# Human choriocarcinomas: Placental growth factor-dependent preclinical tumor models

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**Abstract.** Choriocarcinomas are a rare form of cancer that develops in the uterus from tissue which would normally become the placenta. Choriocarcinomas are a trophoblastic gestational disease and have been studied largely to investigate conditions related to pregnancy such as preeclampsia. Choriocarcinomas are highly angiogenic and produce high levels of placental growth factor (PlGF) to promote the development of blood vessels. Upregulation of PlGF expression also occurs during the development of other human malignancies such as breast cancer and melanoma. Both tumor specimens and plasma samples have higher levels of PlGF than normal tissues. Hence, PlGF has emerged as a valid target for anti-angiogenic therapy. The cell lines BeWo, JAR and JEG-3, derived from human choriocarcinomas, were investigated *in vitro* and *in vivo* for suitability as PlGF-dependent models. BeWo, JAR and JEG-3 cells were characterized in culture and were implanted into immunodeficient mice to generate subcutaneous tumors. The PlGF and VEGF angiogenic profiles of the choriocarcinoma cells and tumors were investigated by ELISA and by immunohistochemical methods. Double immunofluorescence methods were applied to choriocarcinoma xenograft sections to characterize the cellular components of the blood vessels. sFLT01, a fusion protein that neutralizes PlGF, was assessed in cell culture experiments and xenograft studies. The novel results presented here validate the importance of human choriocarcinoma cell lines and xenografts in further exploring the role of PlGF in tumor angiogenesis, for evaluating PlGF as an anti-angiogenic target, and for developing therapies that may provide clinical benefit.

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*Abbreviations:* PlGF, placental growth factor; VEGF, vascular endothelial growth factor; RCC, renal cell carcinoma; AML, acute myeloid leukemia

*Key words:* placental growth factor, choriocarcinoma, angiogenesis, vascular endothelial growth factor, preclinical model, tumor microenvironment

## Introduction

Angiogenesis is essential for embryonic development and physiological functions. Aberrant angiogenesis can contribute to pathological conditions such as cancer, diabetic retinopathy, and wet age-related macular degeneration. Choriocarcinoma is a rare cancer characterized by highly angiogenic tumors that normally would have formed the placenta but can also arise from mutated germ cells or from dedifferentiation of uterine carcinomas (1). Choriocarcinoma is a gestational trophoblastic disease that often, but not always, appears within one year antecedent pregnancy and can be treated by surgery or by standard cytotoxic chemotherapeutic drugs (2). Published case reports of metastatic choriocarcinomas are not uncommon (1,3-5).

The cell lines derived from human choriocarcinomas have been instrumental in research for numerous scientific endeavors. The earliest report of human choriocarcinoma cells used to establish a xenograft model dates back to 1959 (6). The malignant cells were isolated from the cerebral metastasis of a choriocarcinoma post-surgical excision and transferred to the cheek pouch of the hamster followed by serial transplantation *in vivo* over a period of 8 years and were later designated BeWo (6). The BeWo cells were subsequently established as a cell line in culture to study endocrine function *in vitro* (7,8). In addition to the synthesis of hormonal markers, the BeWo choriocarcinoma line is characterized by the production of an extracellular matrix rich in laminin but not fibronectin (9). BeWo cells have also been useful in investigations into preeclampsia (10). BeWo cells served as a model of the placenta for *in vitro* studies involving iron and iodide transport (11,12). *In vivo*, BeWo xenograft tumors have been useful in studies involving radio-labeled antibodies against stage-specific embryonic antigen-1 (SSEA-1) to explore the kinetics of tumor localization of the radio-label (13).

JAR and JEG-3 choriocarcinoma cell lines were established in the late 1960s. Like the BeWo line, the JEG-3 line was developed from a clone derived from a tumor that had been maintained as a xenograft in the hamster cheek pouch (14). JAR expresses the epidermal growth factor receptor at the cell membrane and β-human chorionic gonadotropin (HCG) in the cytoplasm and has been used to study the regulation of HCG secretion (15,16). JAR is characterized by cells of varying stages of differentiation with larger, multinuclear cells arising in cells with higher levels of HCG transcripts (17). JAR cells express heparanase that promotes invasive potential but not to

the same extent as JEG-3 choriocarcinoma cells which express higher heparanse levels and display greater invasive properties in culture (18). RNA interference techniques applied to JEG-3 cells demonstrated a role for homeobox proteins in apoptosis (19). A characteristic common to the BeWo, JAR, and JEG-3 cell lines is the expression of nuclear p53 protein (20). JEG3 and BeWo cells also express melatoninergic receptors at the plasma membrane and in the cytoplasm (21).

Despite the knowledge that choriocarcinomas are highly angiogenic, are associated with the placenta, and that choriocarcinoma cell lines can generate xenograft tumors (22,23), there is a dearth of information on the implementation of human choriocarcinoma cell lines as a research tool for investigation of anti-angiogenic therapies that neutralize placental growth factor (PlGF) for cancer. With the FDA approval of several anti-angiogenic drugs (bevacizumab, sorafenib, sunitinib, and pazopinib) that are directed towards VEGF and its receptors, PlGF has emerged as an anti-angiogenic target against which new drugs may provide clinical benefit. PlGF expression is upregulated in numerous human cancers including melanomas and breast, cervical, lung, prostate, and renal cell carcinomas (24-29). Both PlGF and VEG-A bind to VEGFR1/Flt-1.

The potential clinical benefit of neutralizing PlGF was demonstrated in standard syngeneic and xenograft models using traditional cell lines such as B16F10 melanoma, Panc02 pancreatic, and MCF-7 and MDA-MB-231 breast carcinomas (30-32). We have taken an innovative approach by selecting human choriocarcinoma cell lines to serve as PlGF-overexpressing models for preclinical studies. We utilized sFLT01, a novel fusion protein, as the PlGF-neutralizing agent (33,34). The production of PlGF and VEGF-A by BeWo, JAR, and JEG-3 cells in culture was quantified by ELISA and was altered upon exposure to sFLT01. The growth kinetics of BeWo, JAR, and JEG-3 tumors in immunodeficient mice were characterized. PlGF and VEGF-A production in the tumors was assessed by immunohistochemistry and serum levels were measured by ELISA. sFLT01 significantly inhibited the growth of JAR tumors in an efficacy study thereby validating choriocarcinoma xenografts as a useful model for the development of anti-angiogenic agents that target PlGF.

## Materials and methods

*Materials*. The structure of sFLT01 has been previously reported (35). Briefly, sFLT01 is a novel fusion protein which has a molecular weight of approximately 80 kDa and consists of the VEGF/PlGF binding domain of VEGFR-1/Flt-1 fused to the Fc portion of human IgG1 through a polyglycine linker (9Gly) (33,34).

*Cell culture*. The choriocarcinoma cell lines, BeWo, JAR, and JEG-3 choriocarcinomas, and human 786-O and mouse RENCA RCC, human A673 Ewing's sarcoma, mouse B16F10 melanoma, human H460, HT29, and SW480 colon carcinomas, Karpas 299 lymphoma, and MOLM-13 AML were purchased from ATCC (Manassas, VA). All cell lines were grown in RMPI-1640 media with 10% FBS (Invitrogen, Carlsbad, CA) in a humidified 5% $CO_2$ atmosphere. Confluent monolayers were overlayed with serum-free RPMI-1640 for 24 h to collect conditioned medium.

Human JAR and JEG-3 choriocarcinoma cells were grown in RPMI-1640/10% FBS (Invitrogen) ± 150 $\mu$g/ml sFLT01 for 9-36 days. At several time-points, an aliquot of the cells were grown to confluency in a T25 flask and were washed twice with serum-free RPMI-1640. The cells were overlayed with 4 ml of serum-free medium for 24 h in the absence of sFLT01. The conditioned medium was collected and centrifuged to remove any loose cells and transferred to a new tube. The remaining cells were collected by trypsin/EDTA exposure (Invitrogen), total cell count was taken. After centrifugation the resulting cell pellet was lysed with lysis buffer (Roche Diagnostics, Indianapolis, IN) and 2 mM sodium orthovanadate (New England Biolabs, Ipswich, MA). The conditioned medium and lysed cell pellets of the human cell lines were analyzed for secreted and intracellular levels of human PlGF and human VEGF-A by ELISA (R&D Systems, Minneapolis, MN). The conditioned medium from mouse B16F10 and RENCA cells was analyzed for secreted levels of PlGF and VEGF-A by ELISA specific for mouse PlGF and mouse VEGF-A (R&D Systems).

*In vivo models*. BeWo, JAR, and JEG-3 choriocarcinoma cells were cultured as described above. Female Balb/c nude mice (nu/ nu) (Charles River Labs, Willmington, MA) were implanted subcutaneously with $1x10^6$ cells in 200 $\mu$l of a 1:1 mixture of RPMI-1640 and Matrigel (Invitrogen) (n=10 per group). sFLT01 (25 mg/kg) or vehicle (PBS) was administered by intraperitoneal injection two or three times per week beginning when tumors were ~100 $mm^3$ in volume. Tumors were measured with calipers. Tumor volume was calculated using the formula $width^2$ x length x 0.52. Mice were euthanized when tumors measured 20 mm in diameter or became ulcerated. Studies were blinded. Blood was collected from the ocular sinus and serum was assayed by ELISA (R&D Systems). All procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee in accordance with the Federal Animal Welfare Act (9 CFR, 1992) and were conducted in an AAALAC accredited facility.

*Immunohistochemistry*. Tumors were fixed in 10% neutral buffered formalin and embedded in paraffin. For PlGF immunohistochemistry (IHC), heat-induced epitope retrieval was performed on deparaffinized tissue sections using Dako citrate buffer (Dako, Carpinteria, CA) and then the sections were incubated with a 1:50 dilution of a rabbit polyclonal anti-PlGF antibody (Abbiotec, San Diego, CA). Dako Envision kit (Dako) and DAB as the chromogen were used. For VEGF-A IHC, a 1:200 dilution of a rabbit polyclonal anti-VEGF-A antibody (Thermo Fisher Scientific, Pittsburgh, PA) was used as the primary antibody, a biotinylated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) was used as the secondary antibody, and ABC-Elite peroxidase was used for detection with DAB as the chromogen. For IHC on negative control sections, the primary antibody was replaced with rabbit universal negative control antibody (Dako). Images were captured at magnification x400.

For CD31 IHC, five-micron sections were incubated with proteinase-K (Dako) for 10 min, blocked with 10% goat serum/TBS for 10 min, and incubated with 20 $\mu$g/ml rat anti-mouse CD31 antibody (Fitzgerald Industries, Acton, MA) for 1 h. After 2 washes in TBS for 2 min each, 1.25 $\mu$g/ml biotinylated anti-rat antibody (Jackson Immunoresearch) was applied for 30 min followed by 2 washes as described above. ABC-Elite peroxidase (Vector

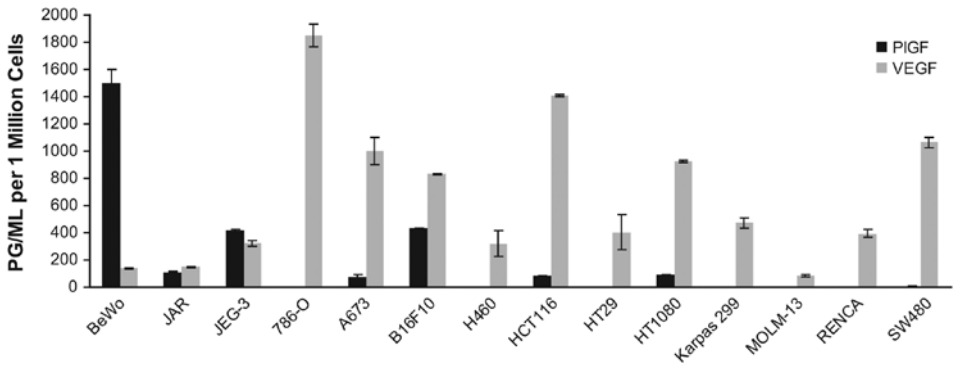


Figure 1. Cell lines were cultured for 24 h under serum-free conditions. The conditioned medium was analyzed by ELISA for PlGF and VEGF-A levels. BeWo, JAR, and JEG-3 choriocarcinoma cells secreted higher levels of PlGF than all cancer cell lines tested except B16F10 which secreted higher levels of PlGF than JAR and comparable levels to JEG-3. In contrast, the choriocarcinoma cell lines secreted lower levels of VEGF-A than most cancer cell lines tested except for MOLM-13 AML.

Laboratories, Burlingame, CA) was applied for 20 min and after 2 washes the DAB (Dako) chromagen was applied for detection for 2 min. Tumors were counterstained with hematoxylin and whole image scans were generated at magnification x150 using the Aperio ScanScope XT scanning system. Microvessel density (MVD) represents the total number of $CD31^+$ vessels/$mm^2$ of tissue area as quantified using Aperio's MVD algorithm.

*Immunofluorescence*. Tumors were flash frozen in OCT compound, cut into 4-5-micron sections, and fixed in 10% zinc formalin (Electron Microscopy Sciences, Hatfield, PA) for 10 min. After a 10-min block in 10% goat serum/TBS, 10 $\mu$g/ml rat anti-mouse CD31 antibody (BD Biosciences, Woburn, MA) and 5 $\mu$g/ml rabbit anti-mouse NG2 antibody (Chemicon/Millipore, Temecula, CA) were applied for 1 h to detect endothelial cells and pericytes, respectively. After 2 washes, secondary goat anti-rat-Cy3 and goat anti-rabbit-Cy2 antibodies (Jackson Immunochemicals) at 5 $\mu$g/ml were applied simultaneously for detection for 20 min. Images were captured at magnification x400.

*Statistical analysis*. *In vitro* data are expressed as mean ± SD. Comparisons between treatment and control groups were made using Student's t-test. Tumor volumes are expressed as mean ± SEM and were analyzed by ANOVA test. Kaplan-Meier survival curves were analyzed by the log-rank test. Graphpad Prism 5.0 (Graphpad Software, Inc., La Jolla, CA) was used for analysis. $P<0.05$ was considered statistically significant.

## Results

The levels of PlGF and VEGF-A secretion from three human choriocarcinoma cell lines (BeWo, JAR, and JEG-3) and 11 human and mouse cancer cell lines in culture were measured by ELISA (Fig. 1). ELISAs that measure human PlGF and human VEGF-A were used to assay the conditioned medium from human cell lines; ELISAs that measure mouse PlGF and mouse VEGF-A were used to analyze the conditioned medium from the mouse B16F10 and RENCA cells. BeWo cells secreted approximately 10-fold higher levels of PlGF than VEGF-A and the highest levels of PlGF of all the cell lines that were evaluated. In contrast, the levels of PlGF and VEGF-A were not significantly different in the conditioned medium collected from the JAR and JEG-3 cells and were lower than the levels of PlGF and VEGF-A secreted by the BeWo cells. The BeWo, JAR, and JEG-3 cell lines secreted higher levels of PlGF than the 11 other cell lines with the exception of the mouse B16F10 melanoma cells that produced PlGF levels that were similar to that of the JEG-3 cells. PlGF was not detectable in the conditioned medium from the following cell lines: 786-O renal cell carcinoma, H460 non-small cell lung carcinoma, HT29 and SW480 colon carcinomas, Karpas 299 lymphoma, MOLM-13 acute myeloid leukemia, and mouse RENCA renal cell carcinoma. VEGF-A levels were lower in the conditioned medium of the three choriocarcinoma cell lines than the other cell lines with the exception of the MOLM-13 AML.

To investigate the effect of PlGF and VEGF-A neutralization on the choriocarcinoma cells in culture, the JAR and JEG-3 cell lines were exposed to sFLT01 for 9-36 days. At several intervals, a portion of the cells were seeded in a separate flask and cultured in serum-free medium in the absence of sFLT01 for 24 h. PlGF and VEGF-A levels were measured in the conditioned medium and in cell lysates. JAR and JEG-3 cells ceased production of VEGF-A following exposure to sFLT01 with undetectable levels of VEGF-A in both the conditioned medium and the cell lysates (Fig. 2A and B). The JAR and JEG-3 cells continued to produce PlGF when sFLT01 was added to the culture but the levels were significantly lower compared to the untreated cells (Fig. 2C and D).

To validate the usefulness of human choriocarcinoma cells as preclinical tumor models of hPlGF overexpression, BeWo, JAR, and JEG-3 cells were implanted subcutaneously into nude mice and tumor volumes were recorded twice per week. JEG-3 tumors grew the fastest and reached ~1500 $mm^3$ in volume in 15 days compared to ~19 and 26 days for JAR and BeWo tumors respectively (Fig. 3A). The serum levels of hPlGF and hVEGF-A in tumor bearing mice were quantified by ELISA. hPlGF was detected in the serum of mice with choriocarcinoma tumors. In the BeWo model the hPlGF levels correlated with tumor size (Fig. 3B). Similar to that observed *in vitro*, the BeWo tumors secreted higher hPlGF levels reaching >2,000 ng/ml *in vivo* compared to the JAR and JEG-3 tumors. Serum hVEGF-A levels were undetectable in the BeWo xenograft model and the hVEGF-A levels were low (<50 ng/ml) in the JAR and JEG-3 models when tumors reached sacrifice criteria. The mPlGF levels ranged from 0-175 pg/ml (data not shown) indicating

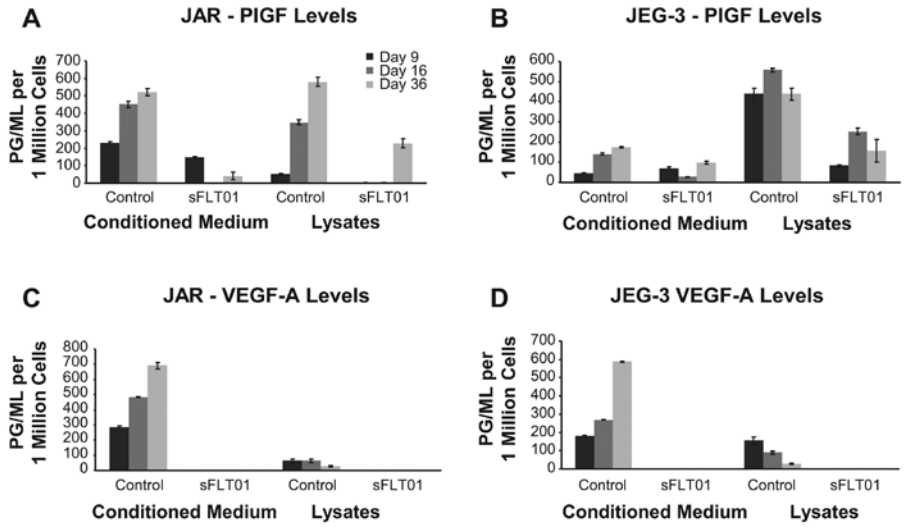


Figure 2. JAR and JEG-3 cells in culture were exposed to sFLT01 (150 $\mu$g/ml) for 9-36 days ± exposure to sFLT01. At several time-points, cells were overlayed with serum-free and sFLT01-free medium. The conditioned medium (CM) was collected after 24 h and the CM and cell lysates were assayed by ELISA for PlGF and VEGF-A. The JAR and JEG-3 cells continued to produce PlGF when sFLT01 was added to the culture but the levels were significantly lower than untreated cells (A and B). JAR and JEG-3 cells ceased production of VEGF-A following exposure to sFLT01 with undetectable levels of VEGF-A in both the CM and the cell lysates (C and D).

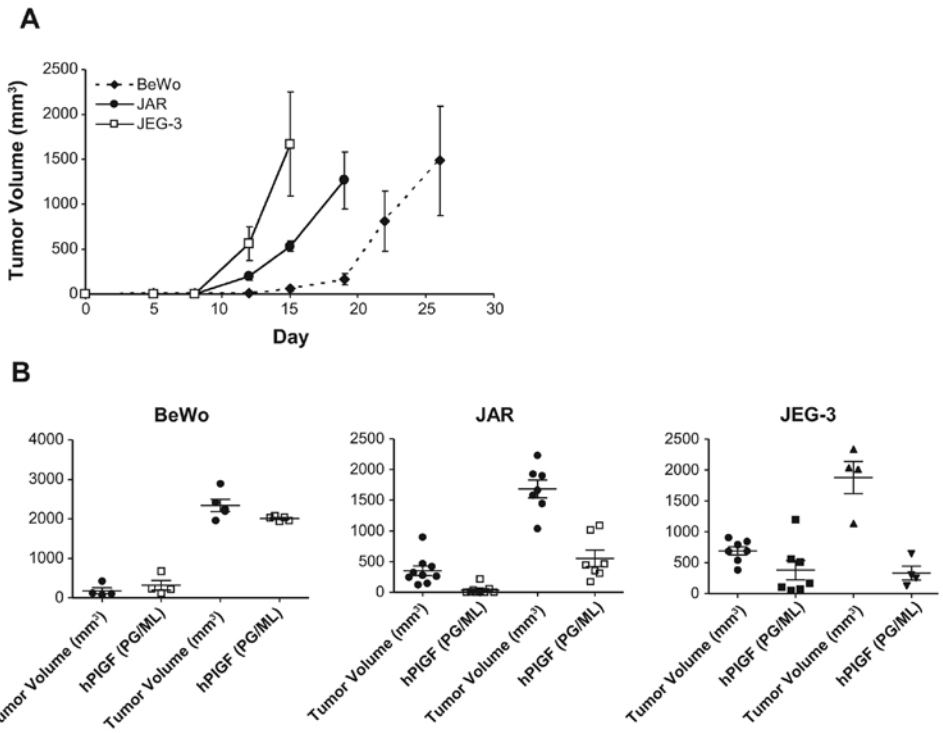


Figure 3. (A) BeWo, JAR, and JEG-3 cells from culture were implanted subcutaneously into nude mice (1x10$^6$ cells/mouse). Tumors were measured with calipers and tumor volume was calculated. Data are expressed as mm$^3$ ± SEM. (B) Serum was collected and the tumor volumes were determined from mice at the time of sacrifice. Similar to observations in culture, the BeWo tumors secreted higher levels of hPlGF *in vivo* compared to the JAR and JEG-3 tumors reaching >2,000 ng/ml. Serum hVEGF-A was undetectable in the BeWo xenograft model and the levels were low (<50 pg/ml) in the JAR and JEG-3 models. The levels of mPlGF ranged from 0-175 pg/ml (data not shown) indicating that the growth of the choriocarcinoma tumors was driven primarily by hPlGF.

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Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded (FFPE) sections of placentas and choriocarcinomas using anti-VEGFA and anti-PlGF antibodies to investigate the expression of PlGF and VEGF-A in choriocarcinoma xenografts. The primary antibodies applied to tumor sections were cross-reactive for mouse and human PlGF and VEGF-A. Human and mouse placental tissues served as positive controls. In human and mouse placental tissue, the most intense labeling for VEGF-A was observed in the cytoplasm of trophoblasts, trophoblastic giant cells and maternal decidual cells (Fig. 4A). VEGF-A immunolabeling was evident but variable in the neoplastic cells of the choriocarcinoma xenografts. In BeWo

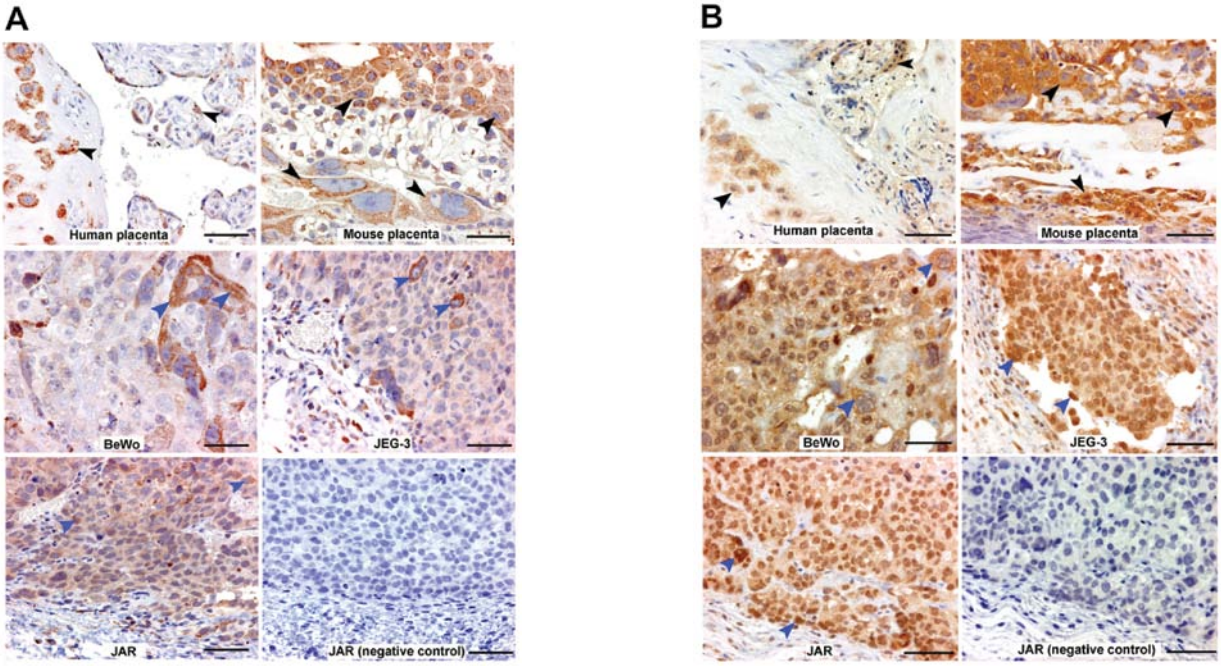


Figure 4. BeWo, JAR, and JEG-3 tumors were formalin-fixed and paraffin-embedded (FFPE). IHC methods were applied to tumor sections using an anti-VEGF-A or anti-PlGF antibody. Both antibodies were cross-reactive for the mouse and human growth factors. Tissue sections of mouse and human placenta served as positive controls. Examples of VEGF-A positive cells in the placenta are indicated by black arrowheads (left panel). hVEGF-A was evident in the neoplastic cells of the choriocarcinoma xenografts. A few intensely labeled neoplastic cells are identified by blue arrowheads. Moderate to strong PlGF immunolabeling was observed in the human and mouse placentas respectively (right panel). A few PlGF immunopositive cells are identified by black arrowheads. The staining for PlGF was more homogeneous across the tumor sections than the staining for VEGF-A. Neoplastic cells with intense immunolabeling are identified with blue arrowheads. JAR tumor sections immunostained with control antibody showed complete lack of immunolabeling following both protocols. Scale bars represent 50 $\mu$m.

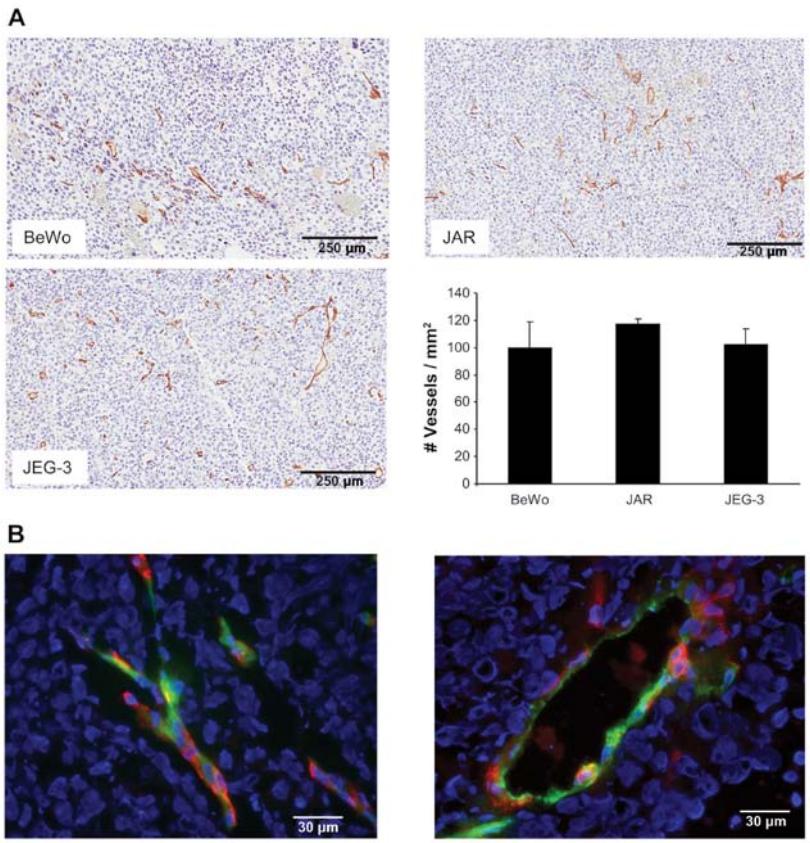


Figure 5. (A) IHC methods were applied to BeWo, JAR, and JEG-3 tumors using an anti-CD31 antibody to visualize the vasculature on FFPE sections of BeWo, JAR, and JEG-3 tumors. Microvessel density was quantified and determined to be similar across the 3 different choriocarcinoma xenografts. Scale bars represent 250 $\mu$m. (B) Double immunofluorescence techniques were employed on frozen sections of JAR tumors. A fluorescently-tagged anti-CD31 antibody (green) and a fluorescently-tagged anti-NG2 antibody (red) revealed the close proximity of and the close cellular interactions between the endothelial cells and pericytes respectively. Scale bars represent 30 $\mu$m.

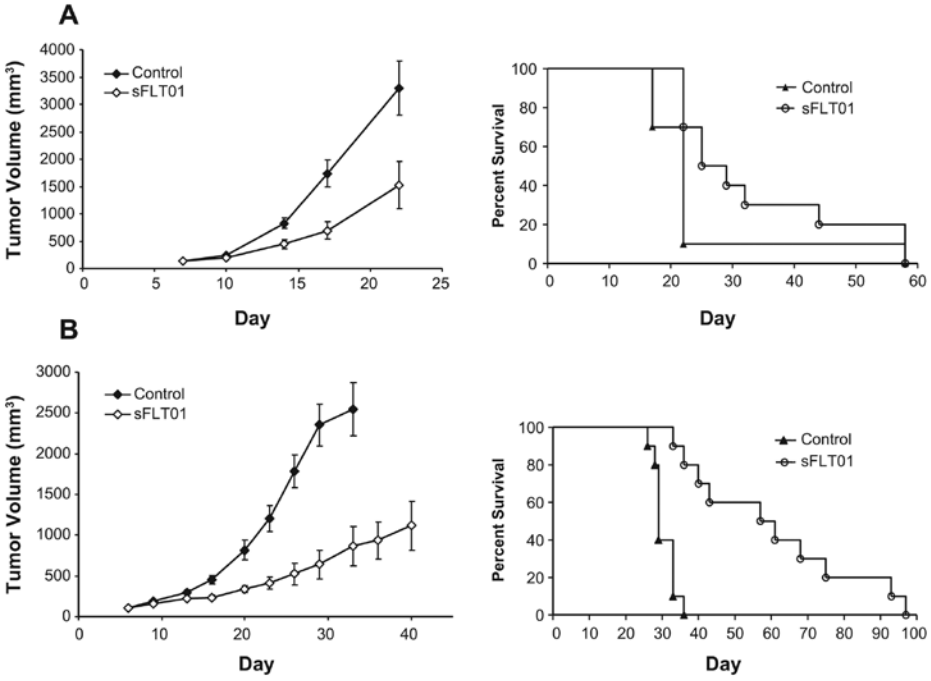


Figure 6. The efficacy of sFLT01 which neutralizes both human and mouse VEGF-A and PlGF was evaluated in two choriocarcinoma tumor models. (A) In the JAR model, the efficacy of sFLT01 (25 mg/kg, IP, 2x/week) was modest resulting in a 6-day tumor growth delay and a 5-day increase in median survival. (B) With the same dosing regimen, sFLT01 was more active in the JEG-3 model resulting in an 18-day tumor growth delay and 34-day increase in median survival ($P<0.05$).

choriocarcinoma most neoplastic cells were weakly positive for VEGF-A and only a few were intensely immunopositive. In JEG-3 tumors most neoplastic cells showed faint labeling for VEGF-A. JAR tumors were more moderately positive for VEGF-A compared to BeWo and JEG-3 tumors. Rare neoplastic cells in JAR tumors were strongly labeled for VEGF-A.

In contrast to the VEGF-A IHC results, the staining for PlGF was more homogeneous across the mouse placenta and choriocarcinoma xenograft sections than for VEGF-A (Fig. 4B). Moderate immunolabeling was observed in the cytoplasm of placental trophoblasts and maternal decidual cells in human placenta. In mouse placenta, intense labeling was observed in trophoblastic giant cell, spongiotrophoblasts and decidual cells in the labyrinth. In the BeWo choriocarcinoma most neoplastic cells were moderately positive and occasionally a few neoplastic cells were intensely immunopositive for PlGF. In JEG-3 tumors most neoplastic cells were moderately positive for PlGF. Mild immunolabeling was observed in most neoplastic cells in JAR choriocarcinoma with rare cells showing moderately intense labeling for PlGF. In all three choriocarcinoma tumors mild to moderate VEGFA and PlGF immunolabeling was also observed in macrophages, a few fibroblast-like cells, and endothelial cells.

To examine the vascular properties of the choriocarcinoma xenografts, an anti-CD31 antibody and IHC methods were applied to FFPE choriocarcinoma xenograft tumor sections to identify the endothelial cells (Fig. 5A). Microvessel density was determined for the BeWo, JAR, and JEG-3 tumors. The vasculature was similar across the 3 tumor types with no significant differences in the size, morphology, or density of the blood vessels. Double immunofluorescence techniques using an anti-CD31 antibody and an anti-NG2 antibody to identify endothelial cells and pericytes respectively were applied to frozen sections from JAR xenograft tumors (Fig. 5B). The images reveal endothelial cells and pericytes in close proximity of one another in nascent blood vessels and in more mature vasculature with patent lumens suggesting cellular interactions between endothelial cells and pericytes in the JAR tumors.

sFLT01 is an anti-angiogenic fusion protein that is efficacious in numerous human xenografts. sFLT01 neutralizes both hPlGF and mPlGF and hVEGF-A and mVEGF-A (33,34). sFLT01 was tested in the JAR and JEG-3 xenograft models but not the BeWo model which produced irregularly shaped and flattened tumors that would not have yielded accurate tumor measurements. sFLT01 (25 mg/kg) administered twice per week by intraperitoneal injection beginning when the tumors were ~100 $mm^3$ in volume inhibited the growth of JEG-3 tumors resulting in a 6-day tumor growth delay as determined when the mean tumor volumes were ~1500 $mm^3$ (Fig. 6A). The increase in median survival of mice bearing the JEG-3 xenograft was modest with a median survival of 22 days for the control group and 27 days for the sFLT01 treatment group. sFLT01 (25 mg/kg) was more active in the JAR model resulting in 18 days of tumor growth delay determined when the mean tumor volumes reached ~1000 $mm^3$ (Fig. 6B). The median survival of mice bearing JAR choriocarcinomas was significantly increased from 34 days in the control group to 64 days in the sFLT01 treatment group ($P<0.05$).

## Discussion

With the clinical validation that VEGF-targeted drugs can improve quality of life and progression-free survival rates in some patients there has been increasing efforts to identify additional potential targets, such as PlGF, that promote tumor

angiogenesis and that when neutralized result in therapeutic benefit. The potential benefit of neutralizing PlGF was demonstrated in preclinical tumor models representing melanoma, pancreatic cancer, colon carcinoma, and bone metastasis (30-32). Increases in serum PlGF after bevacizumab or sunitinib treatment suggest that PlGF may play a role in resistance to some anti-angiogenic therapies (36-38). The fusion protein aflibercept which neutralizes PlGF and VEGF has entered Phase III clinical trials (39,40). The sFLT01 protein described here most closely resembles aflibercept.

Despite the focus on anti-angiogenic agents in oncology, choriocarcinoma xenografts have been overlooked as a relevant tumor model to evaluate VEGF- or PlGF-targeted drugs. Studies investigating VEGF-targeted drugs have validated the utility of numerous tumor models (41). However, preclinical models that are suitable for evaluating anti-PlGF agents have not been widely defined. The data presented here demonstrate that BeWo, JAR, and JEG-3 human choriocarcinoma cell lines endogenously express higher levels of PlGF than VEGF both in culture and when implanted into nude mice. The use of choriocarcinoma cell lines that endogenously overexpress PlGF reduces the need for the addition of exogenous PlGF, development of transfected stable cell lines, or genetically engineered transgenic mice in order to achieve high levels of PlGF in a model system. Furthermore, choriocarcinoma cell lines provide a model that more closely resembles clinical disease. The activity of the fusion protein sFLT01 in the JEG-3 choriocarinoma xenograft further validates the utility of choriocarcinoma cell lines for assessing the potential therapeutic value of anti-PlGF drugs. The phenotypic change observed in the JAR and JEG-3 cells in culture when exposed to sFLT01 further illustrates the ability of choriocarcinoma cells to respond to the neutralization of PlGF.

Human choriocarcinoma cell lines and xenografts have value in not only evaluating anti-PlGF drugs but also for testing other anti-angiogenic agents. The multi-targeted small molecule tyrosine kinase inhibitor sunitinib was tested in preclinical orthotopic models of human testicular germ cell tumors that included the choriocarcinoma cells TGT17 and TGT38 (23). The results indicated that sunitinib, and perhaps other therapies that target the VEGF pathway such as sFLT01, offer a new approach to treating cisplatin-resistant testicular germ cell tumors. Choriocarcinoma tumors also have value for investigating other signaling pathways for cancer such as those that involve epidermal growth factor (16).

The controversy regarding the significance of PlGF to tumor growth (42,43) and the emergence of anti-PlGF drug candidates under development (Lassen *et al*, Mol Cancer Ther 12: abs. 111, 2009; Riisbro *et al*, Mol Cancer Ther 12: abs. 3, 2009) highlight the need for preclinical models where tumor growth is driven primarily by PlGF. The objective of the experiments described herein was not only intended to discern the potential clinical value of an anti-PlGF therapeutic strategy but also to evaluate the value and utility of incorporating human choriocarcinoma cells and tumors into preclinical anti-angiogenic agent assessment studies. The research described here is the culmination of a novel and innovative approach whereby an uncommon and perhaps overlooked malignancy, choriocarcinoma, was exploited and incorporated into traditional study designs that are commonly utilized in research efforts and which may be geared towards the development of the next generation of anti-angiogenic drugs. As with most anti-angiogenic drugs, combination therapy regimens involving standard cytotoxic chemotherapies are necessary for the best clinical outcome; exploring these treatment regimens in PlGF-dependent choriocarcinoma models would provide greater insight into the likelihood that an anti-PlGF drug would offer an improved outcome for cancer patients over the standard of care.

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