

Article

## Inhibition of Stromal PlGF Suppresses the Growth of Prostate Cancer Xenografts

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**Abstract:** The growth and vascularization of prostate cancer is dependent on interactions between cancer cells and supporting stromal cells. The primary stromal cell type found in prostate tumors is the carcinoma-associated fibroblast, which produces placental growth factor (PlGF). PlGF is a member of the vascular endothelial growth factor (VEGF) family of angiogenic molecules and PlGF mRNA levels increase after androgen deprivation therapy in prostate cancer. In this study, we show that PlGF has a direct dose-dependent proliferative effect on human PC-3 prostate cancer cells *in vitro* and fibroblast-derived PlGF increases PC-3 proliferation in co-culture. In xenograft tumor models, intratumoral administration of murine PlGF siRNA reduced stromal-derived PlGF expression, reduced tumor burden and decreased the number of Ki-67 positive proliferating cells associated with reduced vascular density. These data show that targeting stromal PlGF expression may represent a therapeutic target for the treatment of prostate cancer.

**Keywords:** prostate cancer; placental growth factor; xenograft model; tumor-host interaction; tumor angiogenesis

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## **1. Introduction**

Prostate cancer is the second most common cancer in males worldwide and the second most common cause of cancer related death among men [1]. Most tumors are primarily treated with surgery or radiation therapy; advanced-stage or widespread disease at initial diagnosis or disease progression after primary treatment is treated with hormone ablation therapy [2]. Although virtually all prostate cancers are initially androgen sensitive and initial therapy can result in significant long-term remission, the development of advanced, malignant, incurable prostate cancer is frequently associated with loss of androgen-dependence and the formation of distant metastases [3]. The term hormone-refractory prostate cancer was traditionally used for tumors that no longer respond to gonadal androgen deprivation therapy [4] and at this stage of disease treatment options are very limited [4].

Prostate tumor growth is accompanied by neovascularization and stimulation of vessel growth from the existing vasculature in a process known as angiogenesis that is required to ensure adequate supply of oxygen and nutrients and removal of waste products from the growing tumor [5]. Angiogenesis is induced by up-regulation of angiogenic growth signals or the corresponding receptors, down-regulation or loss of angiogenic inhibitors or alterations in the intracellular signaling pathways activated by these molecules [6,7]. In prostate tumors, the microenvironment primarily consists of supporting stromal carcinoma-associated fibroblasts (CAFs) that help promote carcinogenesis by remodeling of the extracellular matrix (ECM) and inducing cellular proliferation and angiogenesis [8,9]. Targeting this reactive stroma is currently under investigation as a potential therapeutic modality in prostate cancer therapy [10].

Placental growth factor (PlGF), also known as PGF and endocrine gland-derived vascular endothelial growth factor [11,12] is a member of the vascular endothelial growth factor (VEGF) family of angiogenic molecules [13]. Many cell types produce PlGF, especially when activated or under stress. In addition to the physiological production of PlGF by endothelial cells, vascular smooth muscle cells, inflammatory cells and fibroblasts, several types of tumors express and secrete PlGF [14]. PlGF homodimers bind FLT-1 homodimers (fms-like tyrosine kinase, also known as vascular endothelial growth factor receptor-1 (VEGFR)-1), as well as homodimers of the co-receptors Neuropilin (NRP)-1 and NRP-2 [11,15]. PlGF is also able to transmit signals through FLT-1, without crosstalk with KDR (kinase insert domain-containing receptor, also known as fetal liver kinase (FLK-1) or VEGFR-2) [16]. It is also possible that PlGF heterodimerizes with VEGF [11,16] to transmit angiogenic signals through binding to and activation of the FLT-1/KDR heterodimer receptor complex [16]. PlGF is involved in endothelial stimulation, pathologic angiogenesis and wound healing [17,18] and directly stimulates neovascularization by stimulating the proliferation, migration and survival of endothelial cells [17]. While PlGF is over-expressed in some cancers such as breast and gastric carcinoma, expression is down-regulated in colon and lung carcinoma [19,20]. PlGF is required for the growth of medulloblastoma [21] and also promotes the growth of chronic myeloid leukemia [22]. However, in other tumors PlGF inhibits tumor growth [23]. A screening program to identify angiogenic biomarkers associated with prostate cancer treatment and progression has recently identified increased levels of PlGF to be associated with androgen deprivation therapy [24].

In this study, we identified a role for fibroblast-derived PIGF in the proliferation of human, androgen independent PC-3 prostate cancer cells and investigated the therapeutic potential of siRNA-mediated PIGF blockade *in vivo*.

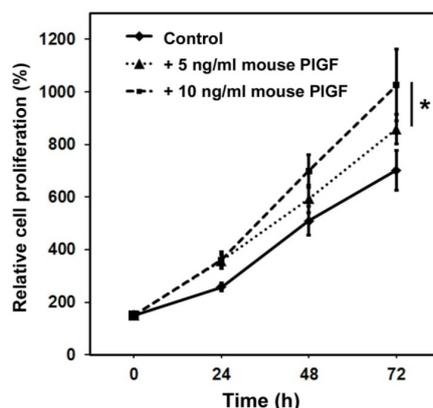
## 2. Results and Discussion

### 2.1. Results

#### 2.1.1. Murine PIGF Stimulates the Proliferation of PC-3 Cells *in Vitro*

Recombinant human PIGF enhances PC-3 prostate cancer cell proliferation (data not shown). To evaluate whether murine PIGF has a direct effect on PC-3 prostate cancer cells and thus constitutes a valid *in vitro* model for the analysis of prostate cancer cell—fibroblast interaction, we cultured cells in 5 ng/mL or 10 ng/mL recombinant murine PIGF and performed a WST-1 cell proliferation assay. Both PIGF treated groups displayed a significantly higher proliferation rate 72 h after treatment compared to untreated controls as shown in Figure 1. No significant difference between groups treated with 5 ng/mL and 10 ng/mL PLGF was observed although proliferation was markedly increased in prostate cancer cells treated with 10 ng/mL compared to 5 ng/mL PIGF. These results show that mouse PIGF enhances PC-3 proliferation in a time-dependent manner, predicting an effect for murine stromal PIGF on PC-3 xenograft growth in mice.

**Figure 1.** Murine placental growth factor (PIGF) enhances the proliferation of human PC-3 prostate cancer cells. PC-3 cells were incubated with 5 and 10 ng/mL recombinant mouse PIGF. Cell proliferation, measured by WST-1 cell proliferation assays after 24, 48 and 72 h was enhanced in a time-dependent manner. Proliferation was expressed as percentage of corresponding untreated control cells on day 0 (mean  $\pm$  SD). \* cells treated with 5 and 10 ng/mL PIGF were significantly different from control,  $p < 0.05$ .

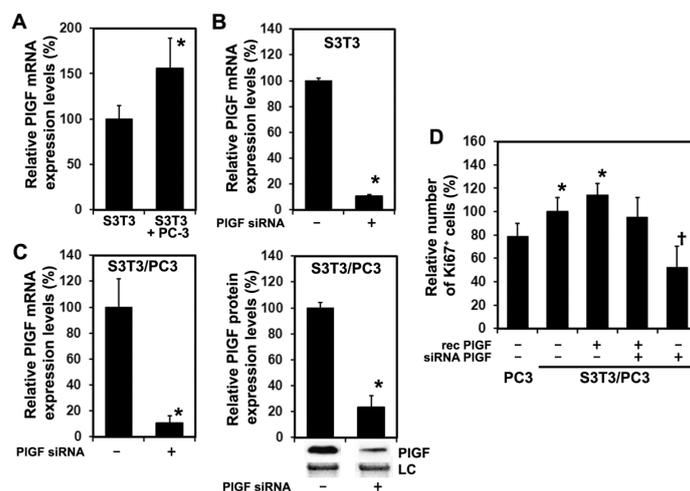


#### 2.1.2. Inhibition of PIGF Expression in Co-Cultured PC-3 and S3T3 Fibroblasts

Human PC-3 cells do not express detectable levels of PIGF as measured by real-time PCR (data not shown). Murine S3T3 fibroblasts express PIGF and expression increases after co-culturing with PC-3 cells (Figure 2A) and PIGF expression can be efficiently reduced by transfection with siRNA targeting murine PIGF (Figure 2B). The role of fibroblast-derived PIGF on the proliferation of PC-3 cells was

then examined in co-culture experiments with S3T3 fibroblasts. Transfection with siRNA reduces expression of murine PIGF in co-cultures on the mRNA (Figure 2C left panel) and protein levels (Figure 2C right panel). Co-culturing prostate cancer cells with fibroblasts increased the number of Ki-67 positive PC-3 nuclei and stimulation with 5 ng/mL murine PIGF further increased Ki-67 positive PC-3 nuclei. Reduced S3T3 PIGF expression is associated with significantly decreased numbers of Ki-67 positive PC-3 nuclei that normalizes in the presence of 5 ng/mL murine PIGF (Figure 2D). These data show that S3T3 derived PIGF has a direct proliferative effect on PC-3 cells which can be attenuated in co-cultures by siRNA targeting murine PIGF.

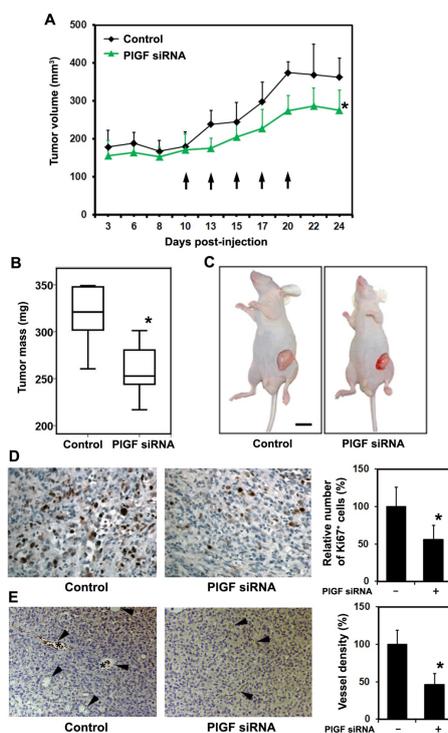
**Figure 2.** Murine PIGF siRNA reduces PIGF expression of S3T3 fibroblasts. **(A)** Co-culturing S3T3 fibroblasts with prostate cancer cells increases S3T3 PIGF expression. \* significantly different from S3T3 alone,  $p < 0.05$ ; **(B)** Transfection with siRNA targeting murine PIGF efficiently reduces endogenous S3T3 fibroblast PIGF expression 24 h following transfection compared to controls transfected with scrambled siRNA. Expression levels are expressed as percentage of corresponding control values (mean  $\pm$  SD). \* significantly different from control,  $p < 0.001$ ; **(C)** In co-cultures of PC-3 with S3T3 fibroblasts transfected with scrambled siRNA, treatment with siRNA targeting murine PIGF reduces murine PIGF expression on the mRNA level 24 h post-transfection as assessed by real time RT-PCR (left panel). \* significantly different from control,  $p < 0.001$ . Quantification of Western blots representatively shown with respective loading control (LC) demonstrates that murine PIGF protein is reduced 24 h post-transfection in co-cultures of PC-3 with S3T3 (right panel). Expression levels are expressed as percentage of corresponding control values (mean  $\pm$  SD). \* significantly different from control,  $p = 0.003$ . **(D)** Co-culturing S3T3 fibroblasts with prostate cancer cells increases the number of Ki-67 positive PC-3 nuclei when compared PC-3 cells alone. Treatment of PC-3/S3T3 co-cultures with siRNA targeting murine PIGF significantly reduces the number of Ki-67 positive PC-3 nuclei when compared to scrambled controls or PIGF siRNA co-cultures supplemented with 5 ng/mL murine PIGF. Proliferation is expressed as percentage of corresponding control values (mean  $\pm$  SD). \* significantly different from PC-3 cells alone  $p < 0.05$ ; † significantly different from controls and PIGF supplemented co-cultures,  $p < 0.05$ .



2.1.3. siRNA Mediated Blockade of Host PIGF Attenuates PC-3 Prostate Cancer Growth *in Vivo*

As shown in Figure 3A, PIGF siRNA treatment markedly suppressed tumor growth compared to the control group treated with scrambled siRNA. Following initiation of therapy on day 10 when tumor volumes were comparable at  $171.6 \pm 41.3 \text{ mm}^3$  in the control group and  $180 \pm 38.6 \text{ mm}^3$  in the PIGF siRNA group, tumor growth in the control group was higher at all subsequent time points measured at  $238.2 \pm 37 \text{ mm}^3$  in the control group compared to  $174.71 \pm 26.5 \text{ mm}^3$  in the PIGF siRNA group (day 13),  $244.9 \pm 51.6 \text{ mm}^3$  vs.  $205.2 \pm 35.2 \text{ mm}^3$  (day 15),  $297.6 \pm 51.5 \text{ mm}^3$  vs.  $227 \pm 50.9 \text{ mm}^3$  (day 17)  $374.6 \pm 28.4 \text{ mm}^3$  vs.  $273.8 \pm 40.4 \text{ mm}^3$  (day 20)  $368.9 \pm 81.1 \text{ mm}^3$  vs.  $287.3 \pm 47 \text{ mm}^3$  (day 22) and at termination on day 24,  $362.4 \pm 50.8 \text{ mm}^3$  vs.  $275.5 \pm 52.5 \text{ mm}^3$ .

**Figure 3.** Stromal PIGF blockade reduces growth of prostate cancer xenografts. (A) Intratumoral injections of siRNA targeting murine PIGF commencing 10 days following PC-3 engraftment and cycled at the indicated time points (arrows) significantly reduced tumor volumes on day 24. \* significantly different from control,  $p = 0.015$ ; (B) Tumor mass was significantly reduced in the PIGF siRNA group on day 24. \* significantly different from control,  $p = 0.008$ ; (C) Representative images of tumor-bearing mice on day 24; (D) Representative images show that siRNA targeting murine PIGF reduces tumor proliferation (Ki-67 staining). \* significantly different from control,  $p < 0.05$ ; (E) Stromal PIGF inhibition reduces microvessel density in the tumors on day 24. Representative images of sections from control tumors and PIGF siRNA treated tumors (arrowheads indicate microvessels; asterisks indicate erythrocytes within vessels) and quantification of microvessel density. Results are expressed as percentage of controls. \* significantly different from control,  $p < 0.05$ .



Tumor weights of the PIGF siRNA treated animals were significantly reduced by 18.6% from  $316.9 \pm 33.3 \text{ mg}$  to  $258 \pm 29.5 \text{ mg}$  when compared to control animals (Figure 3B). Tumor proliferation

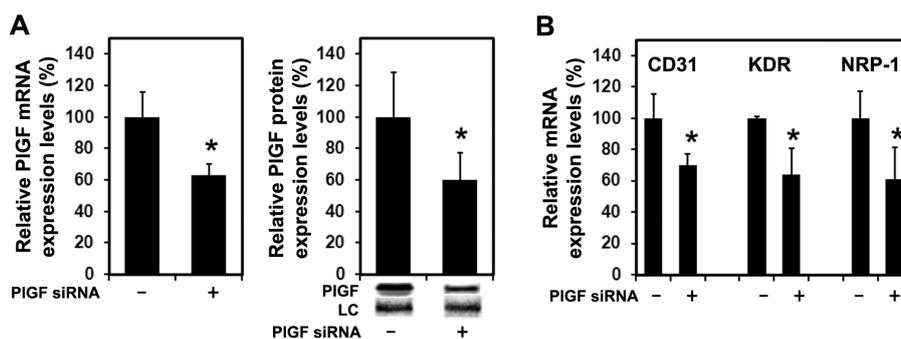
as assessed by Ki-67 antibody staining shown representatively in Figure 3D was significantly decreased following PIGF siRNA treatment by  $44.2\% \pm 19.1\%$  compared to control tumors. In addition, PIGF siRNA treatment reduced capillary density significantly by  $53.9\% \pm 15.7\%$  capillaries compared to control animals, shown representatively in Figure 3E.

These results show that PIGF siRNA treatment retards the growth of PC-3 xenografts and reduces tumor proliferation and vascular density.

#### 2.1.4. PIGF siRNA Treated Tumors Reduce Expression of Angiogenesis-Related Factors in Host Tissue

Treatment of PC-3 tumors with PIGF siRNA significantly reduced the expression of stromal derived murine PIGF mRNA (Figure 4A left panel) and protein (Figure 4A right panel). Expression of human PIGF was not detected by real-time RT-PCR in the tumors indicating PIGF expression is not induced in PC-3 cells within the tumor microenvironment. Since the vascular density in PIGF siRNA treated tumors was significantly reduced, we assessed the expression of a panel of molecules related to PIGF signaling and angiogenesis. As shown in Figure 4B, mRNA levels of murine CD31, KDR and NRP-1 were significantly reduced on the mRNA level whereas the expression of VEGF-A and FLT-1 remained unchanged (data not shown).

**Figure 4.** Stromal PIGF blockade reduces angiogenesis-related factors (A) Treatment of PC-3 xenografts with siRNA targeting murine PIGF reduces expression of intratumoral murine PIGF mRNA (left panel) and protein (right panel) compared to controls. \* significantly different from control,  $p < 0.05$ ; (B) Tumoral expression of murine CD31, KDR and NRP1 mRNA was significantly reduced after PIGF siRNA treatment compared to control tumors on day 24. \* significantly different from control,  $p < 0.05$ .



## 2.2. Discussion

In this study, we identify a direct proliferative effect for PIGF on PC-3 prostate cancer cells and investigated the role of PIGF as a potential target in prostate cancer treatment. Healthy adult prostate cells do not usually express PIGF [25]. The human PC-3 prostate cancer cell line, which is a model for advanced castration-resistant prostate carcinoma, also did not express PIGF mRNA and PIGF protein either *in vitro* or *in vivo*. During prostatic carcinogenesis the stroma undergoes progressive loss of smooth muscle, the primary component surrounding the prostatic ducts of a healthy human prostate, associated with the appearance of carcinoma-associated fibroblasts (CAFs) [8]. CAFs can promote

carcinogenesis in initiated but non-tumorigenic human prostatic epithelial cells by stimulating epithelial proliferation, for example [8]. CAFs are thus major constituents of the prostate tumor stroma and both produce PIGF [14] and respond to PIGF through stimulation of FLT-1 [13]. As a result, PIGF mRNA is also expressed in human prostate cancer tissues and PIGF mRNA levels increase after androgen deprivation therapy [24].

Our data from co-cultured fibroblasts and PC-3 prostate cancer cells show upregulation of PIGF expression in fibroblasts suggesting that a cancer cell-derived factor leads to upregulation of PIGF. Fibroblasts are also a source of PIGF in other pathological conditions such as rheumatoid arthritis [26]. In addition to fibroblasts, PIGF expression has also been detected in cultured endothelial cells whereas monocyte/macrophage lineage cells are not known to produce PIGF [27]. Thus, it is possible that endothelial cells in the tumor stroma also contribute to stromal PIGF production in prostate cancer tissue. The importance of stromal PIGF upregulation is also supported by findings in other cancer types. In medulloblastoma cells, a cancer cell-derived factor stimulates production of stromal PIGF associated with a strong tumor dependence on stromal-derived PIGF [21]. This is in line with our findings, showing that blockade of stromal PIGF reduces PC-3 prostate tumor growth. Blockade of stromal PIGF therefore offers the advantage of disrupting a tumor-stroma interaction that contributes to prostate cancer progression. Bypassing the genetically unstable tumor cell may also reduce the risk of developing therapy resistance. In this context, identifying the prostate cancer cell derived factor, which increases stromal PIGF expression could be additionally important in disrupting tumor-stroma interaction as has been suggested for medulloblastoma [21]. TGF- $\beta$  is a carcinoma-produced factor which has the potential to regulate the stromal compartment during prostate cancer progression [28] and is a potential candidate regulator of PIGF expression. Moreover, TGF- $\beta$  signaling is essential for formation of CAFs in the tumor microenvironment and subsequent tumor promoting effects [29]. Consequently, combined inhibition of PIGF and its cancer cell-derived inducer might offer the maximum benefit in disrupting tumor-stroma interactions. The importance of stromal PIGF is also supported by findings in leukemia, in which loss or inhibition of stromal-derived PIGF prolongs the survival of mice with imatinib-resistant Bcr-Abl1(+) leukemia [22].

PIGF directly stimulates neovascularization by stimulating the proliferation, migration and survival of endothelial cells [17]. Loss of PIGF, although not causing any vascular defects during development, reproduction or normal adult life, impairs angiogenesis in pathological conditions [17]. PIGF<sup>-/-</sup> fibrosarcomas and embryonic stem cell-derived tumors, for example, are smaller and less vascularized than wild type tumors when implanted in nude mice, underlining the role of PIGF in tumor angiogenesis [17]. PIGF regulates many genes involved in angiogenesis [16], the expression of which could be affected by a PIGF-blockade. PIGF is mitogenic and proangiogenic [30] and blockade of PIGF has resulted in inhibition of angiogenesis and tumor cell motility and inhibited growth and metastasis in some models [19], while in others neutralization of PIGF has had no significant effect on tumor angiogenesis [31]. We observed decreased gene expression of the endothelial cell marker CD31 associated with decreased vascular density in tumors, suggesting that the anti-tumor effect of PIGF blockade is at least partly mediated by PIGF stimulated neovascularization in the PC-3 prostate cancer model. Additionally, we found reduced KDR and NRP1 gene expression levels following PIGF therapy. These changes may also interfere with VEGF-driven angiogenesis, since this could affect the transmission of angiogenic signals. By displacing VEGF from FLT-1, PIGF increases the availability

of VEGF which in turn can bind to and activate KDR/VEGFR-2 [16]. Activation of FLT-1 by PIGF can lead to intermolecular transphosphorylation of KDR, thus enhancing KDR-phosphotyrosine levels [16]. It is also possible that PIGF heterodimerizes with VEGF [11,16] to transmit angiogenic signals through binding to and activation of the FLT-1/KDR heterodimer receptor complex [16].

Although PIGF-binding receptors have been extensively studied [19], downstream signaling events and roles in cancer cell survival are not fully understood. A recent study showed that the efficacy of anti-PIGF treatment depends on the presence of functional VEGFR1 (the only known tyrosine kinase receptor for PIGF in tumor cells) but not with antiangiogenesis [32]. In addition, anti-PIGF treatment inhibited growth of a tumor engineered to overexpress the PIGF receptor (VEGFR-1) [31]. Thus, it has been suggested that the role of PIGF in tumorigenesis largely consists of promoting autocrine/paracrine growth of tumor cells expressing a functional VEGFR-1 rather than stimulation of angiogenesis [32]. However, direct inhibition of VEGFR-1 activity did not explain anti-PIGF effects in models pancreatic islet tumors [33] and blockade of VEGFR-1 activity does not affect the rate of spontaneous metastasis formation in different tumor models [34]. Another report showed that genetic ablation of VEGFR-1 signaling in the host did not affect tumor growth [31]. Furthermore, a recent study showed that in medulloblastoma, PIGF signaling via NRP-1 and not VEGFR-1 directly conveys prosurvival signals [21]. These findings suggest that alternative signaling mechanisms for PIGF independent of VEGFR1 exist. Consequently, we cannot rule out that such alternative signaling pathways could account for the PIGF tumor promoting effects in prostate cancer.

Measuring CD31 (also called PECAM-1) revealed decreased mouse mRNA levels in prostate cancer tissue following PIGF blockade. CD31 is highly expressed in the vasculature with approximately one million copies of CD31 on the surface of endothelial cells. CD31 is involved in the initial formation and stabilization of cell-cell contacts at lateral junctions of endothelial cells and transendothelial migration [35]. On the other hand, activated KDR stimulates EC migration survival and growth [36]. The observed reduction of CD31 and KDR mRNA thus provides evidence of less EC and/or less mature vessels within the tumor following PIGF blockade. In addition, mRNA levels of NRP-1, which is not only a co-receptor that enhances the binding of VEGF to KDR but also a PIGF receptor and is thought to play a role in the formation of cell-cell contacts and the permeabilization of blood vessels [37], were also reduced in tumor tissue following PIGF siRNA treatment. From these results, it may be concluded that the reduction of tumor weight by PIGF blockade does involve down-regulation of tumor angiogenesis in prostate cancer.

The role of PIGF in tumor growth and tumor angiogenesis seems to be dependent on the tumor type. In some tumors, PIGF may be important as an angiogenic factor whereas in other tumors it may represent a vital growth factor for tumor survival [21]. Our data support the critical importance of host PIGF in PC-3 prostate cancer and reveals PIGF as a necessary factor for prostate cancer cell growth and in promoting tumor angiogenesis.

Stromal PIGF blockade by siRNA did not lead to complete eradication of PC-3 prostate cancer xenografts. This could be due to low siRNA delivery efficiency and/or the development of unknown mechanisms of resistance after anti-PIGF therapy in prostate cancer. PIGF upregulation has been shown to be a host response to anti-angiogenic therapy [38]. Consequently, a similar mechanism can be proposed for any anti-PIGF therapy. In this study, VEGF-A levels were unchanged following PIGF-blockade. It is possible that a compensatory upregulation of angiogenic factors other than VEGF-A might occur,

which could contribute to the observed moderate effect of PIGF blockade on tumor growth in this model. However, since PIGF blockade by an antibody inhibited growth and metastasis of various tumors, including those resistant to VEGF receptor inhibitors, and enhanced the efficacy of chemotherapy [19], PIGF antibody could be used for therapy. Recent phase I, dose-escalation clinical studies of humanized anti-PIGF antibody TB403 also showed that anti-PIGF therapy was well tolerated with minimal side effects [39,40]. Furthermore, anti-PIGF therapy in prostate cancer would likely be used in combination with standard therapies. Nevertheless, recent technological advances in the use of lipid nanoparticles (LNPs) to deliver siRNAs into target cells observed improved anticancer efficacy suggesting that nanomedicine provides novel opportunities to safely deliver genes to treat cancer [41]. In line with this, down-regulation of regulators of the major intracellular recycling pathways of LNP-delivered siRNAs show enhanced cellular retention of LNPs inside late endosomes and lysosomes and increased gene silencing of the target gene. These data suggest that siRNA delivery efficiency might be improved by designing delivery vehicles that can escape recycling pathways [42]. Thus, technologies promoting siRNA delivery to silence PIGF involved in prostate cancer might be beneficial in treatment of this disease, although tumor subtype, stage and the tumor microenvironment certainly will affect the outcome of any PIGF inhibition therapy.

### 3. Experimental Section

#### 3.1. Cell Culture and Transfection

Human PC-3 (CRL-1435) and mouse S3T3 fibroblasts (CCL-92; mouse embryonic fibroblasts) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified eagle medium (DMEM; PAA, Pasching, Austria) supplemented with 10% fetal calf serum (PAA), 50 U/mL penicillin and 250 µg/mL streptomycin (PAA) in a fully humidified air atmosphere containing 5% CO<sub>2</sub> at 37 °C. PC-3 cells were co-cultured with S3T3 at a ratio of 1:1 (2.5 × 10<sup>4</sup> cells per cell line per 6-well plate for RNA isolation and proliferation assays or 2.5 × 10<sup>5</sup> cells per cell line per 55 cm<sup>2</sup> plate for protein isolation). Co-cultures were incubated for 24 h in the absence of antibiotics in culture medium and were then transfected with siRNA (50 nM) directed against mouse PIGF (target sequence 5'-GUGUACAUCUUGGAUGAAU-3') or a scrambled siRNA (sequence 5'-GAAGCAGCACGACTTCTTC-3') using Lipofectamine reagent (Life Technologies, Grand Island, NY, USA). RNA and protein for real-time RT-PCR or Western blot analysis were isolated 24 h following transfection. All experiments were performed in triplicate.

#### 3.2. Cell Proliferation Assays

PC-3 cells were seeded in 96-well plates at a density of 5 × 10<sup>4</sup> cells/well and starved for 24 h before treatment with 5 ng/mL or 20 ng/mL recombinant mouse PIGF (Sigma-Aldrich, St. Louis, MO, USA). Cell proliferation was determined at 24, 48 and 72 h after treatment using the WST-1 reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. Proliferation differences were expressed as percentages of untreated control cells. All measurements were performed in triplicate.

### 3.3. Real Time RT-PCR

Total RNA was isolated from cell cultures in TRIzol reagent (Life Technologies, Grand Island, NY, USA). Tumor samples were snap frozen in liquid nitrogen and then homogenized in TRIzol for RNA isolation. Reverse transcription was performed with MMLV reverse transcriptase (Fermentas, Burlington, ON, Canada) and PCR with FastStart DNA Master SYBR Green mix (Roche Diagnostics, Indianapolis, IN, USA).

The 5'-sense-3'/5'-antisense-3' primers (VBC Genomics, Vienna, Austria) utilized were mouse  $\beta$ -2 microglobulin CCTCACATTGAAATCCAAATGC/CGGCCATACTGTCATGCTTAAC, mouse PIGF GAGCTTCGGCTTGGGAAGAAG/GTTCCAGAGAGGGGACAAAGG, mouse CD31 CAAAGAAAAGGAGGACAG/GATGACCACTCCAATGAC, mouse KDR GGAGATTGAAAGAAGGAAC/ACTTCTCTTCCATAC and mouse NRP-1 CCAGAAAACATCCGTCTGGT/CCTTGTTTTCTCGGTGCTTC. LightCycler Software Version 3.5.3 (Roche, Mannheim, Germany, 2001) was used for PCR data analysis. The relative quantification of the signals was done by normalizing the signals of the different genes to  $\beta$ -2 microglobulin as described [43]. Measurements were performed in triplicate.

### 3.4. Western Blotting

Cell lysates were prepared [44] and proteins (50  $\mu$ g/lane) were separated by 9% SDS-PAGE prior to electrophoretic transfer onto Hybond C super (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were probed sequentially with goat anti-mouse PIGF (Santa Cruz, Dallas, TX, USA). Proteins were immunodetected by chemiluminescence (Supersignal-West-Femto, Pierce, Rockford, IL, USA) and quantified by Fusion-CAPT-Software 16.07 (Vilber Lourmat, Marne-la-Vallée, France, 2012). Reversible Ponceau S staining was used as a loading control in Western blotting [45].

### 3.5. Histology and Immunohistochemistry, Immunofluorescence

S3T3 cells were labeled with DilC<sub>18</sub> (Molecular probes, Life Technologies, Grand Island, NY, USA), co-cultured with PC-3, and cytopins prepared and fixed in acetone at 4 °C for 8 min. Tissue samples were fixed in formalin and paraffin embedded. Sections were rehydrated in graded alcohols and antigen retrieval was performed in a microwave in 0.1 M sodium citrate (pH 6.5). Sections and cytopin slides were blocked in PBS supplemented with 5% horse serum and stained with monoclonal rabbit anti-human Ki-67 (DAKO, Glostrup, Denmark) or a rabbit polyclonal von Willebrand Factor antibody (vWF; Abcam, Cambridge, UK) to evaluate the density of endothelial cells (ECs). Primary antibody was detected by biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) and either Alexa Fluor 488 conjugated streptavidin (Molecular Probes, Life Technologies, Grand Island, NY, USA), then rinsed with PBS and stained with 0.1  $\mu$ g/mL 4'-6-Diamidino-2-phenylindole (DAPI), mounted in Cityfluor and analyzed on a fluorescence microscope (Zeiss, Thornwood, New York, NY, USA) or horseradish peroxidase-conjugated streptavidin (DAKO), then developed with DAB chromogen (Vector Laboratories, Burlingame, CA, USA). Ki-67 positive proliferating cells and capillaries were counted in 10 consecutive (magnification,  $\times$ 20) fields per slide and results are expressed as percentages of controls. Co-culture

cytopins were analyzed for Ki-67 positive nuclei in DiIC<sub>18</sub> negative cells determined by immunofluorescence.

### 3.6. Xenograft Model

Experiments were approved by the Institutional Animal Care and Use Committee at the Medical University of Vienna. Pathogen-free male athymic nu/nu (nude) mice (Harlan-Winkelmann, Borchon, Germany), 5 weeks of age, were weighed and coded and randomly assigned to two experimental groups ( $n = 6$ ). PC-3 ( $10^7$ ) cells in 100  $\mu$ L Ringer solution were then injected subcutaneously (s.c.) in the left flank. Tumor volumes were calculated as length  $\times$  width<sup>2</sup>  $\times$  0.5. After 10 days when tumors had developed average volumes of approximately 175 mm<sup>2</sup>, mice were anesthetized (ketamine hydrochloride/xylazine at 55:7.5 mg/kg, s.c.) and 10  $\mu$ g PIGF siRNA or scrambled siRNA dissolved in 20  $\mu$ L Lipofectamine reagent (Life Technologies, Grand Island, NY, USA) was injected intratumorally. The treatment was cycled on days 13, 15, 17 and 20. On day 24, the animals were sacrificed and the tumors isolated, weighed and prepared for molecular analyses.

### 3.7. Statistical Analysis

We used the Student's *t* test and ANOVA to compare the data between the groups. All statistical tests were two-sided. Statistical tests were done with the use of SPSS software (version 20, SPSS Inc., Chicago, IL, USA, 2011). Data are expressed as means  $\pm$  SD. *p* values <0.05 were considered to indicate statistical significance.

## 4. Conclusions

Our findings indicate that targeting stromal PIGF by siRNAs can cause effective suppression of tumor cell proliferation associated with reduced vessel density and a reduction of tumor weight. Furthermore, this study indicates the importance of therapeutics targeting the tumor stroma by direct inhibition of a growth factor specifically expressed in the tumor microenvironment. Therefore, inhibition of PIGF may provide an attractive target for the design of novel anticancer therapeutics for prostate cancer. Future applications of siRNAs, however, are largely dependent on the development of appropriate delivery vehicles with optimal pharmacokinetics and intracellular stability.

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## Conflicts of Interest

The authors declare no conflict of interest.

## References

1. Siegel, R.; Naishadham, D.; Jemal, A. Cancer statistics, 2013. *CA: A Cancer J. Clin.* **2013**, *63*, 11–30.

2. Beltran, H.; Beer, T.M.; Carducci, M.A.; de Bono, J.; Gleave, M.; Hussain, M.; Kelly, W.K.; Saad, F.; Sternberg, C.; Tagawa, S.T.; *et al.* New therapies for castration-resistant prostate cancer: Efficacy and safety. *Eur. Urol.* **2011**, *60*, 279–290.
3. Pagliarulo, V.; Bracarda, S.; Eisenberger, M.A.; Mottet, N.; Schroder, F.H.; Sternberg, C.N.; Studer, U.E. Contemporary role of androgen deprivation therapy for prostate cancer. *Eur. Urol.* **2012**, *61*, 11–25.
4. Carles, J.; Castellano, D.; Climent, M.A.; Maroto, P.; Medina, R.; Alcaraz, A. Castration-resistant metastatic prostate cancer: Current status and treatment possibilities. *Clin. Transl. Oncol.* **2012**, *14*, 169–176.
5. Weidner, N.; Carroll, P.R.; Flax, J.; Blumenfeld, W.; Folkman, J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am. J. Pathol.* **1993**, *143*, 401–409.
6. Hanahan, D.; Weinberg, R.A. The hallmarks of cancer. *Cell* **2000**, *100*, 57–70.
7. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **2011**, *144*, 646–674.
8. Cunha, G.R.; Hayward, S.W.; Wang, Y.Z. Role of stroma in carcinogenesis of the prostate. *Differentiation* **2002**, *70*, 473–485.
9. Cirri, P.; Chiarugi, P. Cancer associated fibroblasts: The dark side of the coin. *Am. J. Cancer Res.* **2011**, *1*, 482–497.
10. Franco, O.E.; Hayward, S.W. Targeting the tumor stroma as a novel therapeutic approach for prostate cancer. *Adv. Pharmacol.* **2012**, *65*, 267–313.
11. Tammela, T.; Enholm, B.; Alitalo, K.; Paavonen, K. The biology of vascular endothelial growth factors. *Cardiovasc Res.* **2005**, *65*, 550–563.
12. Tjwa, M.; Luttun, A.; Autiero, M.; Carmeliet, P. VEGF and PlGF: Two pleiotropic growth factors with distinct roles in development and homeostasis. *Cell Tissue Res.* **2003**, *314*, 5–14.
13. Luttun, A.; Autiero, M.; Tjwa, M.; Carmeliet, P. Genetic dissection of tumor angiogenesis: Are PlGF and VEGFR-1 novel anti-cancer targets? *Biochim. Biophys. Acta* **2004**, *1654*, 79–94.
14. De Falco, S. The discovery of placenta growth factor and its biological activity. *Exp. Mol. Med.* **2012**, *44*, 1–9.
15. Neufeld, G.; Kessler, O.; Herzog, Y. The interaction of Neuropilin-1 and Neuropilin-2 with tyrosine-kinase receptors for VEGF. *Adv. Exp. Med. Biol.* **2002**, *515*, 81–90.
16. Autiero, M.; Waltenberger, J.; Communi, D.; Kranz, A.; Moons, L.; Lambrechts, D.; Kroll, J.; Plaisance, S.; de Mol, M.; Bono, F.; *et al.* Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat. Med.* **2003**, *9*, 936–943.
17. Carmeliet, P.; Moons, L.; Luttun, A.; Vincenti, V.; Compernelle, V.; de Mol, M.; Wu, Y.; Bono, F.; Devy, L.; Beck, H.; *et al.* Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat. Med.* **2001**, *7*, 575–583.
18. Dewerchin, M.; Carmeliet, P. PlGF: A multitasking cytokine with disease-restricted activity. *Cold Spring Harb Perspect. Med.* **2012**, doi:10.1101/cshperspect.a011056.
19. Fischer, C.; Jonckx, B.; Mazzone, M.; Zacchigna, S.; Loges, S.; Pattarini, L.; Chorianopoulos, E.; Liesenborghs, L.; Koch, M.; de Mol, M.; *et al.* Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. *Cell* **2007**, *131*, 463–475.

20. Xu, L.; Jain, R.K. Down-regulation of placenta growth factor by promoter hypermethylation in human lung and colon carcinoma. *Mol. Cancer Res.* **2007**, *5*, 873–880.
21. Snuderl, M.; Batista, A.; Kirkpatrick, N.D.; Ruiz de Almodovar, C.; Riedemann, L.; Walsh, E.C.; Anolik, R.; Huang, Y.; Martin, J.D.; Kamoun, W.; *et al.* Targeting placental growth factor/neuropilin 1 pathway inhibits growth and spread of medulloblastoma. *Cell* **2013**, *152*, 1065–1076.
22. Schmidt, T.; Kharabi Masouleh, B.; Loges, S.; Cauwenberghs, S.; Fraisl, P.; Maes, C.; Jonckx, B.; de Keersmaecker, K.; Kleppe, M.; Tjwa, M.; *et al.* Loss or inhibition of stromal-derived PIGF prolongs survival of mice with imatinib-resistant Bcr-Abl1(+) leukemia. *Cancer Cell* **2011**, *19*, 740–753.
23. Xu, L.; Cochran, D.M.; Tong, R.T.; Winkler, F.; Kashiwagi, S.; Jain, R.K.; Fukumura, D. Placenta growth factor overexpression inhibits tumor growth, angiogenesis, and metastasis by depleting vascular endothelial growth factor homodimers in orthotopic mouse models. *Cancer Res.* **2006**, *66*, 3971–3977.
24. Matsumoto, K.; Suzuki, K.; Koike, H.; Hasumi, M.; Matsui, H.; Okugi, H.; Shibata, Y.; Ito, K.; Yamanaka, H. Placental growth factor gene expression in human prostate cancer and benign prostate hyperplasia. *Anticancer Res.* **2003**, *23*, 3767–3773.
25. Marrony, S.; Bassilana, F.; Seuwen, K.; Keller, H. Bone morphogenetic protein 2 induces placental growth factor in mesenchymal stem cells. *Bone* **2003**, *33*, 426–433.
26. Tu, H.J.; Lin, T.H.; Chiu, Y.C.; Tang, C.H.; Yang, R.S.; Fu, W.M. Enhancement of placenta growth factor expression by oncostatin M in human rheumatoid arthritis synovial fibroblasts. *J. Cell Physiol.* **2013**, *228*, 983–990.
27. Fujii, T.; Yonemitsu, Y.; Onimaru, M.; Inoue, M.; Hasegawa, M.; Kuwano, H.; Sueishi, K. VEGF function for upregulation of endogenous PIGF expression during FGF-2-mediated therapeutic angiogenesis. *Atherosclerosis* **2008**, *200*, 51–57.
28. Barron, D.A.; Rowley, D.R. The reactive stroma microenvironment and prostate cancer progression. *Endocr. Relat. Cancer* **2012**, *19*, R187–R204.
29. Shangguan, L.; Ti, X.; Krause, U.; Hai, B.; Zhao, Y.; Yang, Z.; Liu, F. Inhibition of TGF-beta/Smad signaling by BAMBI blocks differentiation of human mesenchymal stem cells to carcinoma-associated fibroblasts and abolishes their protumor effects. *Stem Cells* **2012**, *30*, 2810–2819.
30. Ziche, M.; Maglione, D.; Ribatti, D.; Morbidelli, L.; Lago, C.T.; Battisti, M.; Paoletti, I.; Barra, A.; Tucci, M.; Parise, G.; *et al.* Placenta growth factor-1 is chemotactic, mitogenic, and angiogenic. *Lab. Invest.* **1997**, *76*, 517–531.
31. Bais, C.; Wu, X.; Yao, J.; Yang, S.; Crawford, Y.; McCutcheon, K.; Tan, C.; Kolumam, G.; Vernes, J.M.; Eastham-Anderson, J.; *et al.* PIGF blockade does not inhibit angiogenesis during primary tumor growth. *Cell* **2010**, *141*, 166–177.
32. Yao, J.; Wu, X.; Zhuang, G.; Kasman, I.M.; Vogt, T.; Phan, V.; Shibuya, M.; Ferrara, N.; Bais, C. Expression of a functional VEGFR-1 in tumor cells is a major determinant of anti-PIGF antibodies efficacy. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 11590–11595.

33. Casanovas, O.; Hicklin, D.J.; Bergers, G.; Hanahan, D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* **2005**, *8*, 299–309.
34. Dawson, M.R.; Duda, D.G.; Fukumura, D.; Jain, R.K. VEGFR1-activity-independent metastasis formation. *Nature* **2009**, *461*, E4.
35. Jackson, D.E. The unfolding tale of PECAM-1. *FEBS Lett.* **2003**, *540*, 7–14.
36. Shibuya, M.; Claesson-Welsh, L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp. Cell. Res.* **2006**, *312*, 549–560.
37. Migdal, M.; Huppertz, B.; Tessler, S.; Comforti, A.; Shibuya, M.; Reich, R.; Baumann, H.; Neufeld, G. Neuropilin-1 is a placenta growth factor-2 receptor. *J. Biol. Chem.* **1998**, *273*, 22272–22278.
38. Bagley, R.G.; Ren, Y.; Weber, W.; Yao, M.; Kurtzberg, L.; Pinckney, J.; Bangari, D.; Nguyen, C.; Brondyk, W.; Kaplan, J.; *et al.* Placental growth factor upregulation is a host response to antiangiogenic therapy. *Clin. Cancer Res.* **2011**, *17*, 976–988.
39. Lassen, U.; Nielsen, D.L.; Sorensen, M.; Winstedt, L.; Niskanen, T.; Stenberg, Y.; Pakola, S.; Stassen, J.M.; Glazer, S. A phase I, dose-escalation study of TB-403, a monoclonal antibody directed against PlGF, in patients with advanced solid tumours. *Br. J. Cancer* **2012**, *106*, 678–684.
40. Martinsson-Niskanen, T.; Riisbro, R.; Larsson, L.; Winstedt, L.; Stenberg, Y.; Pakola, S.; Stassen, J.M.; Glazer, S. Monoclonal antibody TB-403: A first-in-human, Phase I, double-blind, dose escalation study directed against placental growth factor in healthy male subjects. *Clin. Ther.* **2011**, *33*, 1142–1149.
41. Singh, S. Nanomaterials as non-viral siRNA delivery agents for cancer therapy. *Bioimpacts* **2013**, *3*, 53–65.
42. Sahay, G.; Querbes, W.; Alabi, C.; Eltoukhy, A.; Sarkar, S.; Zurenko, C.; Karagiannis, E.; Love, K.; Chen, D.; Zoncu, R.; *et al.* Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling. *Nat. Biotechnol.* **2013**, *31*, 653–658.
43. Abraham, D.; Zins, K.; Sioud, M.; Lucas, T.; Aharinejad, S. Host CD147 blockade by small interfering RNAs suppresses growth of human colon cancer xenografts. *Front. Biosci.* **2008**, *13*, 5571–5579.
44. Zins, K.; Abraham, D.; Sioud, M.; Aharinejad, S. Colon cancer cell-derived tumor necrosis factor-alpha mediates the tumor growth-promoting response in macrophages by up-regulating the colony-stimulating factor-1 pathway. *Cancer Res.* **2007**, *67*, 1038–1045.
45. Romero-Calvo, I.; Ocon, B.; Martinez-Moya, P.; Suarez, M.D.; Zarzuelo, A.; Martinez-Augustin, O.; de Medina, F.S. Reversible ponceau staining as a loading control alternative to actin in Western blots. *Anal. Biochem.* **2010**, *401*, 318–320.