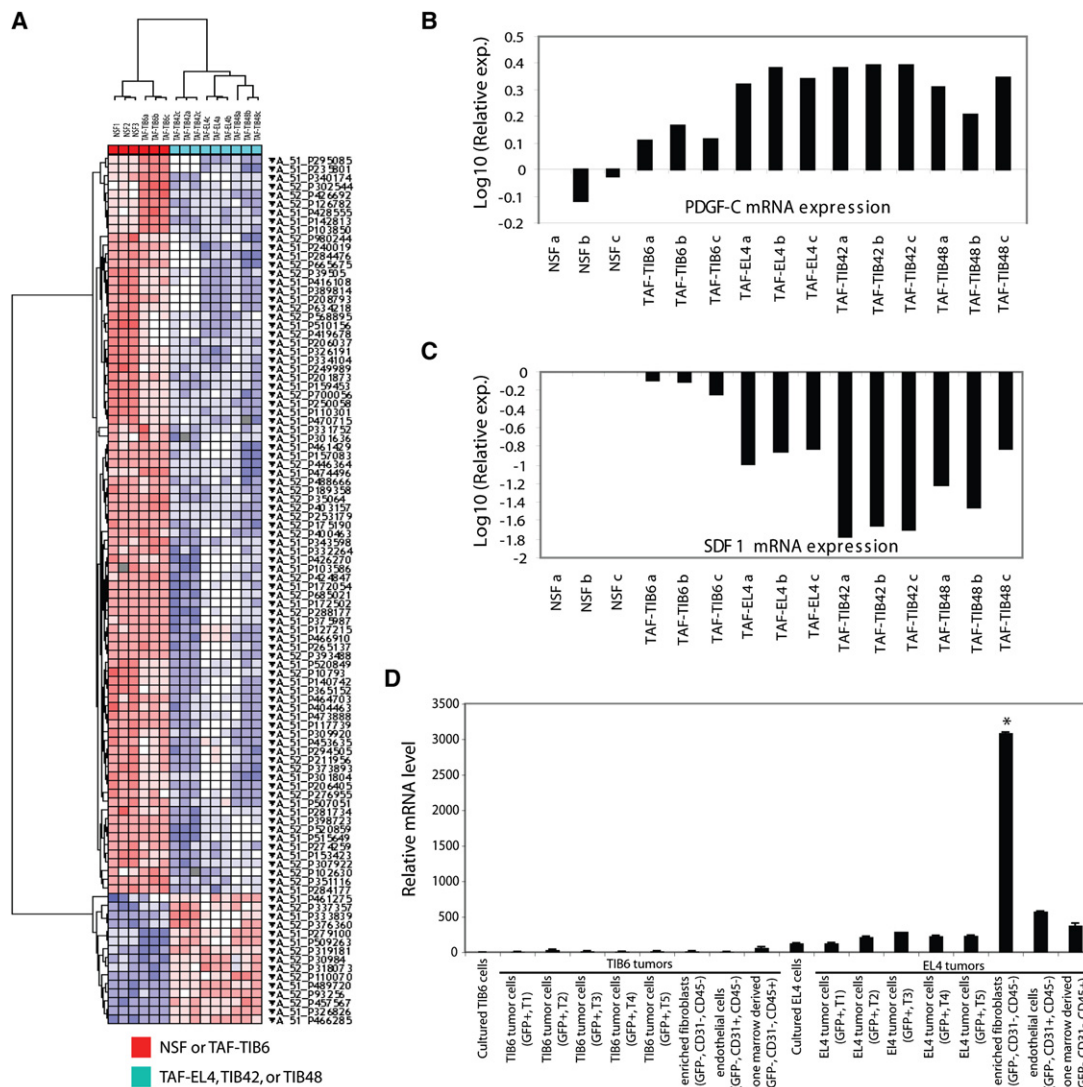


Figure 5. PDGF-C Is Upregulated in TAFs from Anti-VEGF-Resistant Tumors

(A) Clustering analysis for genes that exhibited statistically significant changes in TAFs from anti-VEGF-resistant tumors (EL4, TIB42, and TIB48) versus NSFs and TAF-TIB6.

(B and C) PDGF-C (B) and SDF-1 α (C) expression in NSFs, TAF-TIB6, TAF-EL4, TAF-TIB42, and TAF-TIB48 cells.

(D) PDGF-C expression in different cell types within TIB6 and EL4 tumors. Tumors ($\sim 1000 \text{ mm}^3$; $n = 5$ each) were sorted into four different populations based on expression of GFP, CD31, and CD45: tumor cells (GFP $^+$), enriched fibroblasts (GFP $^-$ CD31 $^-$ CD45 $^-$, pooled), endothelial cells (GFP $^-$ CD31 $^+$ CD45 $^-$, pooled), and bone marrow-derived cells (GFP $^-$ CD31 $^+$ CD45 $^+$, pooled). Enriched fibroblasts from EL4 tumors expressed ~ 200 -fold more PDGF-C mRNA than those from TIB6 tumors (* $p < 0.0001$). Data are shown as means \pm SEM.

growth even when VEGF signaling is inhibited, suggesting that TAFs can modulate tumors' responses to anti-VEGF therapy. Our analysis revealed both VEGF-dependent and -independent mechanisms for TAF-EL4-mediated tumor promotion. Other studies investigating tumor resistance/refractoriness to antiangiogenic therapies with VEGF blockers have identified additional potential mechanisms, including expression of alternative angiogenic factors (Bergers and Hanahan, 2008; Fischer et al., 2007), selection of hypoxia-resistant tumor cells (Yu et al., 2002), and recruitment of myeloid cells (Shojaei et al., 2007a).

TAFs have been reported to upregulate CXCL14 and SDF-1 (CXCL12), which may directly stimulate tumor cell proliferation

(Allinen et al., 2004; Orimo et al., 2005). However, in our study, we did not find any evidence that TAF-EL4 potentiate tumor growth by promoting tumor cell proliferation. Rather, TAF-EL4 were able to stimulate tumor angiogenesis. Interestingly, in admixture tumors containing both TAF-EL4 and TIB6 cells, we also observed a close association between PDGFR β -positive cells and blood vessels, while in TIB6 control tumors, some PDGFR β -positive cells were associated with vessels and some were dispersed throughout the tumor. This observation suggests that TAF-EL4, which also express PDGFR β , may be able to support the survival and/or stability of newly formed vessels when VEGF is inhibited. Furthermore, TAF-EL4 or TAFs isolated

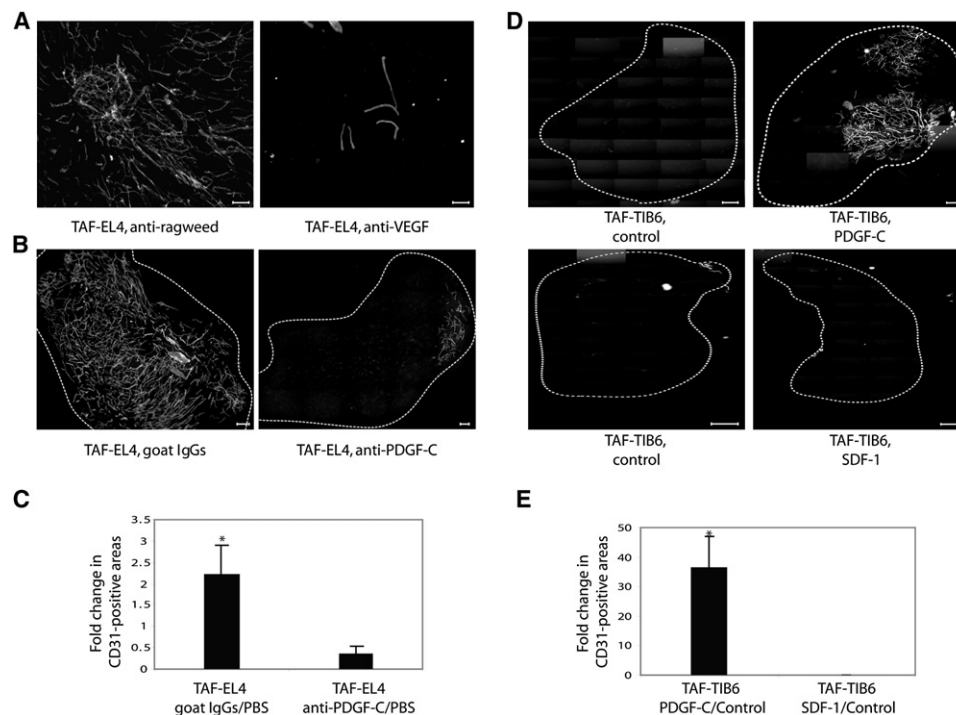


Figure 6. Both VEGF and PDGF-C Are Required for TAF-EL4-Induced Angiogenesis In Vivo

(A) Anti-VEGF mAb impairs angiogenesis induced by TAF-EL4. Vessels were stained with anti-CD31 antibody, and images were taken at 10 \times . Scale bars = 200 μ m.

(B) Anti-PDGF-C IgG significantly reduces angiogenesis in TAF-EL4 Matrigel implants. Images shown here are tiled pictures of 20–120 images taken at 10 \times . Antibodies were administered once at the time of implantation (see [Experimental Procedures](#)). Three independent experiments were performed. Dotted line marks the edge of Matrigel implants. Scale bars = 200 μ m.

(C) Fold change in vascular density of implants containing control IgG or anti-PDGF-C IgG after normalization to the simultaneous PBS control implants (* $p < 0.02$; $n = 9$ each). Data are shown as means \pm SEM. Each mouse was implanted with two Matrigel plugs with control PBS on the left flank and goat IgG or anti-PDGF-C IgG on the right flank.

(D) Addition of PDGF-C, but not SDF-1, leads to angiogenesis in TAF-TIB6 Matrigel implants. The images are tiled pictures of 30–90 images combined. Dotted line marks the edge of implants. Scale bars = 500 μ m.

(E) Fold change in vascular density of implants containing PDGF-C or SDF-1 versus controls (* $p < 0.01$; $n = 6$ each). Data are shown as means \pm SEM.

from other resistant tumors were able to induce angiogenesis in vivo, demonstrating that the ability to induce such activity is maintained regardless of the presence of tumor cells. These data suggest that epigenetic and/or genetic modulations may have occurred in such TAFs. In this context, previous studies have reported genetic alterations in tumor-associated stromal cells (Pelham et al., 2006). In other cases, epigenetic modulations may be involved (Qiu et al., 2008). However, we did not observe any statistically significant copy number gains or losses in any fibroblasts used in this study (Figure S12).

VEGF-A is a major regulator of angiogenesis and is expressed by fibroblasts (Dong et al., 2004). Therefore, it is possible that the tumor-promoting effects of TAF-EL4 in animals treated with anti-VEGF result at least in part from upregulation of VEGF-A. In vivo, VEGF-A concentrations were highest in TAF-EL4 implants. VEGF was indeed important for angiogenesis, since treatment with anti-VEGF antibodies significantly reduced CD31-positive areas in such implants. However, the concentrations of VEGF in TAF-EL4 implants were low and only about 2-fold higher than that in NSF implants, which did not trigger any angiogenesis. This observation, together with the finding that 5 μ g/ml of VEGF alone did not induce angiogenesis in the Matrigel plug assay over the

same time period (data not shown), suggests that one or more other factors from TAF-EL4 synergize with VEGF. SDF-1 has been characterized as a mediator of TAF-induced tumor angiogenesis (Orimo et al., 2005). However, in our system SDF-1 does not appear to be involved, highlighting the complexity and multiplicity of pathways that regulate angiogenesis.

Our analysis shows that fibroblasts from EL4 tumors upregulate PDGF-C compared to fibroblasts from TIB6 tumors. The largest difference was observed when comparing freshly isolated fibroblasts from EL4 and TIB6 tumors. Among different cell populations within EL4 tumors, fibroblasts expressed the highest level of PDGF-C mRNA. Moreover, PDGFR α and β were upregulated in fibroblasts, endothelial cells, and bone marrow-derived cells from EL4 tumors, suggesting possible autocrine and paracrine effects of PDGF-C during EL4 tumor progression. The receptors for PDGF-C, PDGFR α and β , (Cao et al., 2002), have been detected in various cell types including endothelial precursor cells, tumor endothelial cells, pericytes, and fibroblasts (Bergers et al., 2003; Dong et al., 2004; Marx et al., 1994; Pietras et al., 2003; Rolny et al., 2006). Our data point to tumor-specific upregulation of PDGFR α and β in TAFs, endothelial cells, and bone marrow-derived cells.

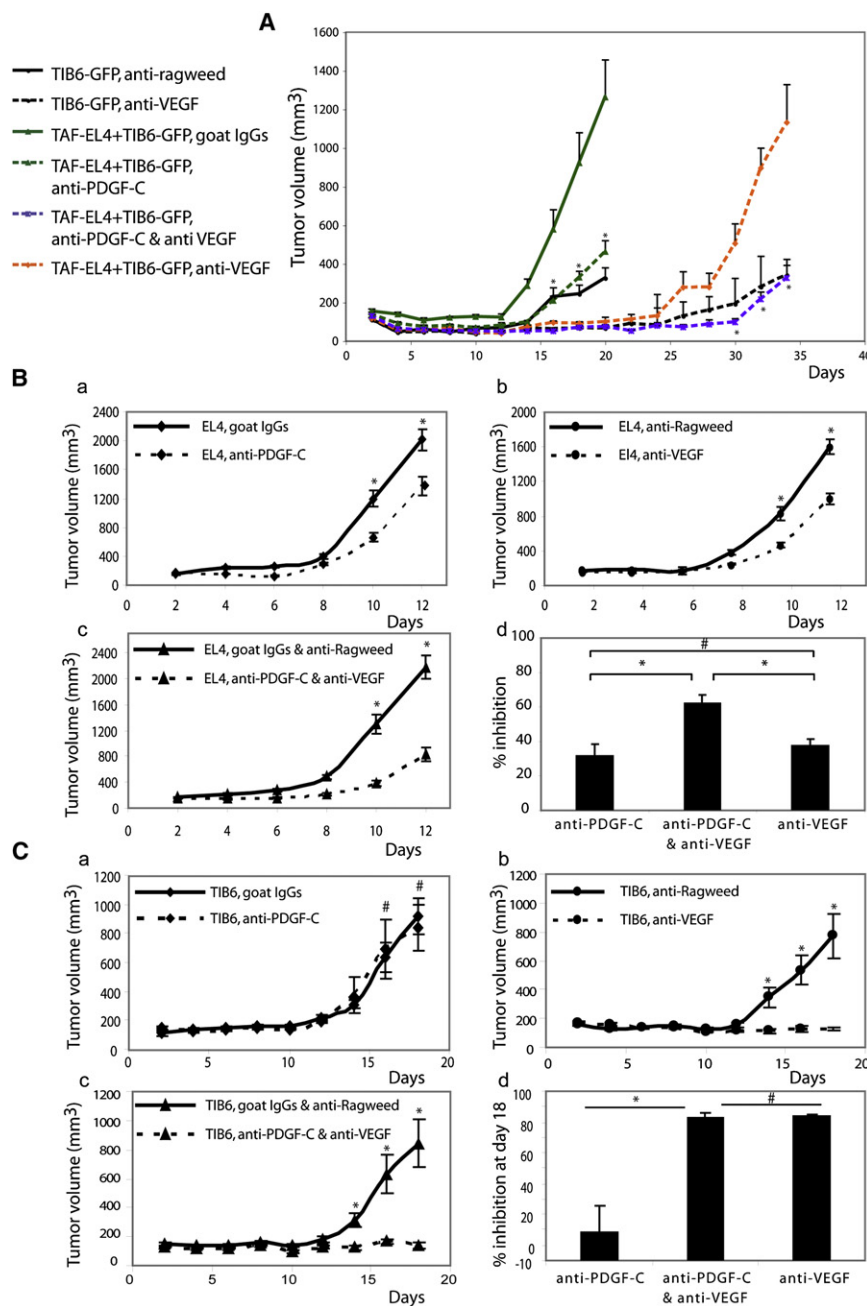


Figure 7. Effects of Anti-PDGF-C and Anti-VEGF Antibodies on Tumor Growth

(A) Anti-PDGF-C IgG reduces growth of admixture tumors as a single agent ($p < 0.006$, 0.005 , and 0.003 , respectively; $n = 10$ each). Combination of anti-PDGF-C and anti-VEGF antibodies induced greater inhibition of admixture tumors than anti-VEGF treatment alone ($p < 0.001$, < 0.001 , and < 0.001 , respectively; $n = 10$ for combination treatment group, $n = 5$ for anti-VEGF-treated group). Data for all control treatment groups are included in Figure S9.

(Ba) Anti-PDGF-C IgG reduces EL4 tumor growth in vivo ($p < 0.001$ and $p < 0.005$, respectively; $n = 10$ each).

(Bb) Anti-VEGF mAb reduces EL4 tumor growth in vivo ($p < 0.001$ and $p < 0.0001$, respectively; $n = 10$ each).

(Bc) Combination of both anti-PDGF-C and anti-VEGF blocking antibodies leads to a further inhibition of EL4 tumor growth ($p < 0.0005$ and $p < 0.0001$, respectively; $n = 10$ each).

(Bd) Percent of tumor growth inhibition for single or combination treatments ($p < 0.001$, anti-PDGF-C treatment group versus combination treatment group; $p < 0.001$, anti-VEGF treatment group versus combination treatment group; $\#p = 0.42$, anti-PDGF-C treatment group versus anti-VEGF treatment group).

(Ca) Anti-PDGF-C IgG has no effect on TIB6 tumor growth ($\#p = 0.79$ and 0.7 , respectively; $n = 10$ each).

(Cb) Anti-VEGF mAb suppresses TIB6 tumor growth ($p < 0.001$; $n = 10$ each).

(Cc) Combination treatment does not further reduce TIB6 tumor growth ($p < 0.001$; $n = 10$ each).

(Cd) Percent of tumor growth inhibition for single or combinational treatments at day 18 ($p < 0.0006$, anti-PDGF-C treatment group versus combination treatment group; $\#p = 0.93$, combination treatment group versus anti-VEGF treatment group).

Data are shown as means \pm SEM.

Given that CD11b⁺Gr1⁺ myeloid cells have been shown to confer refractoriness to anti-VEGF treatment (Shojaei et al., 2007a), we examined the possibility that PDGF-C may recruit and/or activate such cells. However, PDGF-C did not stimulate migration of CD11b⁺Gr1⁺ cells from naive or tumor-bearing animals, nor did it induce transcriptional upregulation of proangiogenic genes. Consistent with these findings, CD11b⁺Gr1⁺ cells expressed relatively low levels of PDGFR α and β . Potentially, other bone marrow-derived cell types within EL4 tumors that express higher levels of PDGFR α and β might respond to PDGF-C signaling and contribute to PDGF-C-mediated tumorigenesis.

Unlike CD11b⁺Gr1⁺ cells, endothelial cells demonstrated a chemotactic response to PDGF-C, comparable to that induced

by VEGF-A. These findings are also consistent with other studies reporting direct chemotactic effects of PDGF-C on human endothelial cells (Li et al., 2005).

Together, these observations support the hypothesis that PDGF-C can directly act on endothelial cells, providing a rationale for its ability to promote angiogenesis.

Intriguingly, upregulation of PDGF-C occurs in several human tumors (Lokker et al., 2002; Ostman and Heldin, 2001; Zwerner and May, 2001), including pancreatic carcinomas (Genentech Gene Logic analysis, data not shown). Pancreatic cancers are enriched in fibroblastic stroma and have shown a very limited response to most therapies, including combinations of anti-VEGF antibodies and chemotherapy (Ellis and Hicklin, 2008; Ferrara and Kerbel, 2005; Kerbel, 2008). Ectopic expression of PDGF-C in mice induces fibrosis and carcinogenesis (Campbell et al., 2005). PDGF-C may also act directly on tumor cells to promote their survival and/or proliferation (Lokker et al., 2002).

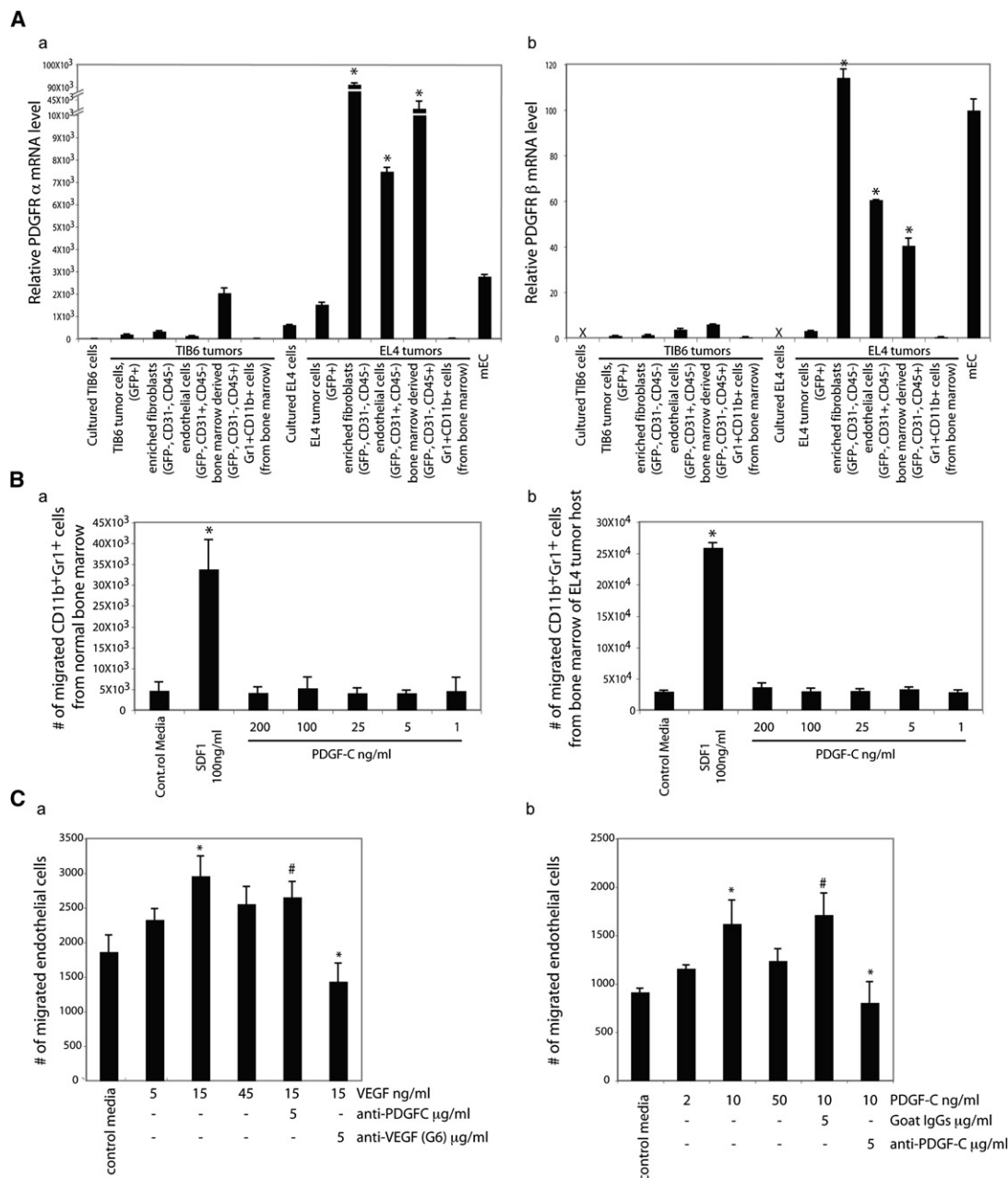


Figure 8. PDGF-C Stimulates Migration of Endothelial Cells, but Not of CD11b⁺Gr1⁺ Myeloid Cells

(Aa) Relative PDGFR α mRNA levels. Enriched fibroblasts, endothelial cells, and bone marrow-derived cells in EL4 tumors express higher levels of PDGFR α than their counterparts in TIB6 tumors (* $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively). mEC, mouse endothelial cells.

(Ab) Relative PDGFR β mRNA levels. Enriched fibroblasts, endothelial cells, and bone marrow-derived cells in EL4 tumors express higher levels of PDGFR β than their counterparts in TIB6 tumors (* $p < 0.001$, $p < 0.0001$, and $p < 0.009$, respectively). X, undetectable levels.

(Ba and Bb) PDGF-C does not induce migration of CD11b⁺Gr1⁺ myeloid cells isolated from normal mice (Ba) or EL4 tumor-bearing mice (Bb).

(Ca) VEGF stimulates mEC migration (* $p < 0.02$, VEGF versus control media). Anti-VEGF mAb blocks VEGF-mediated mEC migration (* $p < 0.004$), whereas anti-PDGF-C antibodies do not (# $p = 0.2$).

(Cb) PDGF-C induces mEC migration (* $p < 0.03$, PDGF-C versus control). Anti-PDGF-C antibodies block PDGF-C-mediated mEC migration (* $p < 0.036$), whereas control IgGs have no effect (# $p = 0.4$).

Data are shown as means \pm SEM.

EL4 tumor cells express PDGFR α . However, PDGF-C did not stimulate EL4 tumor cell proliferation or migration in vitro (Figure S13).

In this study, we demonstrate that an anti-PDGF-C neutralizing antibody can inhibit the angiogenic effects of TAFs isolated from tumors refractory to anti-VEGF treatment. A combination

therapy utilizing both anti-PDGF-C and anti-VEGF antibodies was more effective than anti-VEGF treatment alone. However, this combination did not completely block tumor growth, suggesting that additional mechanisms are involved. Indeed, we have previously reported that CD11b⁺Gr1⁺ myeloid cells stimulate tumor angiogenesis via another secreted protein, Bv8, and contribute to tumor resistance to anti-VEGF treatment (Shojaei et al., 2007a, 2007b). These findings suggest that, within the same tumor, multiple molecular and cellular mechanisms resulting in VEGF-independent angiogenesis may coexist. While our analysis here focused on PDGF-C, array data identified additional potential candidates such as Angptl2, which has been implicated in angiogenesis (Oike et al., 2004). Whether Angptl2 plays a role in tumor refractoriness to anti-VEGF therapy remains to be determined.

Previous studies suggested that a combination of VEGF antagonists with PDGFR inhibitors, such as imatinib, might be a particularly effective anticancer strategy (Bergers et al., 2003; Erber et al., 2004; Jo et al., 2006). However, recent studies suggest that such an approach is associated in some cases with marked toxicity (Hainsworth et al., 2007). It is tempting to speculate that a combination therapy utilizing a more selective inhibition of the PDGF axis, such as that achieved by targeting PDGF-C, may offer some advantages, at least in some circumstances.

In conclusion, we have demonstrated that activation of VEGF-independent proangiogenic programs in TAFs is tumor specific. Once activated by the tumor environment, TAFs can maintain their ability to induce angiogenesis independent of tumor cells, thus suggesting an irreversible process. We have also identified PDGF-C as an important mediator of TAF-induced angiogenesis and tumorigenesis *in vivo*.

EXPERIMENTAL PROCEDURES

Cell Lines

The EL4, TIB6 (J558), TIB42 (R1.1), and TIB48 (BW5147.G.1.4) cell lines were purchased from the American Type Culture Collection and cultured in DMEM supplemented with 10% fetal bovine serum.

Isolation and Culture of Normal or Tumor-Associated Fibroblasts

Normal skin fibroblasts (NSFs) were isolated from dorsal skin of beige/nude/xid mice. Tumor-associated fibroblasts (TAFs) were isolated from tumors reaching ~1000 mm³ in size from the same genetic background. To generate TAFs, 1 × 10⁶ cells were injected subcutaneously into the dorsal flank of 8- to 10-week-old beige/nude/xid mice. Tissues were digested with RPMI with 0.037% hyaluronidase (Sigma), 1 mg/ml collagenase/dispase (Roche), 10% FBS, and antibiotics for 1–2 hr at 37°C. Dissociated cells were filtered through both 100 µm and 40 µm filters. Washed filtrates were plated onto 1% gelatin-coated plates and cultured in 10% FBS-containing medium in an atmosphere of 3% O₂. Suspension tumor cells were removed after vigorous washing or flow sorting. All NSFs and TAFs used in this study had undergone fewer than ten population doublings in culture.

TaqMan Analysis

All TaqMan primers used were from Applied Biosystems. All analyses were carried out on a standard ABI 7500 machine using the SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's recommended protocols.

Antibodies

Anti-VEGF-A mAb G6-31 was derived from human Fab phage libraries (Liang et al., 2006). The variable domains were grafted into the murine IgG2a constant

domain. The ability of mAb G6-31 to potentially neutralize mouse or human VEGF-A *in vitro* and *in vivo* has been previously documented (Gerber et al., 2007; Yu et al., 2008). Anti-ragweed mAb was used as a control for mAb G6-31 (Liang et al., 2006). Neutralizing, affinity-purified anti-mPDGF-C goat IgG was purchased from R&D Systems (AF1447). Goat IgG was used as a control.

Western Blot

Human and murine PDGF-C (R&D Systems), murine VEGF-A₁₆₄ (R&D Systems), human VEGF-A₁₆₅ (Genentech, Inc.), murine PDGF-A and PDGF-B (Antigenix America Inc.), and human PDGF-D (R&D Systems) were loaded into duplicate 4%–20% SDS-PAGE (Invitrogen). All proteins were purified from *E. coli*, except murine VEGF-A, which was expressed in insect cells. Five hundred nanograms of each protein was loaded. PVDF membranes were then incubated overnight with 0.2 µg/ml anti-mouse PDGF-C IgG (R&D Systems, AF1447) or with 10 µg/ml mAb G6-31. The blots were incubated with ImmunoPure HRP-conjugated rabbit anti-goat IgG or goat anti-mouse IgG (Pierce) at a dilution of 1:10,000 and detected using a western blotting detection system (GE Healthcare Bio-Science).

Immunohistochemistry and Flow Cytometry

Live NSFs and TAFs were stained with FITC- or APC-conjugated anti-CD45 and anti-CD31 antibodies (BD Pharmingen). For α -SMA staining, cells were fixed in 4% PFA prior to staining with FITC-conjugated α -SMA (Sigma). The percentage of cells expressing CD45, CD31, and α -SMA was quantified using flow cytometry.

In Vivo Recombination Experiments

Animal studies were approved by the Genentech animal care committee and performed according to regulatory standards. 8 × 10⁵ TIB6 tumor cells in growth factor-deprived Matrigel (BD Biosciences) or admixtures of TIB6 cells and NSFs or TAFs at a ratio of 1:2.5 were injected subcutaneously in 8- to 10-week-old beige/nude/xid mice. Treatment with anti-ragweed or anti-VEGF-A mAb G6-31 was initiated 48 hr after cell inoculation and was administered once a week thereafter at a dose of 5 mg/kg. *In vivo* recombination experiments were divided into three groups using NSFs, TAF-TIB6, or TAF-EL4. Each group contained both control and anti-VEGF-treated arms.

In Vivo Matrigel Plug Assay

NSFs or TAFs were mixed with growth factor-deprived Matrigel and injected subcutaneously in beige/nude/xid mice at a density of ~2 × 10⁶ cells/100 µl of Matrigel. The Matrigel implants were cleared, harvested, and cryofrozen at day 8 for detection of VEGF and SDF-1 levels and at day 10 for vascular staining. In some cases, fibroblasts were mixed with control solutions, goat IgGs (3.3 µg), anti-PDGF-C IgG (3.3 µg), recombinant PDGF-C (3.3 µg), or SDF-1 (3.3 µg) prior to injection. Neutralizing anti-mouse PDGF-C IgG (AF1447) and recombinant PDGF-C and SDF-1 were from R&D Systems. PDGF-C stock solution contained 4 mM HCl. All control experiments for PDGF-C contained the same amount of diluted HCl.

Coculture Studies

NSFs or TAFs were plated onto 1% gelatin-coated 24-well plates and cultured under 3% O₂. TIB6 and EL4-GFP cells were added to the fibroblast cultures, which were in 1% FCS, the next day. The number of TIB6 or EL4 cells after 3 days of coculture was counted using a Z2 particle counter and size analyzer (Beckman).

Vascular Staining in Cryofrozen Samples

Cryofrozen tumor tissues or Matrigel plugs were sectioned at 80 µm thickness. Each slide contained two 80 µm sections that were at least 1000 µm apart in the tumor samples. Slides were stained with primary antibodies including hamster anti-mouse CD31 antibody (Chemicon), rat anti-mouse PDGFR β antibody (eBioscience), or rabbit anti-mouse collagen IV antibody (Cosmo Bio Co., Ltd.). Samples were then stained with secondary antibodies including FITC-, Cy3-, or Cy5-conjugated goat anti-hamster or anti-rat from Jackson ImmunoResearch.

Vascular Density Quantification

Vascular density was assessed by measuring CD31⁺ areas per field using ImageJ software. To quantify vascular branching points, segmental length, vessel length, vessel area, and vessel volume, 3D confocal images of the vascular network were reconstructed (80 μ m in depth) using FilamentTracer and quantified using Imaris software. Each intersection of two or more branches was identified automatically for quantifying the number of branching points and vessel segments between intersections.

VEGF and SDF-1 Assays

VEGF concentrations were measured using a mouse VEGF ELISA kit (R&D Systems) according to the manufacturer's instructions. SDF-1 concentrations were determined by ELISA (Genentech).

Microarray Experiments and Analysis

Microarray experiments were performed using Agilent Whole Mouse Genome expression arrays using Agilent-recommended protocols. Universal mouse reference RNA was applied in each array (Stratagene). Three replicates were prepared for each sample. Raw ratio values were log2 transformed, and a Cyber t test (Baldi and Long, 2001) was performed to identify probe sets differentially expressed between all sensitive and all resistant samples.

In Vivo Tumor Growth Studies with Anti-PDGF-C or Anti-VEGF Antibodies

Recombination experiments using TAF-EL4 and TIB6-GFP cells were carried out as described above. 1×10^6 EL4 or TIB6 cells were injected subcutaneously. All treatments were initiated at day 2. Thirty micrograms of anti-PDGF-C neutralizing IgG or control goat IgGs in 50 μ l solutions was administered every 2 days intratumorally, given the limited availability of such antibody. Anti-VEGF mAb or anti-ragweed antibodies were administered every 6 days at 5 mg/kg intraperitoneally.

Expression Analysis in Cells within TIB6 or EL4 Tumors and in Primary Mouse Endothelial Cells

TIB6-GFP and EL4-GFP tumors (~ 1000 mm³; $n = 5$ each) were dissociated and sorted by flow cytometry into four populations of cells based on expression of GFP, CD31, and CD45. APC or FITC-conjugated anti-CD31 or CD45 antibodies were from BD Pharmingen. Primary mouse endothelial cells were isolated from normal BALB/c mouse lungs using a MACS Cell Separation kit (Miltenyi Biotec) according to the manufacturer's protocol. CD45 beads were used for negative selection, while CD31-FITC and anti-FITC beads were used for positive selection. Cells were cultured in EGM-2MV growth medium (Lonza) and were sorted again for CD31 expression. Postsorting analysis revealed that >99.5% of cells were CD31⁺. Gene expression analysis was determined by TaqMan as described above.

In Vitro Migration Assays

Mouse endothelial and EL4 cells were exposed to EBM or DMEM media with 0.1% BSA and then plated onto either fibronectin-coated or noncoated upper chambers (8 μ m and 5 μ m pore size, respectively) of migration assay plates (BD Biosciences). CD11b⁺Gr1⁺ cells were isolated from the bone marrow of naive or EL4 tumor-bearing mice by flow cytometry. FITC- and PE-conjugated anti-Gr1 and anti-CD11b antibodies were from BD Biosciences. Noncoated inserts with 5 μ m pore size were used for the experiment. Endothelial cells that migrated across the upper chamber after 6 hr of cytokine stimulation were stained with Sytox green nucleic acid stain (Invitrogen) and imaged. Nuclei numbers were counted using ImageJ software. Numbers of migrated EL4 and myeloid cells were determined using a Z2 particle counter and size analyzer. Cytokines or serum were added to the bottom chambers; antibodies or control IgGs were added to both chambers.

Array CGH

Agilent Mouse Genome CGH Microarray 244A was used for array CGH analysis according to Agilent-recommended protocols. Normal genomic DNA was used as the control channel on all arrays (strain 000664, The Jackson Laboratory). The resulting Agilent expression ratios were log2 transformed, centered to a median of zero, and segmented using GLAD (Hupe et al.,

2004). All statistical analysis was performed using Partek software (Partek Inc., version 6.08.0414).

Statistical Analysis

Statistical analysis was performed by t test except where otherwise noted in figure legends or elsewhere in the [Experimental Procedures](#).

ACCESSION NUMBERS

The microarray data reported herein are available at the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) with the accession number GSE13549.

SUPPLEMENTAL DATA

The Supplemental Data include thirteen figures and can be found with this article online at [http://www.cancer.org/supplemental/S1535-6108\(08\)00407-8](http://www.cancer.org/supplemental/S1535-6108(08)00407-8).

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