Cancer Therapy: Preclinical

Clinical Cancer Research

PlGF/VEGFR-1 Signaling Promotes Macrophage Polarization and Accelerated Tumor Progression in Obesity

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Abstract

Purpose: Obesity promotes pancreatic and breast cancer progression via mechanisms that are poorly understood. Although obesity is associated with increased systemic levels of placental growth factor (PlGF), the role of PlGF in obesity-induced tumor progression is not known. PlGF and its receptor VEGFR-1 have been shown to modulate tumor angiogenesis and promote tumor-associated macrophage (TAM) recruitment and activity. Here, we hypothesized that increased activity of PlGF/VEGFR-1 signaling mediates obesity-induced tumor progression by augmenting tumor angiogenesis and TAM recruitment/activity.

Experimental Design: We established diet-induced obese mouse models of wild-type C57BL/6, VEGFR-1 tyrosine kinase (TK)-null, or PlGF-null mice, and evaluated the role of PlGF/VEGFR-1 signaling in pancreatic and breast cancer mouse models and in human samples.

Results: We found that obesity increased TAM infiltration, tumor growth, and metastasis in pancreatic cancers, without affecting vessel density. Ablation of VEGFR-1 signaling prevented obesity-induced tumor progression and shifted the tumor immune environment toward an antitumor phenotype. Similar findings were observed in a breast cancer model. Obesity was associated with increased systemic PlGF, but not VEGF-A or VEGF-B, in pancreatic and breast cancer patients and in various mouse models of these cancers. Ablation of PlGF phenocopied the effects of VEGFR-1-TK deletion on tumors in obese mice. PlGF/VEGFR-1-TK deletion prevented weight gain in mice fed a high-fat diet, but exacerbated hyperinsulinemia. Addition of metformin not only normalized insulin levels but also enhanced antitumor immunity.

Conclusions: Targeting PlGF/VEGFR-1 signaling reprograms the tumor immune microenvironment and inhibits obesity-induced acceleration of tumor progression. *Clin Cancer Res; 22(12); 2993–3004. ©2016 AACR.*

Introduction

Excess body weight has become a major public health problem worldwide (1). Furthermore, a number of large-scale studies have demonstrated that obesity leads to an increase in cancer-related mortality in multiple cancer types including pancreatic and breast (2–4). However, the mechanisms underlying obesity-induced tumor progression are not completely understood (5). An excessive gain in adipose tissue in obesity is associated with angiogenesis, inflammation, and immune cell infiltration (6–9). Similarly, obesity may affect the vasculature and immune cell infiltration in cancer to facilitate tumor progression (10, 11).

We and others have shown that VEGFR-1 is expressed on endothelial cells (EC) and macrophages. Binding of VEGF and placental growth factor (PlGF) to VEGFR-1 promotes tumor angiogenesis, and stimulates the recruitment and/or activation (e.g., cytokine production) of tumor-associated macrophages

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

With the current worldwide obesity epidemic, the majority of pancreatic and breast cancer patients are either overweight or obese at diagnosis. Importantly, obesity is associated with poor prognosis in these patients. Hence, uncovering the cellular mechanisms underlying this poor outcome is imperative. We revealed a significant correlation between plasma placental growth factor (PlGF) and adiposity in pancreatic and breast cancer patients and described how PlGF/VEGFR-1 signaling contributes to obesity-induced tumor progression using clinically relevant mouse models. We found that blocking the PlGF/VEGFR-1 signaling axis is effective in obese but not in lean condition. Therefore, anti-VEGFR-1 agents may prove therapeutically beneficial in pancreatic and breast cancer patient populations stratified by body mass index. On the other hand, ablation of PlGF/VEGFR-1 signaling worsened the impaired systemic glucose metabolism associated with obesity, which could be reverted by the antidiabetic drug metformin. Controlling the metabolic status of patients may enhance the clinical translation of an anti-PlGF/VEGFR-1 strategy.

(TAM; refs.12–17). However, the role of VEGFR-1 in tumor progression remains controversial, with some, but not all studies showing an effect of VEGFR-1 in promoting tumor growth and systemic metastasis in mouse models (12, 14, 18, 19). A recent study, in particular, demonstrated a specific effect of VEGFR-1 in metastasis-associated macrophages (12). In addition, VEGFR-1 signaling may regulate energy metabolism, as mice genetically deficient for PlGF gain less weight on a high-fat diet (HFD) than wild-type (WT) animals (20), but develop insulin resistance and hyperinsulinemia (21). These findings indicate that VEGFR-1 activity may have important roles in both tumor and adipose tissue, making it a potential target in cancers specifically in the obesity setting.

In this study, we evaluated whether VEGFR-1 signaling mediates the adipose tissue expansion and accelerated tumor growth in obesity, and whether this occurs via augmented angiogenesis and immune cell recruitment/activation. For this purpose, we used murine pancreatic ductal adenocarcinoma (PDAC) and breast cancer models orthotopically implanted in syngeneic lean and obese WT or VEGFR-1 tyrosine kinase (TK) knockout (*Flt1*$^{TK-/-}$) mice. Specifically in obese mice, VEGFR-1-TK deletion led to a reduction in tumor progression associated with a shift in tumor cytokine profile and TAM polarization toward the M1 phenotype. Furthermore, VEGFR-1-TK deletion prevented body weight gain in mice fed HFD. However, this was associated with worsened hyperinsulinemia. Metformin not only normalized insulin levels but also further improved the effects of VEGFR-1-TK ablation on tumor progression and the immune environment. In addition, we assessed which ligands, PlGF or VEGFs, are implicated in the effects of VEGFR-1 in tumors. As mentioned above, PlGF has been shown to regulate tumor angiogenesis, immune cell infiltration, and tumor progression and, importantly, preclinical and clinical evidence suggests a relationship between PlGF and obesity (20, 22–24). We found that plasma PlGF but not VEGF-A or VEGF-B was associated with obesity in PDAC and breast cancer patient samples as well as multiple mouse models. Consistent with this, PlGF-null mice (*Plgf*$^{-/-}$) phenocopied *Flt1*$^{TK-/-}$ mice on improving the immune environment, systemic metabolism, and reducing tumor growth in the obese setting.

With the majority of pancreatic and breast cancer patients being overweight or obese at diagnosis (4, 25, 26), uncovering potential therapeutic targets within the mechanisms associating obesity with worsening cancer prognoses is the first step toward developing remedies that could disrupt this association and significantly improve patient outcome. This study describes the mechanisms by which PlGF/VEGFR-1 signaling mediates obesity-induced pancreatic and breast cancer progression.

Materials and Methods

Flt1$^{TK-/-}$, *Plgf*$^{-/-}$, *ob/ob*, C57BL/6, and FVB mice were used. At 6/7 weeks of age, all mice except *ob/ob* switched from standard chow to either low- (10%) or high-fat (60%) diet. At 10 weeks, we evaluated glucose and insulin tolerance, weighed fat pads. Then, we implanted PAN02, E0771 (C57BL/6), or AK4.4 (FVB) tumors. Diet was maintained. Three weeks later, tumor burden, immune cell infiltration, inflammatory and metabolic markers in tumors, and plasma levels of insulin, insulin-like growth factor-1 (IGF-1) and angiogenic/inflammatory factors were determined.

Metformin 300 mg/kg (drinking water) was administered to some animals.

Using human samples, a correlation between body mass index (BMI) or visceral adipose tissue (VAT) and systemic levels of PlGF and VEGF-A was determined in 73 pancreatic cancer patients and 61 breast cancer patients.

For statistical methods and detail description of the methods, please see Supplementary Methods.

Results

Obesity alters the tumor immune microenvironment and promotes disease progression

As reported previously (27, 28), we observed that diet-induced obesity promotes tumor progression in PAN02 pancreatic tumors. In this model, the tumor weight (Fig. 1A) and metastatic burden (Fig. 1B) were approximately twice as high in obese mice than in lean at 3 weeks after tumor implantation. This was associated with increased tumor cell proliferation (Fig. 1C) and reduced apoptosis in tumors (Fig. 1D). Despite no increase in tumor vessel density in obese mice (Supplementary Fig. S1A), obesity was associated with increased infiltration of TAMs (Fig. 2A) and expression of protumor cytokines IL1β, IL4, and IL5 (trend for IL10) in tumors (Fig. 3D). We also observed an increase in IL2, but no difference in the other tumor-associated cytokines measured (IL6, TNFα, IL12, INFγ, CXCL-1; not shown). Taken together, we found that obesity does not affect vessel density in tumors, but is associated with TAM infiltration, increased expression of M2 cytokines, and accelerated tumor progression.

Ablation of VEGFR-1 signaling prevents obesity-induced tumor progression without affecting blood vessels of recruitment of immune cells

Immunosuppressive (M2-like) TAMs have been shown to promote tumor progression (29). We and others have uncovered a role for VEGFR-1 signaling in the recruitment and activity of TAMs (12, 14). Therefore, we evaluated whether ablation of

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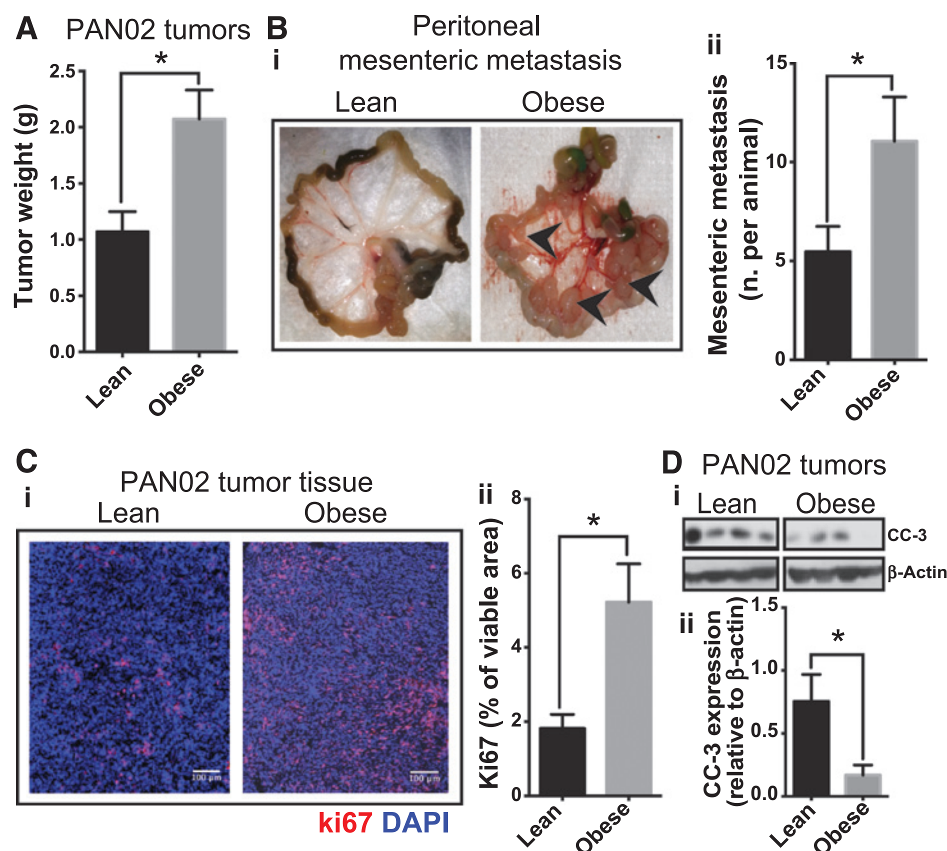

Figure 1.
Effect of diet-induced obesity on pancreatic cancer progression. Following the establishment of diet-induced lean and obesity state in C57BL/6 mice (10 weeks of diet with 60% fat vs. 10% fat diet), PAN02 tumors were implanted orthotopically. Tumors were collected 3 weeks later, and tumor weight, metastases, proliferation, and apoptosis were determined. A, the effect of obesity on tumor weight ($n = 5$–9/group). B, representative images of mesenteric metastases (black arrowheads) in lean and obese mice (i). Average number of mesenteric metastasis per mouse (ii). C, representative images of Ki67 staining (immunofluorescence) in PAN02 tumors removed from lean and obese mice (i). Scale bars: 100 μm. Quantification of the expression of Ki67 proliferation marker in tumors from lean and obese mice ($n = 3$–5/group; ii). D, protein level of cleaved caspase-3 (CC-3) apoptotic marker in tumors from lean and obese mice, determined by Western blot analysis (i). Each band represents an individual tumor. Densitometric analysis normalized to β-actin. *, $P < 0.05$. Error bars, SEM (ii).

VEGFR-1 signaling could prevent the increased infiltration of TAMs in obese mice and obesity-accelerated tumor progression. For this purpose, we used *Flt1*$^{TK-/-}$ mice lacking the signaling domain of VEGFR-1 receptor (30). PAN02 tumors grown in *Flt1*$^{TK-/-}$ lean mice were similar in size to tumors grown in WT lean mice. However, tumors grown in *Flt1*$^{TK-/-}$ obese mice were significantly smaller compared with those in obese WT mice (Fig. 2B). Similarly, ablation of VEGFR-1 signaling reduced the number of mesenteric metastases per animal in obese but not lean mice (Fig. 2C). Furthermore, there was a trend toward a reduction in both the incidence of mesenteric metastasis (i.e., number of mice with metastasis; Fig. 2D, $P = 0.064$) and the extent of retroperitoneal abdominal wall invasion (Fig. 2E, $P = 0.09$) in obese *Flt1*$^{TK-/-}$mice compared with obese WT. The loss of body weight from the time of implantation until tumor extraction (a measure of health and systemic disease burden, in an extreme case, cachexia) was also significantly improved in obese *Flt1*$^{TK-/-}$ mice (approximately four times less body weight loss than obese WT mice; Fig. 2F). Consistent with reduced tumor progression, tumor cell proliferation was reduced in VEGFR-1-TK deletion in obese mice (Fig. 2G), despite no change in apoptosis (Fig. 4H). Interestingly, ablation of VEGFR-1 signaling did not affect the number of CD45^{+} leukocytes, CD11b^{+}F4/80^{+} TAMs (Fig. 2A), NK, CD4^{+}, CD8^{+}, or T regulatory lymphocytes (Fig. 4F) in tumors in either lean or obese mice. Considering that the expression of VEGFR-1 in tumor tissues is not restricted to TAMs, but also extends to ECs, and that VEGFR-1 can regulate tumor angiogenesis, we determined whether the observed effects on tumor progression could be related to inhibition of angiogenesis. We confirmed that both EC and macrophages express VEGFR-1 *in vitro*, with tumor cell lines showing comparatively lower levels of expression (Fig. 3A). However, VEGFR-1-TK deletion was not associated with changes in tumor vessel density or hypoxia (Supplementary Fig. S1B). Taken together our data reveals a role for VEGFR-1 in obesity-induced tumor progression that does not appear to be via modulation of TAM infiltration or angiogenesis.

Ablation of VEGFR-1 signaling reverts the obesity-associated abnormal tumor immune environment and reprograms TAMs

Although the number of TAMs did not appreciably change in with VEGFR-1-TK deletion in obese mice, we found that VEGFR-1 appeared to be predominantly associated with TAMs *in vivo*, with approximately 50% of these cells expressing VEGFR-1 in PAN02 tumors (Fig. 3B and Supplementary Fig. S3A). This suggests that VEGFR-1 might play a role in TAM activity. In fact, it was recently shown that VEGFR-1 regulates TAM inflammatory response without affecting infiltration of these cells in tumors (12). Thus, we determined whether the TAM phenotype and the immune environment was altered in *Flt1*$^{TK-/-}$ mice. We found that VEGFR-1-TK deletion in obese animals was associated with upregulation of M1 genes and concomitant downregulation of M2 genes including *Il1b*β and *Il10* in PAN02 tumors (Fig. 3C). Subsequently, at the protein level, we confirmed a reduction in protumor M2 cytokines IL10, IL4, IL5, and of IL1β in tumor tissue (Fig. 3D), and of IL1β and IL2 (trend for IL10, $P = 0.13$) in plasma (Supplementary Table S1) in obese *Flt1*$^{TK-/-}$ mice. IL1β, in particular, which showed the most dramatic change (50% decrease in obese *Flt1*$^{TK-/-}$ vs. obese WT mice), was robustly expressed by 65% to

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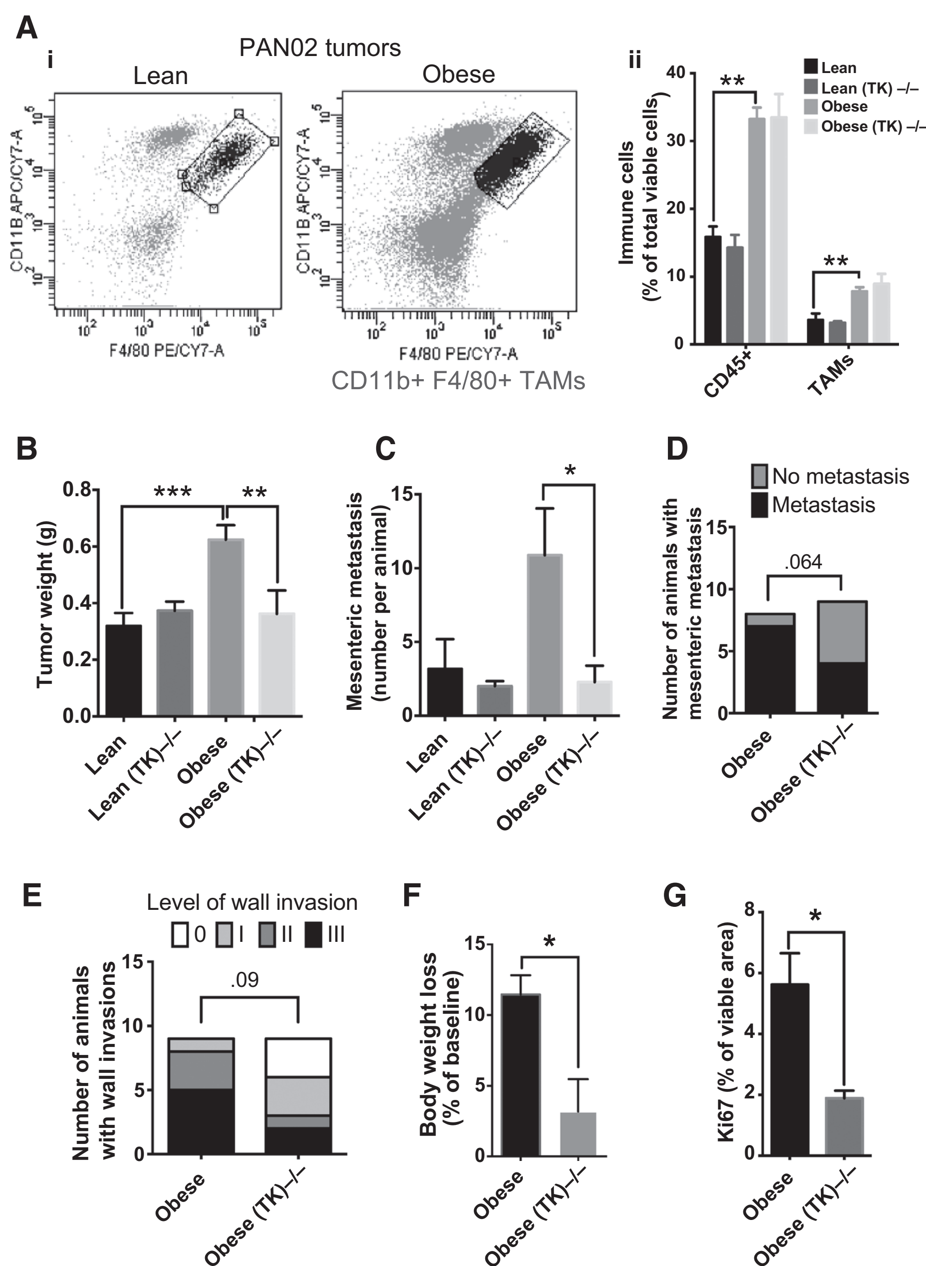

Figure 2.
Effect of VEGFR-1 signaling ablation on obesity-induced TAM infiltration and pancreatic cancer progression: A, flow cytometry was used to determine macrophage infiltration in PAN02 tumors from lean and obese WT and *Flt1*$^{TK-/-}$ mice (i). Representative figures of CD11b (myeloid) and F4/80^{+} double positive cells indicating macrophages. Quantification of CD11b^{+} F4/80^{+} cells within total viable cells ($n = 3$–6/group; ii). B, tumor weights of PAN02 tumors collected 3 weeks after tumor source implantation in lean and obese WT or *Flt1*$^{TK-/-}$ mice ($n = 8$–12 mice/group). C, average number of mesentery metastasis. Mesenteries collected at the time of primary tumor removal. D, number of mice affected with mesentery metastasis in obese WT or *Flt1*$^{TK-/-}$ mice. E, number of mice that presented with different levels of retroperitoneal wall invasion in obese WT or *Flt1*$^{TK-/-}$ mice. Animals were given scores of 0 to 3 based on the extent of invasion: 0 = no invasion, 1 = less than 3 metastases, 2 = less than 6 metastases, 3 = more than 6 metastases. F, body weight loss between tumor implantation and extraction in obese WT or *Flt1*$^{TK-/-}$ mice. G, quantification of Ki67 in tumors from obese WT and *Flt1*$^{TK-/-}$ mice ($n = 3$–6/group). *, $P < 0.05$; **, $P < 0.01$. Error bars, SEM.

80% of TAMs and colocalized with the expression of VEGFR-1 in these cells (Supplementary Figs. S3A, S3B and Supplementary Table S2): about 75% to 90% of VEGFR-1-positive TAMs coexpressed IL1β, whereas about 55% to 70% of IL1β-positive TAMs coexpressed VEGFR-1. This leads to a total of 40% to 50% of all TAMs being positive for both VEGFR-1 and IL1β. In addition, obese WT animals tended to have increased expression of IL1β in TAMs compared with their lean counterparts, but this difference was abrogated in *Flt1*$^{TK-/-}$ mice (Supplementary Fig. S3A and Supplementary Table S2). Consistent with the expression of M1/M2 cytokines, in tumors from obese *Flt1*$^{TK-/-}$ mice, we observed an enrichment of M1-TAMs (F4/80^{+}CD86^{+} and F4/80^{+}Ly6C^{+}) within the leukocyte (CD45^{+}) population compared with tumors in obese WT mice (Fig. 3E), indicating a shift in TAM phenotype. Finally, we also found a decrease in the gene expression of immune checkpoint receptors/ligands such as *Ctla4* (9-fold) and *Pdcd1lg2* (PD-L2) (4-fold) in obese *Flt1*$^{TK-/-}$ mice, suggesting alleviation of T-cell exhaustion (31; Fig. 4G). Collectively, these findings show that ablation of VEGFR-1 signaling in obese mice shifts TAMs toward an M1 phenotype, and promotes antitumor immunity.

Ablation of VEGFR-1 signaling prevents weight gain, but the effect on tumor progression is not dependent on its metabolic effects

Blockade of the VEGFR-1 ligand PlGF has been shown to affect adipose tissue expansion (20). As prevention of obesity itself could also indirectly affect tumor progression, we determined

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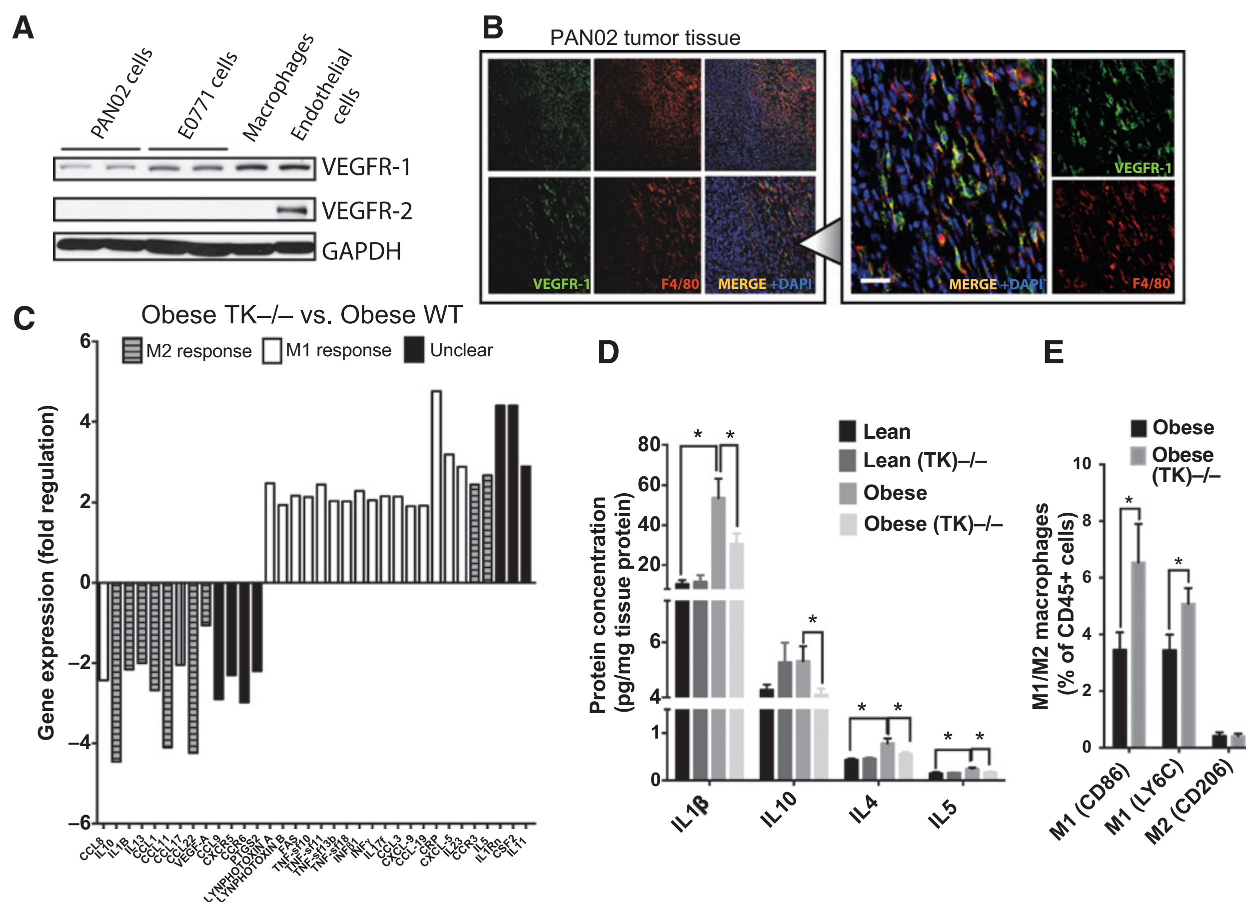

Figure 3.

Effect of VEGFR-1 signaling ablation on obesity-altered tumor immune microenvironment: A, Western blot analysis of VEGFR-1 and VEGFR-2 expression in cell lysates of cultured PAN02, E0771, macrophages (RAW 264.7), and endothelial (HUVECs) cells (samples in duplicate). B, expression of VEGFR-1 and F4/80 in two representative PAN02 tumors obtained by immunofluorescence (left). Right: enlargement of a region of interest. Scale bar: 100 μm. C, gene expression of M1/M2 markers comparing tumors from obese *Flt1*$^{TK-/-}$ to obese WT. Four samples were pooled into each PCR array plate. D, ELISA was used to determine the cytokine expression in PAN02 tumors from lean and obese WT and *Flt1*$^{TK-/-}$ mice ($n = 3$–10/group). E, flow cytometry was performed to determine the expression of the M1 (CD86 and LY6C positive) and M2 (CD186^{+}) macrophages within the total leukocyte population in tumors from obese WT and *Flt1*$^{TK-/-}$ mice ($n = 6$/group). *, $P < 0.05$. Error bars, SEM. Data shown in D also presented as part of a more comprehensive table (Supplementary Table S1).

whether VEGFR-1-TK deletion attenuates body weight gain. Indeed, we found that *Flt1*$^{TK-/-}$ mice on a HFD (but not on a low-fat diet) gained significantly less body weight than WT mice (body weight at the time of tumor implantation in Fig. 4A, body weight gain kinetics in Supplementary Fig. S4A). The reduction in weight gain was associated with reduced perigonadal fat and adipocyte size (Supplementary Figs. S4B and S4C). Remarkably, these effects in adipose tissue occurred without a reduction in the number of leukocytes, macrophages, macrophage-rich crown-like structures, or vessel density (Supplementary Figs. S4D–S4J), similarly to what was observed in the tumor setting. To rule out the possibility that the effect of VEGFR-1 signaling on tumor progression could be solely due to changes in body weight, we performed body weight–matched (*Flt1*$^{TK-/-}$ mice with the same body weight as obese WT at the time of tumor implantation) subset group analysis. We still found significantly decreased tumor growth in body weight–matched *Flt1*$^{TK-/-}$ mice obese mice compared with WT obese mice (Supplementary Fig. S5A). Similar findings were observed for mesenteric and wall metastases (Supplementary Figs. S5B and S5C), and weight loss (cachexia; Supplementary Fig. S5D). In fact, the body weights of tumor-bearing obese WT and *Flt1*$^{TK-/-}$ mice were similar at the end of the experiment, because WT mice lost body weight whereas *Flt1*$^{TK-/-}$ mice maintained it (Supplementary Fig. S5E). These results indicate that blockade of VEGFR-1 signaling induces body weight–independent effects on the tumor microenvironment.

Ablation of VEGFR-1 signaling aggravates glucose metabolism

We found that despite preventing weight gain, VEGFR-1-TK deletion in obese mice (but not in lean) led to elevated fasting glucose level and impaired glucose tolerance test (GTT; Fig. 4B; Supplementary Fig. S6A). Interestingly, insulin tolerance remained similar between the two genotypes in obese mice (Supplementary Fig. S6B). Of note, these changes in glucose metabolism were not due to macrophage infiltration, altered pancreatic β-cell, insulin production, or an overall change in pancreatic tissue mass (Supplementary Fig. S6C–S6G). We then investigated whether the altered systemic metabolism observed in obese *Flt1*$^{TK-/-}$ mice also affects tumor metabolism upon

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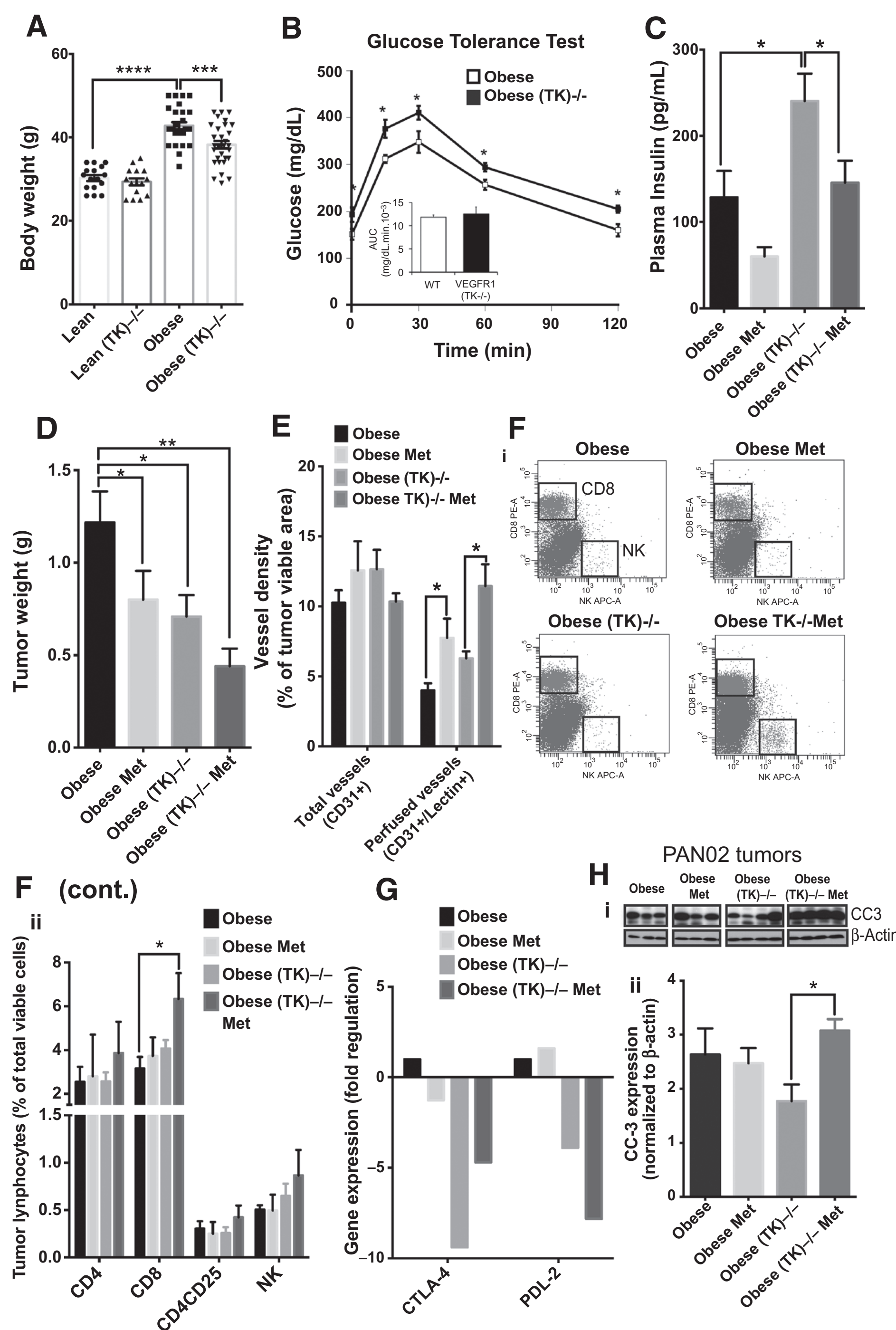

Figure 4.
Effect of metformin on the metabolic consequences of VEGFR1 signaling ablation and on the tumor microenvironment: A, body weight of male C57BL/6 mice after 10 weeks of low-fat or high-fat diet in *WT* or *Flt1*$^{TK-/-}$ background. Number of animals depicted as dots on each column. Low-fat diet generated "lean" mice, and HFD generated "obese" mice. B, GTT in *Flt1*$^{TK-/-}$ versus WT obese mice ($n = 10$/group). C, levels of plasma Insulin in WT, metformin treated, *Flt1*$^{TK-/-}$, or combination of *Flt1*$^{TK-/-}$ + metformin-treated obese mice 3 weeks after implantation of PAN02 tumors ($n = 6$–8/group). D, weights of PAN02 tumors 3 weeks after chunk implantation in same treatment groups as in C ($n = 7$–10/group). E, percentage of vessels (CD31$^+$ expression in total DAPI+ viable area) and perfused vessels (lectin staining in total DAPI+ viable area) in tumors (no less than 10 ROI were analyzed per tumor, $n = 3$–4 tumors/group). F, representative figures of CD8 and NK cells in tumors (i). Quantification of immune cells (% of total viable cells; $n = 3$–6/group; ii). G, gene expression of *Ctla4* and Pdcd1lg2 (PD-L2) in tumors. Data extracted from a PCR array assay, four samples were pooled into each PCR array plate. H, Western blot analysis of cleaved caspase-3 (CC-3) from PAN02 tumors lysates from D (i). Each band represents an individual tumor. Quantification relative to β-actin in the bottom panel (ii). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.001$. Error bars, SEM.

tumor inoculation. In PAN02-bearing mice, we observed that *Flt1*$^{TK-/-}$ mice presented with increased plasma insulin (Fig. 4C) compared with WT animals. The elevation of insulin was associated with increased phosphorylation of insulin receptor (p-IR) and IGF-1 receptor (p-IGF-1R) in PAN02 tumors (Supplementary Fig. 7A). These findings were associated with decreased expression of gluconeogenic genes and decreased autophagy in tumors (Supplementary Fig. S8A and S8B), but with no major changes in genes involved in glycolysis or lipid/protein metabolism (not shown). Collectively, these results indicate that ablation of VEGFR-1 signaling induces adverse effects on systemic metabolism in obese mice, which may have an impact on local tumor metabolism.

Metformin alleviates hyperinsulinemia in *Flt1*$^{TK-/-}$ obese mice and further improves the immune tumor microenvironment

To ameliorate the metabolic aberrations of VEGFR-1-TK deletion in PAN02-bearing obese mice, we administered the antidiabetic drug metformin. As anticipated, metformin prevented the increase in insulin levels observed with VEGFR-1-TK deletion (Fig.

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4C). In addition, we and others have shown that metformin can also affect tumor progression (32–34). Combined VEGFR-1-TK deletion and metformin led to the lowest PAN02 tumor weight in obese mice (Fig. 4D) and these tumors displayed increased perfusion, despite unaltered vessel density in either tumor (Fig. 4E) or adipose tissue (Supplementary Fig. S8C). Similar to what was observed in our previous study with anti-VEGFR2 antibody (35), the increased vessel perfusion in the combination group was associated with increased recruitment of cytotoxic $CD8^+$ T cells (trend for NK cells; Fig. 4F). In addition, downregulation of the immune checkpoint markers CTLA-4 and PD-L2 by VEGFR-1-TK deletion was maintained in the combination group (Fig. 4G). These effects of the combination therapy on the immune environment were associated with increased tumor cell apoptosis compared with VEGFR-1-TK deletion alone (Fig. 4H). Interestingly, metformin restored the expression of major gluconeogenic genes that were downregulated by VEGFR-1-TK deletion in obese tumors (Supplementary Fig. S8A), although no changes were seen in AMPK/ACC activation (Supplementary Fig. S8D) or IR/IGF-1 signaling (not shown). Taken together, our findings indicate that in addition to normalizing systemic metabolism, metformin can be helpful in restoring the abnormal tumor vasculature and immunity in obese $Flt1^{TK-/-}$ mice and in inhibiting tumor progression.

Ablation of VEGFR-1 signaling exerts similar effects in breast cancer

Next, we determined whether the effects of VEGFR-1 blockade in obesity could be seen in another tumor type. Similar to PAN02, VEGFR-1-TK deletion prevented E0771 breast cancer progression in obese, but not lean animals. Lung metastases were significantly decreased by ablation of VEGFR-1 signaling in obese animals (Fig. 5A and B), even though there was no effect on primary tumor growth (Supplementary Fig. S9A). Body weight loss was also significantly reduced in obese $Flt1^{TK-/-}$ mice (Fig. 5C). Again, similar to PAN02, we observed that VEGFR-1-TK deletion produced no effect on vessel density, hypoxia markers, or immune cell infiltration in tumors of obese mice (Supplementary Figs. S9B and S9C). We found no change in tumor levels of IL4, IL5, or IL10 but instead observed an effect on other protumor M2 markers, IL6 and matrix metalloproteinase-9 (MMP-9; ref.36; Supplementary Fig. S9D and S9E): obesity promoted IL6 and MMP-9 expression in tumors, and VEGFR-1-TK deletion decreased expression of these markers in obese but not lean mice. In addition, similar to PAN02 model, ablation of VEGFR-1 signaling prevented body weight gain in obese but not lean animals (Fig. 5D), and was associated with increased systemic insulin levels (Fig. 5E) and increased activation of p-IGF-1R at the tumor level (Supplementary Fig. S7B). Taken together, we observed similar effects of VEGFR-1-TK deletion in the obese setting in two different cancer types.

Obesity is associated with increased systemic PlGF levels in pancreatic and breast cancer patients as well as in preclinical mouse models

PlGF and VEGF-A are ligands of VEGFR-1 with a role in tumor progression (37). Plasma levels of PlGF and VEGF-A were measured in PDAC ($n = 73$) and breast cancer ($n = 61$) patients and were correlated with BMI or, in the case of breast cancer, with VAT. We found that PlGF ($r = 0.35$ and 0.45 for BMI and VAT), but not VEGF-A ($r = 0.09$ and -0.02 for BMI and VAT), correlated with adiposity in both PDAC and breast cancer patients (Fig. 6A). We then confirmed that plasma PlGF was also elevated in WT obese mice compared with lean mice in the PAN02 and E0771 tumor models, as well as in an additional PDAC tumor model (AK4.4; Fig. 6Bi, Supplementary Fig. S10A). Of note, we observed a similar elevation in plasma PlGF in both diet-induced obesity and genetically obese leptin-deficient mice (*ob/ob*; Supplementary Fig. S10A). Consistent with human data, VEGF-A was not increased in plasma (systemic expression) in WT obese mice compared with lean mice in both PAN02 and E0771 models (Fig. 6Bii). On the other hand, the expression of VEGF-A in tumors (local expression; Fig. 6Bii) significantly increased in $Flt1^{TK-/-}$ mice as compared with corresponding WT mice in both tumor models. However, the increase in VEGF-A levels was essentially the same in lean and obese mice. Conversely, another VEGFR-1 ligand, VEGF-B, was increased in circulation in obese mice in the PAN02 model, but not in the E0771 model (Supplementary Fig. S10B). Most importantly, in $Flt1^{TK-/-}$ mice, plasma PlGF, but not VEGF-A or VEGF-B, was dramatically increased compared with WT in both lean and obese settings (more than 10-fold increase in obese PAN02-bearing mice; (Fig. 6B; Supplementary Fig. S10B). This indicates that signaling via VEGFR-1 is particularly associated with PlGF rather than VEGFs in the models used. Taken together, our data shows that PlGF is increased in obese mice and patients, and may mediate the obesity-induced VEGFR-1–dependent effects in tumors.

PlGF deletion reproduces the findings of VEGFR-1-TK deletion in obese mice

To confirm that PlGF is responsible for the observed metabolic and tumor effects of VEGFR-1 signaling, we implanted PAN02 tumors in obese WT and $Plgf^{-/-}$ mice. Similar to the findings in the $Flt1^{TK-/-}$ background, tumors grown in $Plgf^{-/-}$ obese mice were significantly smaller compared with those grafted in obese WT mice (Fig. 6C). The number of TAMs, $CD4^+$, $CD8^+$, and T-regulatory lymphocytes were not altered by PlGF ablation in obese mice (Fig. 6D), but instead we observed an increased enrichment of M1-TAMs ($F4/80^+CD86^+$) in obese $Plgf^{-/-}$ mice, similar to that in obese $Flt1^{TK-/-}$ mice (Fig. 6E). Of note, PAN02 and E0771 cells cultured *in vitro* had no detectable production of PlGF, whereas secreted VEGF-A and VEGF-B (Fig. 6B and Supplementary Fig. S10B, right columns.), suggesting that the stroma has a substantial contribution to PlGF expression *in vivo* compared with other VEGFR-1 ligands. Finally, and again similar to what we observed in obese $Flt1^{TK-/-}$ mice, weight gain in high-fat fed (but not low-fat fed), and consequently body weight at the time of tumor implantation were both reduced in $Plgf^{-/-}$ mice (Supplementary Fig. S11C), and plasma insulin was elevated compared with obese WT mice (Supplementary Fig. S11D). Taken together, these data indicate that PlGF blockade reproduces the effects of VEGFR-1-TK deletion and reduces the obesity-induced alterations in metabolism, immune microenvironment, and tumor progression.

Discussion

We demonstrated here for the first time that obesity is associated with increased systemic levels of PlGF in breast and pancreatic cancer patients as well as in obese tumor mouse models. Furthermore, we revealed that PlGF/VEGFR-1 signaling mediates obesity-induced tumor progression (Fig. 6F).

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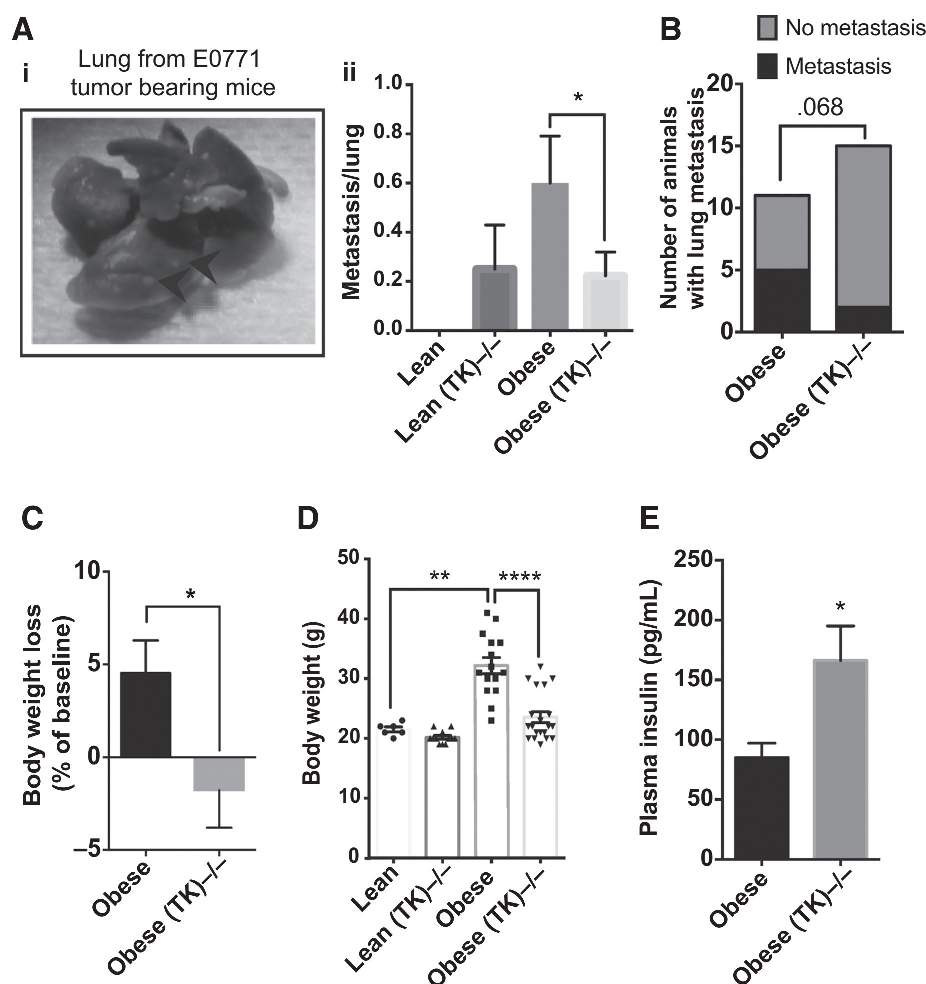

Figure 5.
Effect of VEGFR-1 signaling ablation on a breast cancer model. A, lungs collected 3 weeks after implantation of E0771 tumors in lean and obese WT or $Flt1^{TK-/-}$ mice (n = 6–16/group). Representative image of metastases in the lungs (black arrows; i). Average number of lung metastases per mice (ii). B, incidence (number of mice affected with lungs metastasis) in obese WT or $Flt1^{TK-/-}$ mice. C, body weight loss from implantation until tumor extraction in obese WT or $Flt1^{TK-/-}$ mice. D, body weight of female C57BL/6 mice after 10 weeks of high-fat or low-fat diet in WT or $Flt1^{TK-/-}$ group. Number of animals depicted as dots on each column. E, levels of plasma insulin in WT versus $Flt1^{TK-/-}$ obese mice 3 weeks after implantation of E0771 tumors (n = 7–10/group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.001$. Error bars, SEM.

In a PDAC model, at the tumor level obesity was associated with increased TAM infiltration, expression of M2 cytokines, tumor growth, and metastasis. VEGFR-1 was abundantly expressed in TAMs, and blockade of VEGFR-1 signaling in obese but not lean mice led to a shift in protumor cytokine production (e.g., IL1β) and TAM polarization from an M2 protumor to an M1 antitumor phenotype, ultimately reducing obesity-induced tumor progression. Furthermore, PlGF blockade in obese mice reproduced the effects of VEGFR-1-TK deletion on the tumor immune environment and tumor growth. Rolny and colleagues reported an M1 shift in TAM polarization after inhibition of PlGF in a nonobese setting (38). Furthermore, we and others have shown that PlGF/VEGFR-1 signaling promotes secretion of IL1β by monocytes/macrophages (14, 39). However, this is the first study to demonstrate a specific role for VEGFR-1 in TAM polarization. Similar to previous studies, inhibition of VEGFR-1 signaling did not alter immune cell infiltration in tumors (12, 40–42). However, this effect has been shown to be highly tumor- and context-dependent (14, 15, 18, 43), and we show here that PlGF/VEGFR-1 signaling affects the function rather than the number of infiltrating immune cells. Of note, despite that differences in TAM polarization markers between lean and obese settings were not clear (Supplementary Fig. S2), PlGF/VEGFR-1 inhibition functionally promoted an M1 shift only in the obese setting. Hence this pathway is clearly involved in immunosupression in this metabolic setting. Importantly, plasma PlGF, but not VEGF-A or VEGF-B, was robustly associated with obesity across all models/human samples. In addition, unlike VEGFs, PlGF was not produced by these tumor cells, further supporting that PlGF levels can be more affected by the obese-altered host tumor microenvironment. It is therefore not surprising that stromal deletion of PlGF replicated the effects of VEGFR-1 deletion on metabolism and tumors in obese mice. This is also consistent with our previous study using $Flt1^{TK-/-}$ mice, where we found that VEGFR-1-TK deletion affects primary tumor growth only in the presence of PlGF overexpression, but not of VEGF overexpression (18). Of note, we found that VEGF-A in tumors (local expression) did increase in $Flt1^{TK-/-}$ mice in both the lean and obese setting. The fact that it increased in both settings further validates the lack of any association between VEGF-A with obesity in this study. On the other hand, we have shown before that VEGF-A was decreased in peritoneal macrophages derived from $Flt1^{TK-/-}$ mice compared with those from WT mice (44). This indicates that the effect of VEGFR-1-TK deletion on the expression levels of VEGF in specific cells of the tumor microenvironment may vary, despite an overall increase.

Importantly, similar effects of VEGFR-1-TK deletion on systemic metabolism, tumor progression, vasculature, immune cell infiltration, and TAM polarization were observed not only in PAN02 but also in a breast cancer model (E0771). This is

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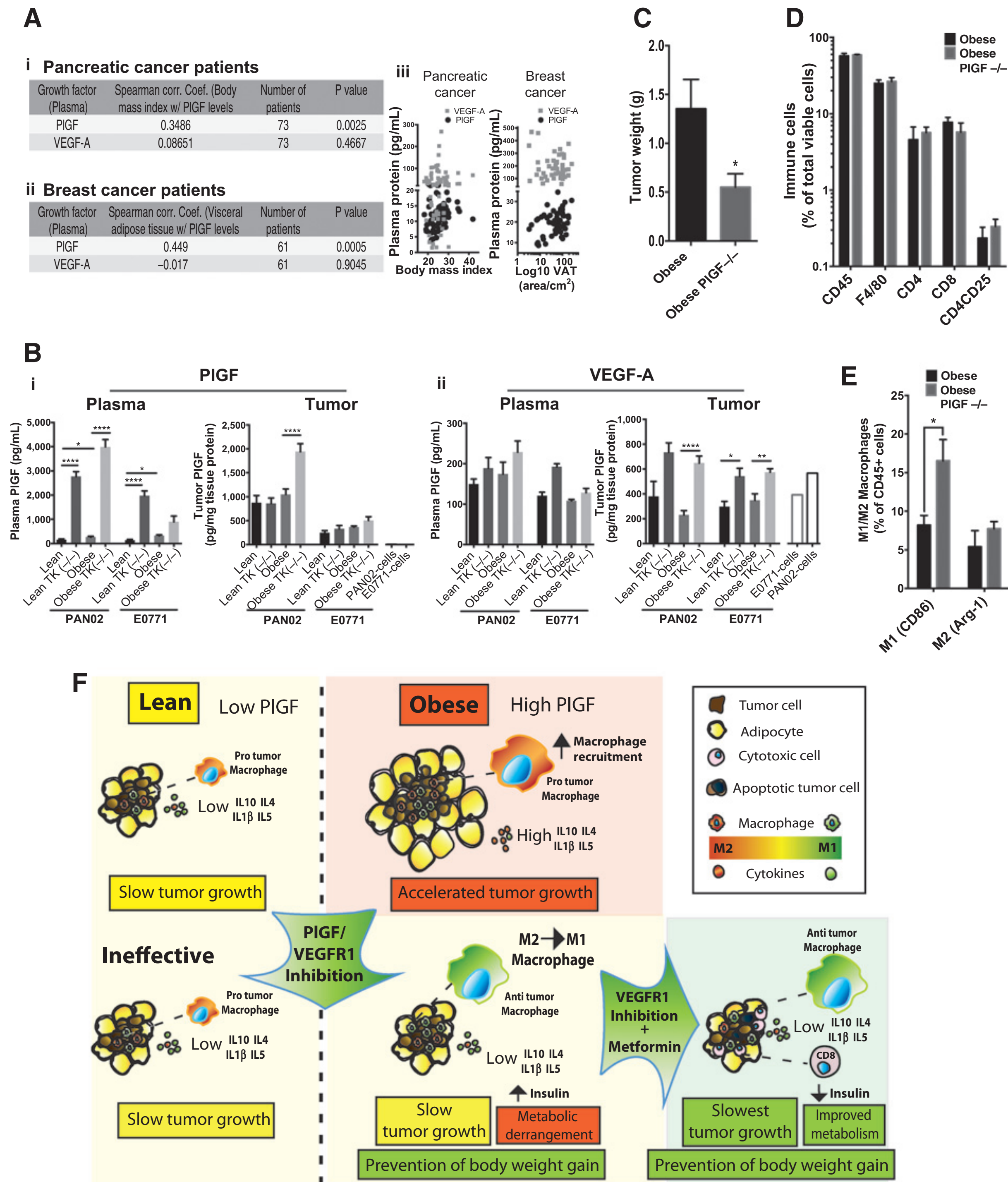

Growth factor (Plasma)	Spearman corr. Coef. (Body mass index w/ PlGF levels	Number of patients	P value
PlGF	0.3486	73	0.0025
VEGF-A	0.08651	73	0.4667

Growth factor (Plasma)	Spearman corr. Coef. (Visceral adipose tissue w/ PlGF levels	Number of patients	P value
PlGF	0.449	61	0.0005
VEGF-A	−0.017	61	0.9045

Figure 6.
Effect of PlGF inhibition on obesity-induced tumor progression. A, correlation between plasma levels of PlGF and VEGF and VAT in (i) pancreatic cancer patients ($n = 61$) or BMI in (ii) breast cancer patients ($n = 73$) that have received no prior treatment (for more information, see Supplementary Methods). B, levels of plasma and tumor PlGF (i) and VEGF-A (ii) in lean and obese WT or *Flt1*$^{TK-/-}$ mice 3 weeks after implantation of PAN02 and E0771 tumors (n = 3–20/group). C, tumor weights of PAN02 tumors collected 3 weeks after tumor source implantation in obese WT or *Plgf*$^{-/-}$ mice (n = 3–6/group). D, flow cytometry was used to determine immune cell infiltration in PAN02 tumors from obese WT and *Plgf*$^{-/-}$ mice (n = 3–6/group). E, flow cytometry was performed to determine the expression of M1 (CD86^{+}) and M2 (Arg1 positive) macrophages within the total leukocyte population in tumors from obese WT and *Plgf*$^{-/-}$ mice (n = 3–6/group). F, graphical abstract. Increased levels of plasma PlGF in obese mice associate with increased recruitment of TAMs, M2-like cytokine profile, and accelerated tumor progression compared with lean setting. Although ineffective in the lean mice, in obese setting PlGF/VEGFR-1 signaling ablation did not affect TAM recruitment or vessel density, but shifted TAM polarization and the immune environment toward an M1 phenotype, and prevented the obesity-induced tumor progression. This occurred with an aggravation of the systemic metabolism and increase in plasma Insulin levels. Metformin not only normalized the systemic metabolism, but also promoted increased tumor vascular perfusion, cytotoxic cell recruitment, tumor cell apoptosis, and reduced tumor growth. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.001$. Error bars, SEM.

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consistent with the elevated systemic levels of PlGF in the obese setting also in this model. Despite inducing a smaller impact on the tumor immune environment compared with PAN02 tumors, we found that VEGFR-1-TK deletion reduced the expression of IL6 and MMP-9 in the obese setting. This may explain the effect of VEGFR-1-TK deletion on metastasis in this model, in line with previous findings that IL6 and VEGFR-1–dependent MMP-9 expression in the stroma (TAMs, in particular) can affect systemic metastasis (14, 45). On the other hand, VEGFR-1-TK deletion did not affect primary tumor growth in obese mice in the E0771 model, contrary to results in the PAN02 model which is consistent with the smaller effects on the tumor immune microenvironment in the former model. We and others have shown that the growth rate of tumors implanted in *Flt1*$^{TK-/-}$ mice compared with WT may be affected differently depending on the tumor model (12, 15). In addition, as mentioned above, VEGFR-1-TK deletion appears to affect primary tumor growth only in the presence of PlGF overexpression (15, 18). Similarly, overexpression of PlGF in colorectal cancer cell lines increased the migration and invasion of tumor cells in a VEGFR-1–dependent manner (46). In fact, the lack of effect on primary tumor growth (and smaller effect on the immune environment) by VEGFR-1-TK deletion in the E0771 tumor model could have been due to the lower levels of PlGF in tumors (local levels) compared with PAN02 tumors, which might have contributed to the lower sensitivity to VEGFR-1-TK deletion. Conversely, the effect of VEGFR-1-TK deletion on metastases may be more dependent on the levels of systemic PlGF, which was increased in obese mice in both PAN02 and E0771 tumor models. Our results on systemic metastasis are in line with our previous work in normal weight *Flt1*$^{TK-/-}$ mice (that have relatively low levels of plasma PlGF), where we showed no effect of VEGFR-1 genetic or pharmacologic inhibition on spontaneous metastases formation (41, 42, 47).

Despite that VEGFR-1 can regulate tumor angiogenesis in a context-dependent manner (14, 37, 48), here we found that obesity did not promote tumor vessel density, and VEGFR-1 signaling ablation did not modify vessel density in either tumors or adipose tissue. Carmeliet and colleagues have shown that the angiogenic role of VEGFR-1 is in part due to the recruitment of TAMs that secrete proangiogenic factors in the tumor microenvironment (37). Hence, the lack of the effect on TAM recruitment observed here may explain at least in part the absence of an effect on angiogenesis in our models.

Beyond the effects on tumors, we showed that PlGF and VEGFR-1 deficiency also reduced weight gain in mice maintained on HFD. This is consistent with previous findings from Lijnen and colleagues in PlGF-deficient mice (20), although previous work with pharmacologic inhibition of PlGF or VEGFR-1 did not demonstrate the metabolic derangement observed here (20, 49). The transient effects of pharmaceutical agents on metabolic parameters may not always be the same as phenotypes caused by lifelong genetic deficiencies. Imperfect delivery/efficacy of pharmaceutical agents may also cause differences in phenotypes compared with complete genetic blockade of the same molecular pathways. The reduction of body weight gain with VEGFR-1-TK deletion was not associated with reduced vasculature, as in Lijnen and colleagues (20), or immune cell infiltration. However, the shift to M1 macrophages and M1 cytokine profile with VEGFR-1-TK deletion is known to be associated with insulin resistance (50) and promote adipocyte cell death (51), which could explain the impaired glucose metabolism, increased levels of insulin and reduced weight gain in *Flt1*$^{TK-/-}$ and *Plgf*$^{-/-}$ obese mice compared with WT. This is consistent with findings from Hemmeryckx and colleagues, who showed that PlGF deficiency in mice fed with a HFD promoted insulin resistance and hyperinsulinemia, presumably via reduced fraction of brown adipocytes and stimulation of white adipocyte hypertrophy (21). Interestingly, however, we observed that VEGFR-1-TK deletion actually reduced adipocyte size. In addition, various pancreatic morphologic parameters including β-cells and insulin production, as well as macrophage infiltration in the pancreas, which can also influence insulin production (52), were unaltered with VEGFR-1-TK deletion. This indicates that the lack of VEGFR-1 signaling did not alter pancreatic insulin production, likely affecting peripheral resistance to this hormone. In fact, inhibition of Pi3K-eNOS signaling by VEGFR-1 inhibition may have been involved, as previously observed in a model of diabetic nephropathy (53). Importantly, previous studies have demonstrated similar glucose metabolism impairments in mice with genetic VEGF deficiencies in the whole pancreas (54), or specifically in β-cells (55) where an impairment is thought to be caused by defective transport of insulin across vascular ECs. Of note, we found that the effects observed on tumor progression were not due to the protective effects on body weight. In fact, in body weight–matched mice, mice that present with same body weight at the time of tumor implantation, VEGFR-1-TK deletion still significantly decreased tumor progression, allowing us to conclude that the bulk of the mechanism of action is via direct impact on the tumor microenvironment. Nevertheless, considering the physical and psychologic beneficial effects of preventing weight gain in breast cancer patients (56, 57), long-term VEGFR-1 inhibition may further improve the quality of life.

The aggravated diabetes-like systemic metabolism in obese *Flt1*$^{TK-/-}$ compared with obese WT mice was associated with increased plasma insulin and activation of insulin/IGF-1 signaling in tumors. This was associated with decreased expression of gluconeogenic genes and autophagy in tumors, but no major changes in other metabolic pathways were observed. Although activation of Insulin/IGF-1 in tumors may be detrimental (5), recent evidence indicate that inhibition of gluconeogenesis may lead to prevention of tumor growth (58). Further studies are necessary to explore the role of VEGFR-1 in local tumor metabolism. Besides normalizing the plasma levels of insulin in obese *Flt1*$^{TK-/-}$, remarkably metformin also normalized pancreatic tumor vasculature and immune microenvironment, by increasing perfusion and recruitment of $CD8^+$ T cells, that were associated with increased cell death and reduced tumor growth. As VEGFR-1-TK deletion decreased expression levels of CTLA-4 and PDL-2, those findings suggest a synergy between enhanced T-cell function by VEGFR-1-TK deletion (hence acting as an immune checkpoint inhibitor) and increased infiltration of T/NK cells by metformin. Of note, the effect of metformin on perfusion is consistent with the ability of this drug to reduce tumor desmoplasia, which we have recently described (32). Contrary to previous studies, we did not find a significant change in activating of major metabolic pathways, including IGF-1 and AMPK, after metformin treatment (33, 34), indicating that the major effects of metformin seem to be on the stromal–vascular–immune tumor microenvironment and not on local metabolism. This is consistent with our previous work showing that metformin can modulate metabolic pathways specifically in TAMs but not at the whole tumor level (32). The positive impact of metformin on tumors are consistent with reports in preclinical models of obesity from our laboratory and

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others (32–34) and in diabetic PDAC patients (59), and multiple trials are ongoing to validate its beneficial effects (59).

Collectively, our findings reveal that PlGF/VEGFR-1 signaling contributes to weight gain and to a protumor immune microenvironment in the obese but not lean setting, which promotes accelerated tumor progression. We found this effect in both PDAC and breast cancer models, suggesting that this may be a common mechanism of tumor induction by obesity that could apply to other cancer types. Furthermore, the increased plasma levels of PlGF observed in obese PDAC and breast cancer patients strongly support the findings of this study, and suggest the PlGF/VEGFR-1 pathway as a potential target for obese cancer patients. As most PDAC and breast cancer patients have excess weight at diagnosis, stratifying these patients by body weight for treatment may enhance the efficacy of anti-VEGFR-1 agents such as multikinase inhibitors, which have failed to show efficacy in unselected populations (60). In addition, this study suggests that PlGF/VEGFR-1 may be a valid target particularly in combination with therapies that control systemic metabolism, such as metformin.

Disclosure of Potential Conflicts of Interest

I.E. Krop reports receiving a commercial research grant from Genentech. D.G. Duda reports receiving commercial research grants from Merrimack and Healthcare Pharma. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Incio, N.N. Rahbari, S.M. Chin, A. Khachatryan, D. Fukumura
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