Supplementary Data

Results

PlGF1-DE/VEGF heterodimer purification.

To purify PlGF1-DE/VEGF heterodimer we used culture medium of H460-PlGF1-DE stable clone since it produces the highest amount of heterodimer (**Table 1**). The extent of purification was evaluated by ELISA and Western blot analysis.

Quantitative ELISAs indicated that the purification procedure allowed obtaining 1026 ng of PlGF1-DE/VEGF heterodimer with a yield of 47%. Only negligible amounts of VEGF (0.85 ng) or PlGF-DE (2.7 ng) homodimers were detectable in the purified fractions.

Furthermore, Western blot analyses performed in non-reducing condition confirmed the purification degree. Proteins were detected using anti-heterodimer and anti-PlGF antibodies (**Figure S1**). The anti-heterodimer antibody recognized specifically only recombinant heterodimer and purified PlGF1-DE/VEGF heterodimer, whereas the anti-PlGF antibody, as expected recognized both PlGF homodimer and the two forms of heterodimer. No contamination of PlGF was detectable in the PlGF1-DE/VEGF preparation. We analyzed the purification with anti-PlGF antibody since PlGF was present at higher concentration compared to VEGF in the purified heterodimer preparation, as determined by ELISA assays.

Differences in vessel lumen in tumors overexpressing PlGF1-DE or PlGF1wt .

We decided to further analyze tumor vasculature to assess which differences are generated by the overexpression of PlGF1wt or PlGF1-DE. First we evaluated the distribution of vessels based on their lumen, dividing the vessels into three groups based on the length of bigger diameter: <8 μm (small), 8-30 μm (medium)

and >30 μm (large) (14). In the first series of tumor analyzed (n=7 per group) (**Fig. S3A**) A2780-PlGF1-DE tumors showed significant percentage increase in small vessels ($p<0.005$ vs A2780 and A2780-pCDNA3; $p<0.0001$ vs A2780-PlGF1wt) and a corresponding significant decrease ($p<0.0001$ vs all other groups) of medium and large vessels. Interestingly, A2780-PlGF1wt tumors showed an opposite vessels distribution, with a significant decrease of small vessels ($p=0.05$ vs A2780; $p\leq0.01$ vs A2780-pCDNA3) and a significant increase ($p<0.0001$ vs A2780 and A2780-pCDNA3) of medium and large vessels.

A similar distribution was found analyzing the vasculature of tumors with the same volume (n=5 per group) (**Fig. S3B**). A2780-PlGF1-DE tumors again showed significant increase in small vessels ($p<0.05$ vs A2780 and A2780-PlGF1-N; $p<0.01$ vs A2780-pCDNA3 and A2780-PlGF1wt) and a significant decrease of medium ($p<0.0001$ vs all other groups) and large ($p<0.001$ vs A2780; $p<0.01$ vs A2780-pCDNA3; $p<0.0001$ vs A2780-PlGF1wt and A2780-PlGF1-N) vessels. About tumors over-expressing active PlGF1wt and PlGF1-N, only tumors over-expressing PlGF1wt displayed a significant decrease of small vessels ($p<0.05$ vs A2780 and A2780-pCDNA3), but both showed a significant increase of large vessels ($p<0.0001$ vs A2780 and A2780-pCDNA3).

Susceptibility of A2780 cells to Ad infection.

We demonstrated that recombinant Ads were able to transduce in vitro the A2780 cells. Indeed after 24 hours from transduction, almost all cells exposed to Ad-GFP with a multiplicity of infection (MOI) = 20 expressed GFP (**Fig. S4**). Moreover, after 48 hours from infection with Ad-PlGF1wt and Ad-PlGF1-DE the presence of VEGF/PlGF heterodimer and the consequent significant reduction of VEGF

homodimer production ($p = 0.01$ vs Ad-GFP) were measurable in the A2780-trasduced cell culture medium (**Table S1**).

Materials and Methods

Cell culture.

All cell lines were cultured at 37°C in the presence of 5% CO_2. The stable cell line 293-hFlt-1 was grown in Dulbecco Modified Eagle Medium, the PAE-KDR stable line was cultured in Ham's F-12, in both cases supplemented with 10 % inactivated fetal bovine serum FBS, 2 mM glutamine, standard antibiotics concentrations plus 0.2 mg/ml of Geneticin. HUVECs were grown in endothelial basal medium containing endothelial growth factor supplements (EBM-2 and EGM-2 bullet kit, Cambrex, Charles City, IA).

Cell proliferation assay.

The growth rate of generated stable clones was evaluated using the CellTiter Aqueous One Cell Proliferation Assay (Promega, Madison, WI), following the manufacturer's procedure. Cells were seeded at different densities in standard (10%) or low serum conditions (1%), and the growth was evaluated each 24 hours up to 72 hours. Each point was done in triplicate and the experiment was repeated two times. The absorbance at 490 nm was measured on a microplate reader (BenchMark, Bio-Rad, Hercules, CA).

ELISA assays

All the reagents used in ELISA were from R&D Systems (Minneapolis, MN). To avoid the interference of the heterodimer in the quantification of PlGF and VEGF homodimers, for PlGF determination samples were pre-incubated with anti-VEGF antibody coated on ELISA plate at 1 µg/ml, while for VEGF determination, samples was pre-incubated with anti-PlGF antibody, coated at 1 µg/ml. To quantify the

VEGF/PlGF heterodimer, antibody anti-PlGF was coated on ELISA plate, while biotinylated antibody anti-VEGF was used in solution. As reference, human recombinant VEGF, PlGF and VEGF/PlGF dimers were used. PlGF, VEGF and VEGF/PlGF concentrations were determined by interpolation with the relative standard curves, using linear regression analysis. For the evaluation of the presence of mPlGF/hPlGF dimer in tumor extracts from xenograft tumor infected with Ads, anti-hPlGF antibody was used in capture while biotinylated antibody anti-mPlGF was used in solution. Due to the absence of recombinant mPlGF/hPlGF dimer availability, standard curves were performed on the same ELISA plate contemporarily for mPlGF and hPlGF homodimers, and the concentration reported were calculated by interpolation on the average of the two curves.

VEGF and PlGF binding to VEGF-R1 and -R2 in ELISA-based assays.

The procedure performed to determine the binding activity of VEGF and VEGF/PlGF to VEGFR-1 and R-2, were performed as recently described (19). The same procedure was used to evaluate the binding of PlGF and VEGF dimers present in the culture medium of A2780-PlGF1-DE stable clone. The culture medium was opportunely concentrated and 2.5 to 10 ng/ml of VEGF was used. Consequently the quantity of PlGF1-DE/VEGF used was between 4.5 and 18 and that of PlGF1-DE between 71.5 and 286 ng/ml (**see Table 1**). Antibodies anti-VEGF were used to detect the VEGF/VEGFR-1 binding, whereas antibody anti-PlGF were used to detect the binding of PlGF1-DE and PlGF1-DE/VEGF dimers. Binding of active PlGF dimers produced by A2780-PlGF1wt was confirmed with similar assay (not shown). For all ELISAs, each point was carried out in triplicate and each experiment was repeated two times.

PlGF1-DE/VEGF heterodimer purification and analysis.

We have collected 1.4 L of serum free conditioned medium from H460-PlGF1-DE stable clone. The concentration of PlGF and VEGF dimers was evaluated in each step of this procedure by sandwich ELISAs as described above. The medium was dialyzed against PBS and concentrated to 50 ml using a Viva Flow 50 system, 10,000 MW cut off polyethersulfone filter (Sartorius AG, Goettingen, Germany). To purify the heterodimer 1 ml of NHS-activated Sepharose 4 fast flow resin (GE Healthcare Europe, Milan, Italy) was used to prepare two affinity columns, the first coupling a mAb anti-PlGF (ADD109, Abbott Diagnostics, Abbott Park, IL), the second coupling an anti-VEGF polyclonal antibody (R&D Systems, Minneapolis, MN) following the procedures suggested by the manufacturer. 15 ml of dialyzed and concentrated medium (containing 9060 ng of PlGF1-DE, 795 ng of VEGF and 2175 ng of heterodimer) were loaded on the anti-VEGF column, in order to capture VEGF homodimer and VEGF/PlG1-DE heterodimer and to remove PlGF-DE homodimer. The bound proteins were eluted with two-column volume of glycine 50mM pH 2.6 , immediately neutralized with sufficient volume of Trizma base 1M. The eluted proteins were loaded on the anti-PlGF column to capture the heterodimer, which was eluted as described before. After the two affinity chromatography steps, 1026 ng of purified heterodimer with a yield of 47% was obtained. Only traces of VEGF (0.85 ng) or PlGF-DE (2.7 ng) homodimers were detectable.

The purification was confirmed also by Western blot analysis. Purified VEGF/PlG1-DE heterodimer (10 ng) and the same amount of recombinant wild type VEGF/PlGF heterodimer (32 kDa), VEGF (42 kDa) and PlGF (29 kDa) homodimers (R&D Systems Minneapolis, MN) were analyzed in non-reducing conditions. To detect the

proteins anti-VEGF/PlGF heterodimer (1µg/ml) (R&D Systems Minneapolis, MN) and anti-PlGF (1 µg/ml) (ADD109, Abbott Diagnostics, Abbott Park, IL) were used.

Receptor Phosphorylation assays

These assays were performed as described elsewhere (19). Briefly, 293-hFlt-1 and PAE-KDR were starved for 16 hours in absence of FBS. HUVECs were starved in absence of growth factors, but in presence of 1% FBS. To induce VEGFR-1 and VEGFR-2 receptors activation, 20 ng/ml of PlGF1 or 50 ng/ml of VEGF, VEGF/PlGF or VEGF/PlGF1-DE heterodimers for 10 minutes were used. In competition experiments, 50 ng/ml of VEGF in the presence of 500 ng/ml of heterodimer was assayed. To detect phosphorylated forms of receptors in western blot experiments performed following standard procedures, antibodies anti-p-VEGFR-1 (R&D Systems, Minneapolis, MN) diluted 1:500 and anti-p-VEGFR-2 (Cell Signaling, Danvers, MA) diluted 1:1000 were used. Normalization was performed using anti-VEGFR-1, 1:500 (Sigma-Aldrich) or anti-VEGFR-2, 1:500 (SantaCrutz Biotechnology, Santa Crutz, CA).

Tumor protein extracts

Frozen tumor samples were disrupted with a Tissue-Lyser (Qiagen, Milan, Italy) in a lysis buffer composed by 10 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton-X 100, 0.1% SDS, 0.5% Na-Deossycolate, 0.2% NaN_3, 100 µM Na_3VO_4 and a mixture of protease inhibitors, 300 µl / 100 mg of tissue, for 5 minutes at 3000 rpm. Samples were then incubated under agitation for 1 hour at 4°C and centrifuged at 12,000 x g for 15 minutes. The supernatants were recovered, aliquoted and stored at -80°C. The protein concentration was determined by the Bradford method (BioRad reagent). 50-

400 µg of extract was used in ELISAs for determination of VEGF and PlGF dimers.

Tumor histomorphometrical and immunohistochemical analyses.

Tumor sections were stained with Hematoxylin & Eosin by standard procedures. Morphometric evaluation of necrosis was performed on images captured by a Hamamatsu camera connected to a Nikon microscope and analyzed by Scion Image software (Scion Corporation, Frederick, MA). Blinded evaluation of mitotic index (mitotic cells per mm^2) at 200X magnification was performed on ten independent fields/animal by two observers, with intervariability less than 5%. Necrotic and hemorrhagic areas were excluded from the mitotic count.

Four µm-thick deparaffined tumor sections were incubated ON at 4°C with the following primary antibodies: rat anti-mouse PECAM-1 (anti-CD31; BD Pharmingen, San Jose, CA) 1:1000, anti-smooth muscle α-actin (DAKO) 1:1000, rat anti-mouse F4/80 (Serotec, Oxford, UK) 1:50. The staining procedure was continued using specific secondary biotinylated antibody (all from DAKO, Glostrup, Denmark). Slides were counterstained with hematoxylin. Images were recorded with a digital camera Leica DC480 (Milano, Italy). Vessel density, vessel diameters and SMA positive vessels were manually measured. Densitometric analysis for F4/80 staining was performed with QwinPro software (Leica).

A2780 cells susceptibility to Ad infection and evaluation of VEGF and PlGF dimers production.

The A2780 cells susceptibility to Ad5 was evaluated by infection with Ad-GFP. Cells were plated at density of $5x10^4/cm^2$ and after 6 hours, when showed about 50% of confluence, were infected with Ad-GFP at different multiplicities of infection (MOI)

ranging from 5 to 100 MOI. Expression of GFP was visualized 24 hours later.

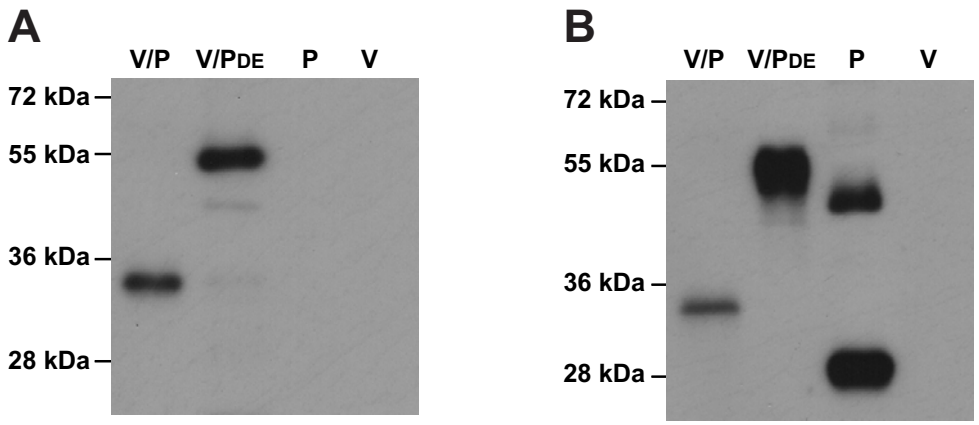
To evaluate the production of VEGF and PlGF dimers A2780 cells were infected with Ad-PlGF1wt or Ad-PlGF1-DE with a MOI of 20. After 24 hours from infection, the culture medium was replaced, and 24 hours later the medium was recovered and submitted to quantitative ELISAs.

Table S1. Quantification of PlGF and VEGF dimers secreted by Ad-transduced A2780 cells.

		VEGF	PlGF/VEGF	PlGF
A2780 cells	Ad-GFP	2.5 ± 0.4	ND	ND
	Ad-PlGF1wt	1.2 ± 0.2[#]	1.8 ± 0.2	24.2 ± 1.9
	Ad-PlGF1-DE	1.3 ± 0.1[#]	1.7 ± 0.3	26.6 ± 3.2

The values, expressed as ng /1x10^6, represent the average ± SEM of two independent experiments, in which each sample was analyzed in triplicate. ND: not detectable. [#]p =0.01 vs Ad-GFP; [¶]p <0.01 vs Ad-GFP.

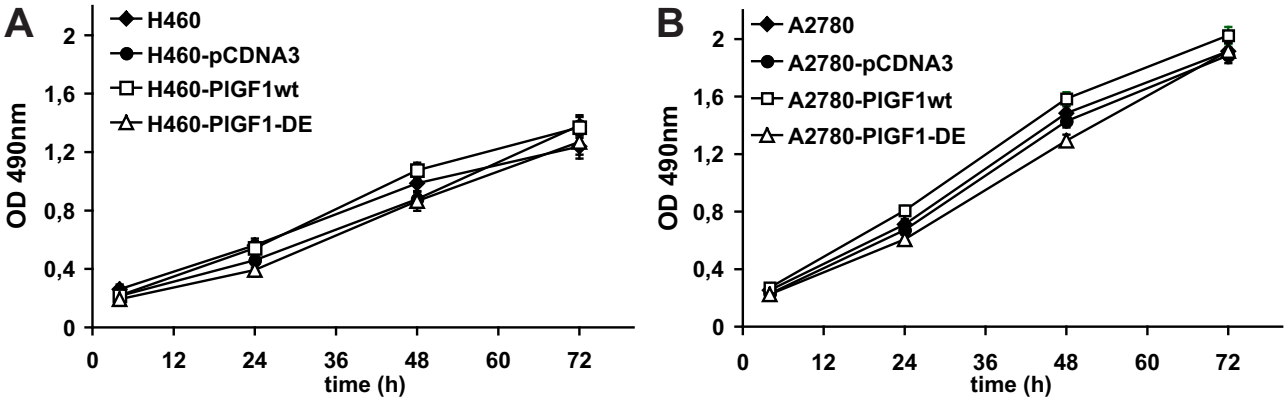
Figure S1



Purification of VEGF/PlGF1-DE heterodimer.

Western blot analyses of purified VEGF/PlGF1-DE (V/P$_{DE}$) heterodimer (10 ng) performed using anti-VEGF/PlGF heterodimer (**A**) or anti-PlGF (**B**) antibodies. As control the same amount of recombinant wild type heterodimer (V/P), PlGF (P) or VEGF-A (V) were loaded. The analyses were performed under non-reducing conditions. Since the wild type V/P is produced in bacteria, the higher molecular weight showed by V/P$_{DE}$, produced in a mammalian cell line, was probably attributable to glycosylation. The additional band observed in PlGF lane, panel B, was probably due to protein aggregation.

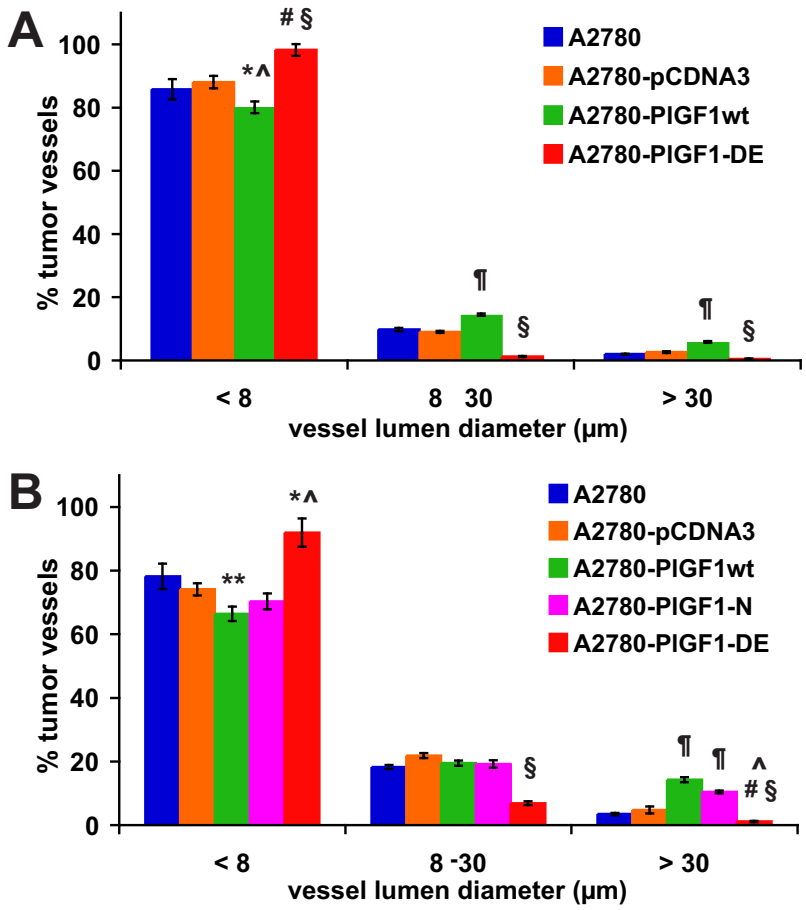
Figure S2



In vitro growth of NCI-H460 and A2780 stable clones.

NCI-H460 (**A**) or A2780 (**B**) stable clones, and as control not-transfected cells, were seeded in 96-wells plate. Cell growth was evaluated using the CellTiter Aqueous One Cell Proliferation Assay (Promega) at indicated time. Each point was carried out in triplicate and the data are represented as the mean of two experiments ± SEM.

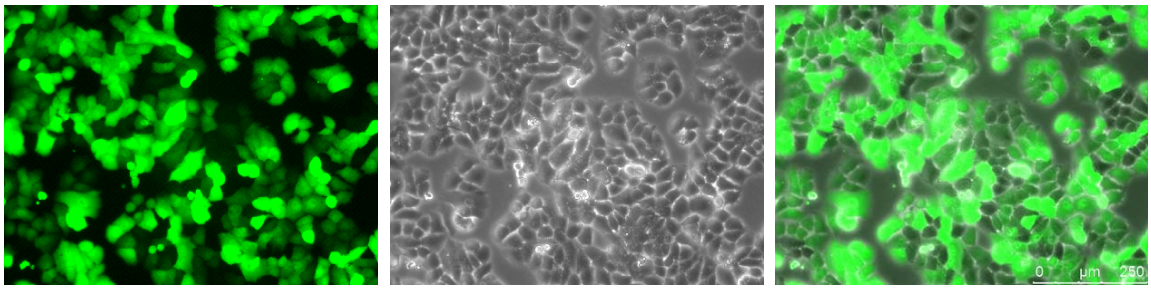
Figure S3



Analysis of vessel dimension of the A2780 xenograft tumors

Vessels were divided into three groups based on the length of bigger diameter: <8 µm (small), 8-30 µm (medium) and >30 µm (large). Data reported as percentage of tumor vessels of different dimension, are represented as the mean ± SEM. (**A**) Analysis of vasculature of tumors with different volume (see table 2). A2780-PIGF1-DE tumors showed increase in small vessels (#p<0.005 vs A2780 and A2780-pCDNA3; §p<0.0001 vs A2780-PIGF1wt) and decrease of medium and large vessels (§p<0.0001 vs all other groups). Conversely, A2780-PIGF1wt tumors showed decrease of small vessels (*p=0.05 vs A2780; ^p=0.01 vs A2780-pCDNA3) and increase of medium and large vessels (¶p<0.0001 vs A2780 and A2780-pCDNA3). (**B**) Analysis of vasculature of tumor with similar volume (see table 2). A2780-PIGF1-DE tumors showed increase in small vessels (*p<0.05 vs A2780 and A2780-PIGF1-N; ^p<0.01 vs A2780-pCDNA3 and A2780-PIGF1wt) and decrease of medium (§p<0.0001 vs all other groups) and large (#p<0.001 vs A2780; ^p<0.01 vs A2780-pCDNA3; §p<0.0001 vs A2780-PIGF1wt and A2780-PIGF1-N) vessels. A2780-PIGF1wt displayed decrease of small vessels (**p<0.05 vs A2780 and A2780-pCDNA3). A2780 -PIGF1wt and –PIGF1-N showed increase of large vessels (¶p<0.0001) vs A2780 and A2780-pCDNA3.

Figure S4



GFP **brigh field** **merge**

A2780 cells susceptibility to Ad infection.

The presence of GFP in A2780 cells exposed to Ad-GFP, MOI = 20, was detected by fluorescence analysis. Images were acquired with a digital camera Leica DFC 420. Scale bar reported on merge picture is 250 µm.