Real Time Cell Analysis of VEGF and PlGF-Induced Endothelial Cell Proliferation in the Presence of Heparin and Growth Factor Inhibitors

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A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

November 2017

Abstract

Real-time Cell Analysis (RTCA) has been made possible by new instruments that exploit the ability of cells to impede electric current to determine cell proliferation. Because impedance measurement does not interfere with cell processes or destroy cells, repeated measurements can be made and used to produce time-dependent cell response profiles (TCRPs). These TCRPs can be used to study time-dependent alterations in cell proliferation.

Heparin has been reported to modulate the proliferative effects of $VEGF_{165}$ and PlGF-2 in endothelial cells. Whether heparin produces time-dependent differences in growth factor signaling occur has been difficult to study using endpoint assays. RTCA was used to determine if heparin induces time-dependent changes in proliferation in the presence of heparin-binding growth factors -- $VEGF_{165}$ and PlGF-2-- and whether heparin alters activity of growth factor inhibitors.

Human umbilical vein endothelial cells (HUVECs) were stimulated with VEGF and PlGF in the absence or presence of heparin. RTCA impedance measurement was used to determine the effective concentration and maximum cell number for each growth factor and the combination of $VEGF_{165}$ and PlGF-2. Unlike endpoint measurements, RTCA measurement was also used to determine kinetic differences by determining the time of maximum cell proliferation and area under the curve. These TCRP-derived measurements were used to determine heparin's time-dependent effects on VEGF and PlGF proliferation responses, and heparin's impact on the effectiveness of their respective inhibitors.

RTCA was shown to provide accurate measurements of proliferation when compared to Bromodeoxyuridine (BrdU) proliferation data. However, unlike BrdU endpoint proliferation measurement, RTCA impedance measurement was also useful for revealing time-dependent differences in proliferation. The effect of soluble heparin binding to $VEGF_{165}$ and PlGF-2 was found to be more consistent with models of stabilized ligand receptor binding and increased ligand internalization. Additionally, comparison of TCRP data from PlGF-2 and $VEGF_{165}$-induced proliferation demonstrated that $VEGF_{165}$ and PlGF-2 have different time-dependent characteristics. RTCA measurements revealed a significant positive correlation between VEGF concentration and T_{max} of VEGF-induced proliferation wherein proliferation is delayed at high concentrations of VEGF, but not for PlGF-2-induced proliferation.

Time-dependent growth factor differences were reflected in inhibition of single or multi-target inhibitors as unique inhibitor signatures. Comparison of time-dependent inhibitor profiles of single versus multi-target inhibitors in $VEGF_{165}$: PlGF-2-stimulated HUVECs confirmed previously observed efficacy differences, but also provided information on the completeness of drug inhibition effects. AUC analysis revealed that uninhibited factors were present when either VEGF or PlGF inhibitors were used, while targeting of both factors demonstrated that no significant endogenous growth factor inhibition was present. Soluble heparin was found only to affect the activity of a multi-targeted inhibitor also reported to bind heparin.

RTCA impedance measurement is an accurate and useful tool for revealing time-dependent differences in proliferation responses that can be used to differentiate growth factors and their inhibitors and to study signal modulation.

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Chapter I

Introduction

Real-time cell analysis (RTCA) impedance technology was used to study the inhibition of VEGF and PlGF-induced proliferation in the presence of heparin in this work. RTCA technology and the novel measurements it enables are described. The prior understanding and potential mechanism of VEGF and PlGF signaling and its modulation by heparin gained by traditional methods are reviewed. Finally, to provide insights into potential applications of this work the use of VEGF and PlGF inhibitors is discussed.

Real-Time Cell Analysis for Measurement of Cell Proliferation

Cell proliferation studies have commonly been performed using endpoint measurements of nucleoside-incorporation or metabolic activity (Romar, Kupper, & Divito, 2016). Endpoint measurements capture information about a single point in time. The measurement of cell proliferation at multiple time points has been made possible by the development of instruments that measure cell-induced changes in electrical impedance. Electrodes embedded in tissue culture plates are used to determine changes in electric current produced by the attachment and spreading of cells on the plate surface. These cell-induced changes in impedance are reported as Cell Index (CI). CI measurements are correlated to changes in cell number, cell spreading and viability. Because CI measurements are non-invasive and do not alter cell response, measurement

can be performed repeatedly within the same cell population at multiple time points (ACEA Biosciences, 2013). Repeated CI measurements can be plotted to generate time-dependent cell response profiles (TCRPs) which can be used for the kinetic study of cell proliferation. From TCRPs the maximum cell index (CI_{max}), time of peak proliferation (T_{max}) and area under the curve (AUC) can be determined and used to characterize and compare cell proliferation responses. The additional information provided by real-time cell analysis (RTCA) enables the differentiation of time-dependent proliferation responses that would be difficult to measure using traditional endpoint measurements. RTCA was used in this study to differentiate $VEGF_{165}$ and PlGF-induced proliferation in endothelial cells. Heparin's ability to modulate proliferation and alter inhibitor effects was also studied.

Proposed Mechanisms for Heparin Modulation of $VEGF_{165}$ and PlGF-2 Induced Endothelial Cell Proliferation

Vascular Endothelial Growth Factor-A (VEGFA) and Placenta Growth Factor (PlGF) differ in their interactions with vascular endothelial growth factor receptors (VEGFRs). Both VEGFA and PlGF bind and activate VEGF receptor 1 (VEGFR-1), but VEGFA is also a ligand for VEGF receptor 2 (VEGFR-2) (Ferrara, Gerber, & LeCouter, 2003; Papadopoulos et al., 2012). Both proteins exist in multiple isoforms produced by alternative splicing (Maglione et al., 1993; Robinson & Stringer, 2001). Two of these isoforms $VEGF_{165}$ and PlGF-2 have been shown to bind heparin. Despite having similar binding affinities for VEGFRs, these heparin-binding isoforms have been shown to behave differently in models of angiogenesis and endothelial cell proliferation than their

shorter non-heparin-binding isoforms (Ashikari-Hada, Habuchi, Kariya, & Kimata, 2005; Keyt et al., 1996; Krilleke, Ng, & Shima, 2009).

The presence of heparin-binding sequences in $VEGF_{165}$ and PlGF-2 increases their potential to interact not only with their respective VEGFRs, but also with heparin's cell surface binding partners. Heparin has its own interactions with cell surface receptors including VEGFR-1 and the heparin-binding receptor, neuropilin-1 (NRP-1) (Maglione, et al., 1993; Teran & Nugent, 2015). Heparin's ability to bind with both VEGF family ligands and receptors provides one mechanism whereby heparin can stabilize or enhance VEGF family ligand binding to VEGFRs. Binding is the initial event in receptor activation that results in increased cell proliferation. By enhancing binding to weaker receptors heparin might serve to enhance functional activity.

VEGFA binds more weakly to VEGFR-2 than VEGFR-1 (Shinkai et al., 1998). Despite tighter binding to VEGFR-1, VEGFA stimulation of endothelial cells results in weak receptor VEGFR-1 phosphorylation and proliferation (Gille et al., 2001). Increased binding and activation of VEGFR-2 may be achieved by formation of multi-receptor complexes which can stabilize weaker VEGFA-VEGFR-2 binding interactions (Whitaker, Limberg, & Rosenbaum, 2001). In this way, heparin-bound VEGF may be stabilized by formation of complexes of VEGFRs with heparin-binding cell surface receptors like NRP-1 leading to enhanced receptor activation and proliferation (Migdal et al., 1998; Teran & Nugent, 2015; Whitaker, et al., 2001). By stabilizing VEGFA binding towards more active VEGFR-2, cell proliferation can be increased. This might also lead to altered timings in activity compared to signaling in the absence of heparin. In the

presence of heparin ligand might be depleted more rapidly and proliferation responses observed earlier.

VEGF family ligands might also bind to cell surface-associated heparin without the formation of complexes between VEGFR and cell surface heparin binding partners. Instead cell surface-associated heparin could bind heparin-binding ligand isoforms and alter the available ligand in the cellular environment (Robinson & Stringer, 2001). This type of mechanism might be expected to produce weaker proliferation with delayed or increased duration. Studies in VEGFR-1 and VEGFR-2-transfected cell lines showed that a non-heparin-binding isoform of VEGFA, $VEGF_{121}$, was observed to have slightly higher activity than the heparin-binding isoform $VEGFA_{165}$ (Papadopoulos, et al., 2012). $VEGF_{121}$ is more diffusible than $VEGF_{165}$ and has reduced interaction with cell surface-associated heparin. If heparin is reducing available ligand then $VEGF_{121}$ could be less impacted by heparin and have higher activity at some observed time points.

Experimental evidence for both heparin inhibition and enhancement has been observed depending on the concentration of heparin used (Robinson, Mulloy, Gallagher, & Stringer, 2006). This indicates that either or both of these mechanisms may be occurring depending on heparin dose. In this study, heparin will be pre-incubated with VEGFR-1 ligands, $VEGF_{165}$ and PlGF-2, to determine effects of soluble heparin. Endogenous cell-surface associated heparin is expected to be present and its effects will be determined in comparison to the addition of soluble heparin. Non-heparin and heparin-binding isoforms of VEGFR-1 ligands will be studied to help elucidate the impact of heparin on cell proliferation. Kinetic differences in heparin effects such as changes in

timing and duration of response can be identified using RTCA as well as changes in cell number.

Studies of PlGF and VEGF on Endothelial Cell Proliferation

Significant efforts have been made previously to characterize the impact of VEGF ligands on endothelial cells, but have produced conflicting results. $VEGA_{165}$- induced cell proliferation has been shown to be enhanced in Human Umbilical Vein Endothelial Cells (HUVECs) in combination with either heparin or PlGF-2 (Ashikari-Hada, et al., 2005; Park, Chen, Winer, Houck, & Ferrara, 1994). However, earlier data using cell counting in the presence of high serum reported only weak proliferation after three days in the presence of VEGF and no enhancement in the presence of VEGF and heparin (Giraux et al., 1998). Another study relying on cell counting to determine HUVEC proliferation observed a dose dependent effect of PlGF-2 in the presence of heparin and $VEGFA_{121}$. In contrast to previous findings, the overall enhancement of VEGF-induced proliferation by PlGF was not observed in these experiments (Migdal, et al., 1998; Park, et al., 1994). The use of cell counting combined with extended growth periods may be expected to produce confounding results as changes in cell number are only in part due to proliferation. Other differences in cell survival or cell death are also reflected in cell count measurements of proliferation (Romar, et al., 2016). The presence of multiple growth factors in serum can also confound measurements if high serum concentrations are used during proliferation experiments. The conflicting reports on the effects of suspected mitogens suggest that study design can impact result interpretation.

Studies have demonstrated small mitogenic effects of PlGF on HUVEC cells alone and these effects were enhanced in combination with VEGF (Park, et al., 1994; Ziche et al., 1997). The ability to detect PlGF mitogenic effects may require carefully structured methodologies as the sensitivity of nucleoside-analog methods is highly dependent on the timing of addition, duration of incorporation and concentration of analog. This study proposes to use an alternative RTCA technology to measure proliferation. The advantages of this technology include label-free measurement and no requirement for time window predetermination. These features may facilitate the collection of more complete information for PlGF-induced endothelial cell proliferation by RTCA than nucleoside labeling. The time-dependent cell growth profile generated using the RTCA instrument will be informative to determine optimal time addition and incorporation of Bromodeoxyuridine (BrdU) incorporation. In turn, BrdU data will be used to verify that observed increases in CI using the RTCA instrument are due to proliferation of cells.

Tolerance to Anti-Angiogenic Drugs in Cancer and Eye Disease

Prolonged treatment with anti-VEGFA drugs is often necessary to prevent tumor progression in cancer and abnormal blood vessel growth resulting in eye disease. In some cases, the effectiveness of these therapies decreases over time. Multiple mechanisms have been suggested for observed drug resistance including the ability of non-VEGFA angiogenic factors to compensate for VEGFA functions (Duda, 2012; Forooghian, Cukras, Meyerle, Chew, & Wong, 2009). The VEGFR-ligand PlGF has been associated with increased angiogenesis and is elevated both in circulating plasma of patients treated with anti-VEGFA drugs and in the eye of patients suffering from

neovascular eye diseases (Chen et al., 2015; Jain et al.). Heparin is found to be associated with cell-surfaces and extracellular matrix throughout the body including in the eye and in tumor microenvironments, and therefore could be modulating angiogenic signaling (Ashikari-Hada, et al., 2005). Effective therapies targeting both PlGF and VEGFA should mitigate potential effects caused by potential up regulation of PlGF in the absence of VEGFA. It is also reasonable to consider a potential role of heparin in modulating these two ligands as heparin is also expected to be present at disease sites. In this study, differences in single target and multi-target drug efficacies for inhibiting endothelial cell proliferation will be examined in the presence of VEGFA, PlGF and heparin. Because time-dependent signatures can be detected using RTCA it may be possible in this study to observe previously unidentified differences in the drug profiles produced by PlGF-compensation and heparin-modulation.

Effects of VEGF-related Angiogenic Drugs

Although most VEGFA-induced cell proliferation signaling appears to operate through VEGFR-2 phosphorylation, blocking of VEGFR-1 signaling also inhibits VEGFA-induced cell proliferation (Autiero et al., 2003). Therefore, blocking of VEGFA interaction with both receptors is desirable to impact endothelial cell proliferation. Comparative studies of an anti-VEGFA targeted drug and a drug targeting both PlGF and VEGFA have revealed differences in these two drug activities (Papadopoulos, et al., 2012; Yu, Liang, & Ferrara, 2011). In VEGFR-1-transfected cells, binding of both PlGF and VEGFA by a VEGFR-1/VEGFR-2 chimera protein inhibited VEGFR-1 phosphorylation by both VEGFR-1 ligands. In contrast, an anti-VEGFA antibody was

unable to prevent PlGF-induced activation of VEGFR-1. In VEGFR-2 transfected cell lines both molecules were able to inhibit receptor activation. In HUVECs the capacity of these molecules was studied for effectiveness in blocking VEGFR-ligand induced cell migration and again the anti-VEGFA drug was unable to prevent PlGF-induced effects (Papadopoulos, et al., 2012). Evidence that anti-PlGF antibodies may also have value as an anti-angiogenic therapy is found in murine models where some efficacy in treatment of cancer and AMD has been observed (Van de Veire et al., 2010). The use of single target and multi-target anti-angiogenic drugs will help to delineate the contribution of ligand neutralization on inhibition of endothelial cell proliferation.

However, neutralization of ligand may not be the only contributing factor to efficacy of anti-VEGFA drugs. These drugs may also differ in their abilities to block heparin's effects. MacDonald *et al.* demonstrated that VEGF:anti-VEGF antibody complexes were still able to bind heparin and at a 100-fold lower concentration than $VEGFA_{165}$:Receptor Chimera complexes (MacDonald et al., 2016). Meanwhile, VEGF-Trap perhaps through its VEGFR-1 domain may bind directly to heparin. Together these observations suggest that the presence of PlGF and heparin have potential to alter the efficacy of anti-VEGF drugs.

Chapter II

Materials and Methods

The materials and experimental methods used in this work are described. Experimental details and analysis are also presented to enable replication of work.

Materials

The source of biological materials is identified.

Growth Factors

Recombinant human $VEGFA_{165}$ was purchased from DiscoverX. $VEGF_{121}$, PlGF-2, and PlGF-1 were purchased from R&D systems. Heparin was obtained from Sigma.

Inhibitors

Polyclonal anti-PlGF was purchased from R&D systems. VEGF-Trap (Zaltrap) was manufactured by Regeneron. Anti-VEGF antibody (Avastin) was manufactured by Genentech.

Methods

Experimental procedures required to maintain cells, perform growth factor proliferation and inhibition studies are explained. Data analysis methods used are described.

Cell Culture and Maintenance

HUVECs (Lonza) between passage 3 and 9 were used as the model endothelial cells for evaluating VEGFA-induced cell proliferation. The procedure for maintaining and culturing HUVECs was based on the manufacturer's recommended conditions. Cells were maintained in Lonza Endothelial Growth Media-2 (EGM-2) growth media containing VEGF, heparin, insulin-like growth factor, fibroblast growth factor and heat-inactivated fetal bovine serum (HI-FBS) prior to seeding for experiment. Cells were grown under standard tissue culture conditions of 37°C, 5% CO_2 and 90% humidity. (Lonza Walkersville, 2015).

Measuring HUVEC Proliferation using Real-time Cell Analyzer

Cells were seeded at 3.0 x 10^4 cells/cm^2 in 0.15 ml of EGM-2 in E-plate-96 PET plates (ACEA Biosciences) for 16-20 hours. Prior to treatment cells were rinsed with Dulbecco's Phosphate Buffered Saline (DPBS) without calcium or magnesium (Hyclone) to remove residual growth factors. Experiments were performed in Lonza Endothelial Basal Media (EBM) media containing 1% HI-FBS with penicillin/streptomycin antibiotic (Life Technologies). The final volume per well was 0.15 ml.

Growth factor titrations were performed in FBS to determine range to be tested (data not shown). Initial ranges were based on information provided by manufacturer regarding expected effective concentration. VEGFA isoforms, $VEGFA_{165}$ and $VEGF_{121,}$ were tested at concentrations ranging from 50 ng/ml to 1.3 ng/ml. PlGF isoforms, PlGF-2 and PlGF-1, were tested at concentrations ranging from 1000 ng/ml to 26 ng/ml. Growth factor proliferation was tested in the presence of exogenous heparin added at 1µg/ml, 10 µg/ml,

and 33 μg/ml. Four-parameter curve fits were used to determine the 50% response (EC_{50}) of each growth factor.

For combination studies a submaximal concentration of VEGF was selected for testing based on VEGF titration experiments. In addition to the combination of three treatments, $VEGFA_{165}$, PlGF-2 and heparin, each of the pairs were also tested-- $VEGFA_{165}$: PlGF-2, $VEGFA_{165}$: heparin, PlGF-2: heparin, VEGF, and PlGF2. Two non-heparin-binding isoforms, PlGF-1 and $VEGFA_{121}$, were also tested in combination with 33 μg/ml heparin as a control for the effect of heparin signaling versus simultaneous activation of VEGFR-1 and VEGFR-2 receptors by PlGF and VEGFA.

The RTCA instrument was used to record changes in cell index at fifteen minute intervals for up to 120 hours post addition of treatment (Figure 1). As cells proliferate impedance increases and can be detected as an increasing slope on the time-dependent cell response profile (TCRP). The time of maximum cell index (T_{max}) and maximum CI (CI_{max}) were measured for each concentration. Cell indices (CI) were normalized after addition of treatment. Temperature fluctuations and the media changes affected the cell index readings; therefore, CI was normalized using the FBS negative control to determine when CI had stabilized. Cell index normalization is a mathematical transformation of the cell index values and does not alter the actual cell index recorded in each well. For all experiments the cell index was normalized no later than 4 hours after addition of treatment. Data is summarized from three experiments. Student's t-test with an alpha-level of 0.05 was used to determine whether there were significant differences among treatments. For dose response curves the concentration inducing 50% cell proliferation (EC_{50}) was calculated from the 4-parameter logistic fit. Additionally, the area under the

curve (AUC) was calculated for different treatments over the duration of the experiment. For AUC calculations the baseline was set to 1 and AUC was calculated using GraphPad Prism software.

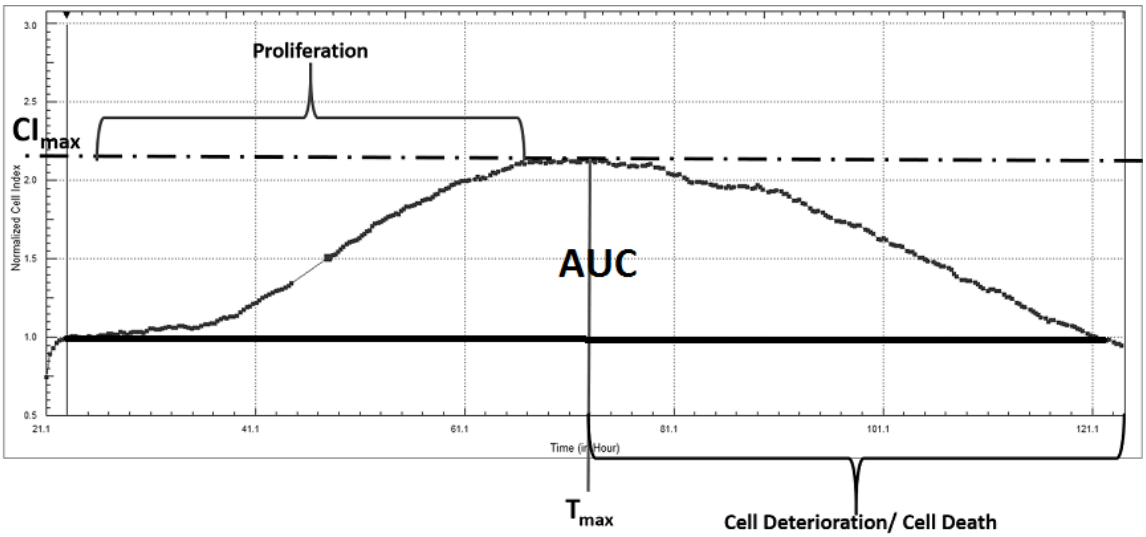

Figure 1: Representative HUVEC Time-dependent Cell Response Profile (TCRP)
An example TCRP for HUVECs stimulated with $VEGF_{165}$ is shown above. Cell Index (CI) was normalized to 1 post addition of growth factor. Proliferation is observed as an increase in CI. CI peaks at approximately 73 hours (T_{max}). The CI at 73 hours is referred to as CI_{max}. After 73 hours cell health begins to deteriorate and results in reduced CI.

T_{max} was determined from TCRPs. To examine concentration dependence of T_{max} the T_{max} was plotted versus log growth factor concentration. A linear regression was performed using GraphPad Prism software. The slope was determined. An F-test was used to determine whether slope was significantly non-zero using an alpha-level of 0.05.

Inhibition of VEGFA:PlGF:Heparin-induced Proliferation by VEGF ligand antagonists

To study the impact of PlGF and heparin in modulation of $VEGFA_{165}$-induced HUVEC proliferation, ligand antagonists of VEGFA only, PlGF only and both PlGF and VEGFA were used in a series of experiments (Table 1). Inhibitors were added to

HUVECs prior to stimulation with the trio of $VEGFA_{165}$, PlGF-2 and heparin or the combination of $VEGF1_{65}$ and PlGF-2 without heparin. TCRPs were generated to determine if differences in the duration of inhibition of cell proliferation were observed over extended time periods (Papadopoulos, et al., 2012). Duration of experiment was determined by TCRP data using FBS treatment as the control profile. Stimulation with VEGFA only and the combination of VEGFA + PlGF were run as comparison for antagonist inhibition of VEGFA or both VEGFA and PlGF-induced cell proliferation.

Table 1: Specificity of Tested Inhibitors

	Target Binding		
Inhibitor	VEGFA	PlGF	Heparin
VEGF-Trap	+	+	+
Anti-VEGF	+	-	-
Anti-PlGF	-	+	-

To compare drug efficacy multiple concentrations of antagonist were tested in three independent experiments. Inhibitors were serially-diluted in the presence of PlGF-2:$VEGF_{165}$ and fit with a 4-parameter logistic fit using GraphPad Prism software. The concentration that inhibited fifty percent of cell proliferation (IC_{50}) of each treatment was calculated. Cell index values at maximum observed inhibition were also compared. Area under the curve for each treatment was calculated and compared to measure the overall effectiveness of inhibition. Student's t-test was used to determine statistical significance.

Validation of RTCA Proliferation using BrdU incorporation

BrdU incorporation into newly formed DNA strands is a well-established, accurate technique for detecting cell proliferation. In order to accurately measure proliferation BrdU needs to be added to cells in a sufficient amount prior to the most active proliferation phase of growth. As RTCA is a new technology and changes in TCRP can potentially be produced by non-cell number changes, the confirmation of the change in cell number was performed using BrdU incorporation assay (Atienza, Yu, Wang, Xu, & Abassi, 2006). A commercially-available enzyme-linked immunosorbent assay (ELISA) kit (Millipore EMD) was used to detect the level of BrdU incorporation.

Correlation of BrdU ELISA and impedance measurements were assessed for proliferation measurement by seeding HUVECs at densities ranging from 1.3e4 cells/cm^2 to 1.0e5 cells/cm^2. BrdU was also used to verify growth factor-induced proliferation. For all BrdU proliferation measurements, BrdU reagent was added to E-96 plate 16 hours post treatment. Incorporation and impedance measurements were performed in the same plate. RTCA measurements were recorded until peak cell index was observed for all treatments. BrdU ELISA measurements and maximum Cell index (CI_{max}) were analyzed for correlation using GraphPad Prism to perform Pearson correlation analysis. A two-tailed t-test was used to determine statistical significance of correlation.

Chapter III

Results

The results of HUVEC proliferation studies using RTCA are presented. RTCA was able to replicate previous findings when proliferation was evaluated using traditional measurements. In addition time-dependent measurements delineated new features of proliferation that allow further description of growth factor and inhibitor responses. RTCA measurements correlate with nucleoside incorporation measurements of proliferations and verify the utility of RTCA for the study of proliferation.

$VEGF_{165}$ and PlGF-2 induced HUVEC Proliferation Measured by Real-Time Cell Analysis

A higher concentration of PlGF-2than of $VEGF_{165}$ was required to induce HUVEC proliferation. Initial experiments were performed in HUVECs to establish effective growth factor concentration ranges. The effective concentration ranges were established for $VEGF_{165}$ and PlGF-2 by titration on HUVECs in the absence of heparin. The maximum Cell Index (CI_{max}) versus the concentration of each growth factor was plotted and fitted with a 4-parameter curve to determine the EC_{50} (Figure 2). PlGF-2 was found to be a weaker mitogen than $VEGF_{165}$. The concentration of PlGF-2 required to induce proliferation was more than 50-fold higher than required concentration of

$VEGF_{165}$. The mean EC_{50} of $VEGF_{165}$ from three experiments was 8 ng/ml while the EC_{50} for PlGF-2 was determined to be 630 ng/ml. PlGF-2's CI_{max} was also much lower than $VEGF_{165}$'s.

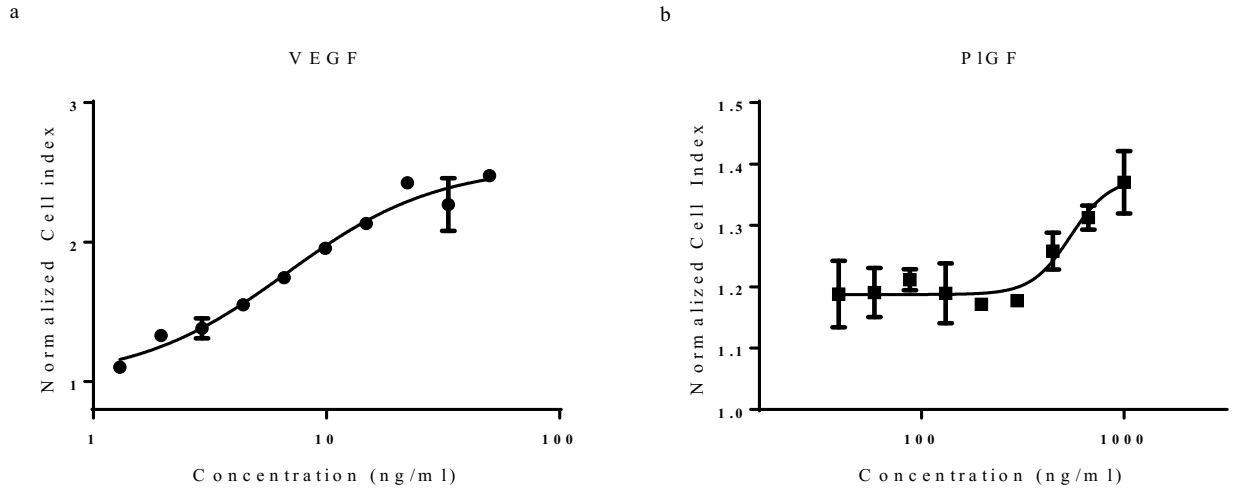

Figure 2: Representative 4-Parameter Curve Fit of CI_{max} Values
$VEGF_{165}$ and PlGF-2 were titrated on HUVECs in the absence of soluble heparin. The CI_{max} was plotted against log concentration for a)$VEGF_{165}$ or b)PlGF-2 and fitted with a 4-parameter curve. $VEGF_{165}$ was titrated from 50 ng/ml to 1.3 ng/ml and PlGF-2 from 1000 ng/ml to 26 ng/ml. EC_{50} values were determined from 4-parameter curve fits.

RTCA Impedance Measurement Confirms Heparin Enhanced Proliferation of $VEGF_{165}$ and PlGF-2 Proliferation

Heparin reduced the concentrations of VEGF165 and PlGF-2 that were needed to induce HUVEC proliferation. $VEGF_{165}$ and PlGF-2 are heparin-binding isoforms. Heparin has previously been reported to both enhance and inhibit proliferation of $VEGF_{165}$ depending on heparin concentration tested (Ashikari-Hada, et al., 2005). The suitability of RTCA impedance measurement for studying HUVEC proliferation was assessed by measuring heparin effects on $VEGF_{165}$ and PlGF-2 proliferative activity. Each growth factor was tested in the presence or absence of soluble heparin (FBS). Three

heparin concentrations were selected: 1 μg/ml, 10 μg/ml or 33 μg/ml. Mean EC_{50} values were determined from three experiments.

The addition of soluble heparin reduced the EC_{50} of both $VEGF_{165}$ and PlGF-2 (Table **2**). The mean EC_{50} of $VEGF_{165}$ ranged from 5.9 – 8.2 ng/ml. The highest $VEGF_{165}$ EC_{50}, 8.2 ng/ml, was observed in the absence of heparin. Slightly lower $VEGF_{165}$ EC_{50} values were observed in the presence of heparin. PlGF-2's EC_{50} was also consistently reduced in the presence of heparin. The maximum reduction was observed with the addition of 1 μg/ml heparin which reduced PlGF-2's EC_{50} from 631 ng/ml 369 ng/ml (Table **2**). Heparin was observed to consistently reduce the EC_{50} in HUVECs of both $VEGF_{165}$ and PlGF-2, but these differences were not statistically significant.

Table 2: Reduction of $VEGF_{165}$ and PlGF-2 EC_{50} in the Presence of Heparin

Heparin Concentration (μg/ml)	$VEGF_{165}$ EC_{50} (ng/ml)	PlGF-2 EC_{50} (ng/ml)
0	8.2	631
1	7.4	369
10	7.5	422
33	5.9	484

Alterations in HUVEC Proliferation by Combination of PlGF-2 and $VEGF_{165}$ in Presence of Heparin

Low concentrations of $VEGF_{165}$ reduced the PLGF-2 concentration that was needed to stimulate HUVEC proliferation. Enhanced HUVEC proliferation activity is reported for the combination of PlGF-2 and low $VEGF_{165}$ concentrations (Park, et al.,

1994). To test the combination of $VEGF_{165}$:PlGF-2-induced proliferation, a 2 ng/ml concentration of $VEGF_{165}$ was used with PlGF-2 titrated from 1000 to 23 ng/ml. The 2 ng/ml $VEGF_{165}$ concentration was selected for use in the combination as it was less than the determined EC_{50} for VEGF (6 - 9 ng/ml), but still induced measurable proliferation. These $VEGF_{165}$:PlGF-2 combinations were tested in the absence or presence of heparin.

In combination with $VEGF_{165}$, the EC_{50} of PlGF-2 was reduced compared to PlGF-2's EC_{50} in the absence of $VEGF_{165}$ (Figure 3). In the absence of heparin, the mean PlGF-2 EC_{50} decreased more than 5-fold from 630 ng/ml without $VEGF_{165}$ to 90 ng/ml in the presence of $VEGF_{165}$. The EC_{50} reduction of PlGF-2 in combination with $VEGF_{165}$ was observed for all concentrations of heparin tested (Figure 3).

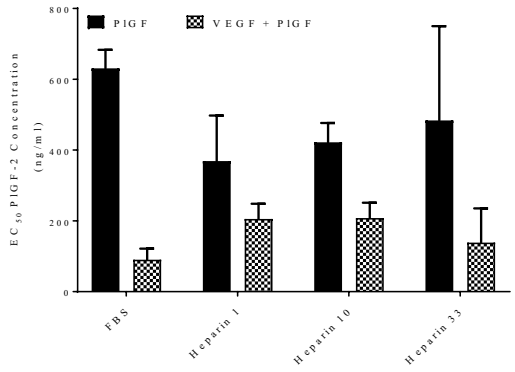

Figure 3: Reduction of PlGF-2's EC_{50} in Combination with $VEGF_{165}$
PlGF-2 was titrated on HUVEC cells alone or in combination with 2ng/ml VEGF165. Heparin effects were determined by addition of 1 µg/ml, 10 µg/ml, or 33 µg/ml exogenous heparin to growth factors. Heparin effects were determined by comparison to PlGF-2 or PlGF-2:VEGF165 in FBS media without addition of heparin. The Mean PlGF EC_{50} concentrations are plotted with SEM values from 3 experiments.

CI_{max} Measurement of Cell number Changes Produced by Combination of Heparin and PlGF-2 with $VEGF_{165}$

The EC_{50} determined during proliferation is only one aspect of growth factor effectiveness and it does not necessarily reflect differences in cell number achieved. In addition to heparin's reduction of EC_{50}, the level of cellular proliferation was also measured using RTCA impedance measurements of maximum cell index (CI_{max}). Changes in CI_{max} reflect differences in peak HUVEC cell number. Measurement of CI_{max} showed that HUVEC proliferation was significantly increased in the presence of 2 ng/ml $VEGF_{165}$ compared to a no growth factor negative control (Figure 4). The addition of 10 μg/ml heparin to $VEGF_{165}$ resulted in a statistically significant increase in HUVEC cell number compared to FBS control ($p=0.02$). Therefore, at lower $VEGF_{165}$ concentrations the addition of soluble heparin was shown to enhance $VEGF_{165}$'s proliferative effect in HUVECs.

The CI_{max} of combined PlGF-2:$VEGF_{165}$ was compared to the corresponding $VEGF_{165}$ response in the absence of PlGF-2. As reported previously, PlGF-2 combined with low concentrations of $VEGF_{165}$ results in significantly increased cell proliferation when compared to $VEGF_{165}$ only treatment (Park, et al., 1994). In combination with $VEGF_{165}$, 296 ng/ml PlGF-2 increased cell indices in all three experimental runs when compared to the VEGF control (Figure 4). The enhancement of proliferative activity was highest in the absence of heparin ($p=0.02$), but was observed at all three heparin concentrations tested.

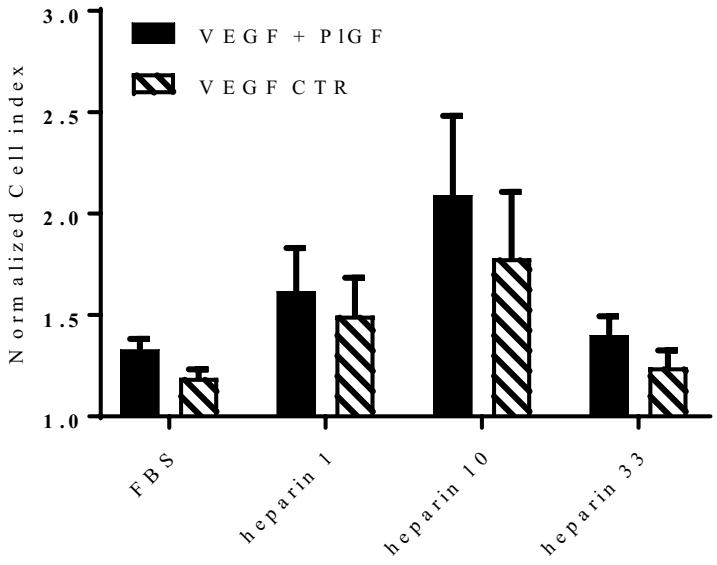

Figure 4: Heparin Alteration of Cell Index of PlGF:VEGF Treated HUVECs
Cell index was normalized to wells containing FBS only (CI=1). The mean normalized cell index is shown for stimulation of HUVECs with 296 ng/ml PlGF-2 in combination with 2ng/ml VEGF (black) and VEGF 2 ng/ml (diagonal stripe).

Time-dependent Study of Heparin and Growth Factor Concentration Effects on HUVEC Proliferation

Determination of EC_{50} and cell number are possible using traditional proliferation readouts at a single time point. RTCA confirmed previous findings related to cell number changes using CI_{max} and EC_{50} analysis. RTCA also enables the study of proliferation at repeated time points; therefore, enabling the determination of additional kinetic parameters including the time of peak proliferation (T_{max}) and area under the curve (AUC) calculations.

Differences in $VEGF_{165}$'s Proliferation T_{max} are Significantly Altered by Increasing VEGF Concentration

The time at which the peak cell index is achieved (T_{max}) was used as another measurement to study alterations in HUVEC proliferation. The T_{max} was calculated for each concentration of $VEGF_{165}$ tested in the presence or absence of heparin. $VEGF_{165}$ simulation of HUVECs in a dose-dependent manner significantly lengthened the T_{max} of HUVEC proliferation (Figure 5a). A large difference in T_{max} is observed across the concentration range with $VEGF_{165}$ concentration appearing to be positively correlated with T_{max}. Some effect of heparin was observed at low VEGF concentrations (Figure 5b). At low concentrations of $VEGF_{165}$ a slight increase in T_{max} is observed in the presence of 1 and 10 μg/ml heparin, but these differences were insignificant.

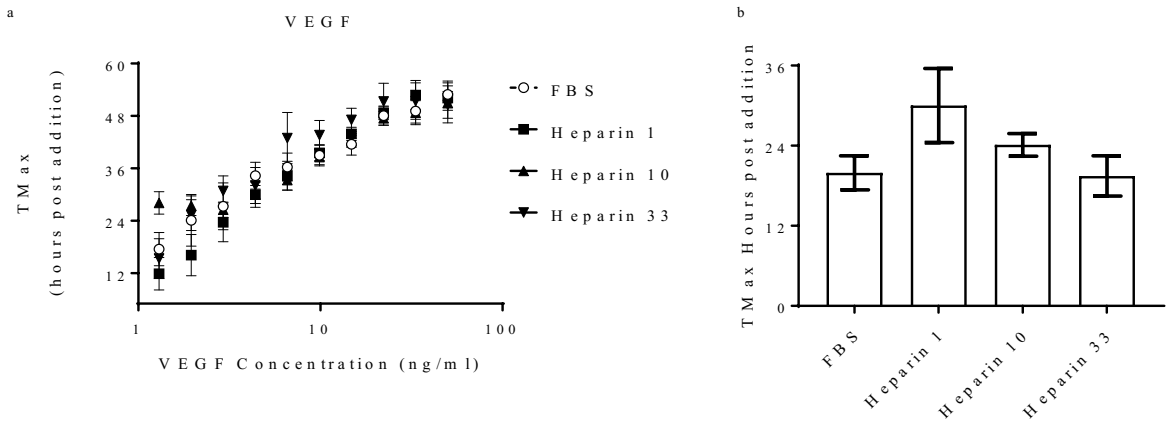

Figure 5: Alteration of VEGF $T_{max\ by}$ Heparin
a) Mean T_{max} values are shown for VEGF titration in the presence of increasing concentrations of heparin. b) A magnified bar graph representation of mean T_{max} with SEM values for VEGF 2ng/ml tested in presence of increasing concentrations of heparin.

Slope of $VEGFA_{165}$ Concentration versus Tmax Linear Regression is Significant. Based on visual analysis of the plot of T_{max} versus $VEGF_{165}$ concentration it appears that VEGF

concentration significantly increases the T_{max} of HUVEC proliferation. To examine this observation mathematically, a linear fit of T_{max} versus $VEGF_{165}$ concentration was made and the significance of the slope of the line tested using an F-test. The linear regression produced a statistically non-zero slope ($p<0.0001$). For $VEGF_{165}$ the slope was approximately 20 h·ml/ng (Table 3).

To further examine the contribution of the heparin binding property of $VEGF_{165}$ to its concentration-dependent T_{max} effect, the non-heparin VEGF isoform, $VEGF_{121}$, was also tested in the presence or absence of heparin. $VEGF_{121}$ is not expected to interact with cell surface-associated heparin that would be present in FBS-cultured HUVECs while $VEGF_{165}$ would. For the $VEGF_{121,}$ non-heparin-binding isoform, the VEGF concentration also significantly altered T_{max} slope ($p<0.0001$). Therefore, the ability to bind heparin is not required for VEGF to increase the T_{max} of HUVEC proliferation. However, heparin may have some effect on T_{max} as the $VEGF_{121}$ linear regression produced shallower slopes than $VEGF_{165}$. $VEGF_{121}$'s mean T_{max} slope was 16.2 h·ml/ng in the absence of heparin and 12.0 h·ml/ng in the presence of 33 µg/ml heparin. Although, T_{max} is positively correlated to VEGF concentration for both isoforms heparin may still have a role in enhancing $VEGF_{165}$ effect on T_{max}. Heparin's effect is most apparent at high concentrations of $VEGF_{165}$ where a consistent trend for longer T_{max} is observed in comparison to $VEGF_{121}$ (Figure 6).

Table 3: Mean Slope of T_{max} versus VEGF Isoform Concentration

	$VEGF_{165}$	$VEGF_{121}$
FBS	21.4	16.2
Heparin 33	22.3	12.0

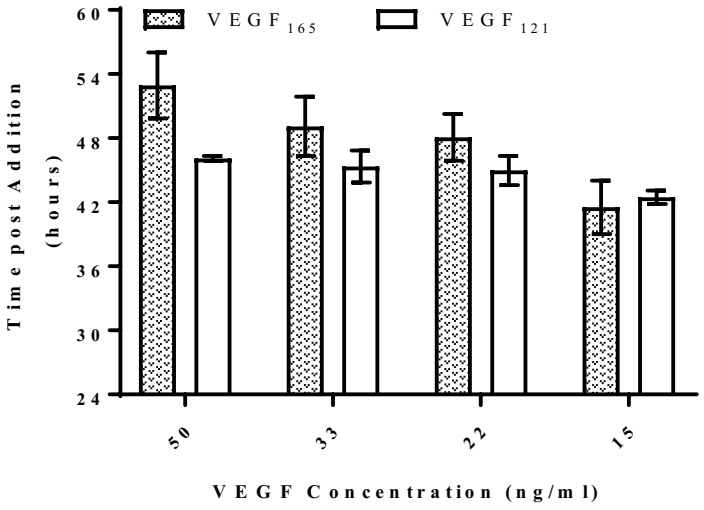

Figure 6: T_{max} of VEGF Isoforms in Absence of Soluble Heparin
Mean T_{max} with SEM values for $VEGF_{165}$ and $VEGF_{121}$ are shown from three experiments.

PlGF-2's T_{max} is Similar at Tested Concentrations and is Not Altered by Heparin

PlGF-2's $T_{max\ is}$ less dependent on PlGF-2 concentration than observed for VEGF isoforms (Figure 7). T_{max} values for PlGF-stimulated HUVECs produced a narrower time window than VEGF over the tested concentrations. The mean T_{max} values of PlGF-induced proliferation varied from 16 - 30 hours post-treatment addition depending on concentration. Unlike for VEGF, the slopes for the linear regression of T_{max} versus PlGF-2 concentration were not statistically significant. The slope of the linear regression ranged from 1.9 in the absence of heparin to 6.5 in the presence of 33 µg/ml heparin. A slight mean T_{max} decrease was observed at low PlGF-2 concentrations in the presence of 33 µg/ml heparin, but T_{max} was highly variable from run to run.

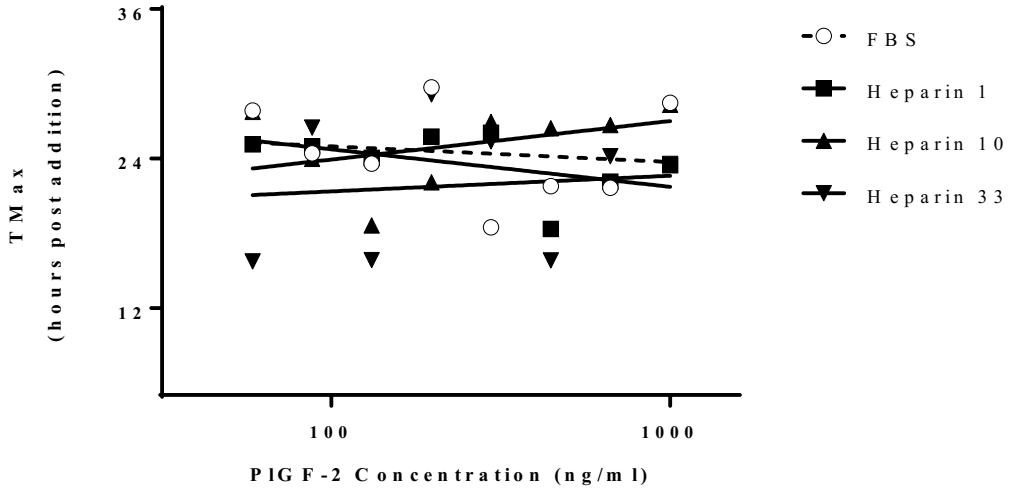

Figure 7: T_{max} of PlGF in HUVECs
The mean T_{max} post-addition of growth factor was calculated and plotted against log10 PlGF-2concentration. A linear regression was performed. Slopes of the line were determined and an F-test used to determine signficance of slope.

Combination of PlGF-2 with $VEGF_{165}$ Results in Later T_{max} than for $VEGF_{165}$. When PlGF-2 was combined with 2 ng/ml VEGF, T_{max} values ranged from 18 to 33 hours depending on PlGF-2 concentration. The mean T_{max} for 2 ng/ml VEGF alone overlapped with PlGF-2 T_{max} ranging from19 - 30 hours (data not shown). The combined effects of $VEGF_{165}$ and PlGF-2 could be expected to be highest at concentrations where the mitogenic activity peaks overlap which may explain why PlGF-2 is shown to enhance VEGF at low, but not high VEGF concentrations. However, within each of the three experiments PlGF-2 combined with VEGF at 2 ng/ml produces a later T_{max} than VEGF alone (Table 4).

Table 4: Difference in T_{max} for Combination versus VEGF control

Heparin Concentration (μg/ml)	$VEGF_{165}$ (2 ng/ml)	PlGF-2 (296 ng/ml) + $VEGF_{165}$	Delta T_{max}
None	18.4	25.6	7.2
1	26.5	29.7	3.3
10	23.4	25.9	2.6
33	16.6	22.1	5.5

Heparin Effects on Combined PlGF-2:$VEGF_{165}$ T_{max}. No statistically significant change in T_{max} was observed compared to FBS control for $VEGF_{165}$:PlGF-2 combination treated cells in the presence of soluble heparin. A slight increase was observed at 1 μg/ml heparin but this effect was variable. Statistically significant increases were observed in T_{max} of the combination of PlGF-2:$VEGF_{165}$ without soluble heparin and at 1 μg/ml heparin (Table 4). At 10 μg/ml the effect is borderline (p=0.06).

Combined PlGF-2:$VEGF_{165}$ stimulation of HUVECs increases both CI_{max} and T_{max}. Both T_{max} and CI_{max} were altered by combination of growth factors. This is observable by examination of TCRPs for the combination compared to corresponding PlGF-2 and $VEGF_{165}$ single growth factor controls. TCRP data shows highest peaks for the combination of $VEGF_{165}$:PlGF-2 and consistently later peaks compared to VEGF and PlGF controls (Figure 8). T_{max} for the combination is similar to the T_{max} for PlGF, but still

later than PlGF alone. The shift in T_{max} is smaller compared to PlGF only but was observed in each experimental run.

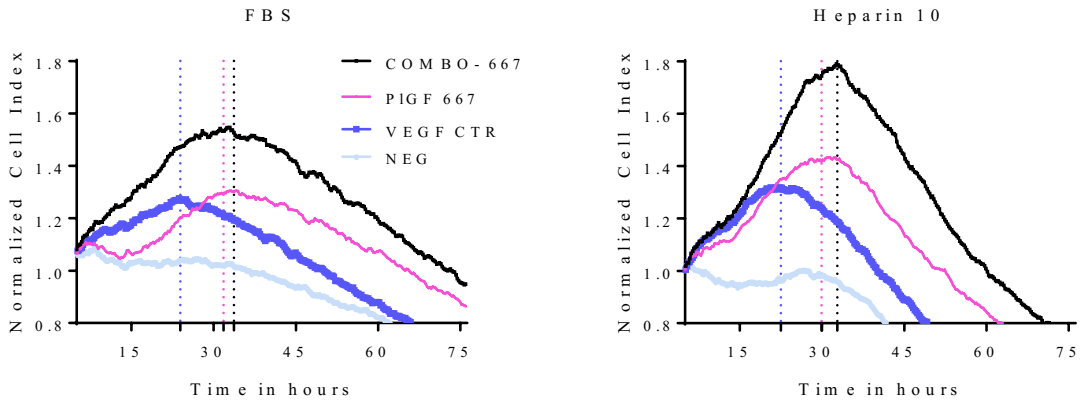

Figure 8: Representative TCRP of HUVEC Proliferation
The normalized cell index observed in response of the combination of 667 ng/ml PlGF with VEGF (Combo-667) is shown compared to the TCRP for individual components 667 ng/ml PlGF (PlGF 667) or 2 ng/ml VEGF (VEGF CTR). FBS only data is shown as the negative control (NEG). T_{max} for growth factors are shown as dotted vertical lines.

PlGF-2 combined with $VEGF_{165}$ increases Area Under the Curve. AUC integration integrates the time-dependent effect and magnitude of cell proliferation. In addition to maximum cell index and T_{max} values, the total area under the curve (AUC) was calculated from TCRP data. In the absence of growth factors, HUVECs undergo cell death. This is observed in the TCRP as a decline below normalized cell index (Figure 8). Both VEGF and PlGF have been reported to have anti-apoptotic effects and promote cell survival (Gerber, Dixit, & Ferrara, 1998; Yoo et al., 2015). To distinguish between anti-apoptotic effects the baseline for area under the curve was set to the normalized cell index value (CI =1) at the start of treatment. Consistent with increases in CI_{max} and T_{max}, the addition

of PlGF-2 significantly increased the AUC of the response compared to $VEGF_{165}$ only control at all concentrations of heparin (Figure 9).

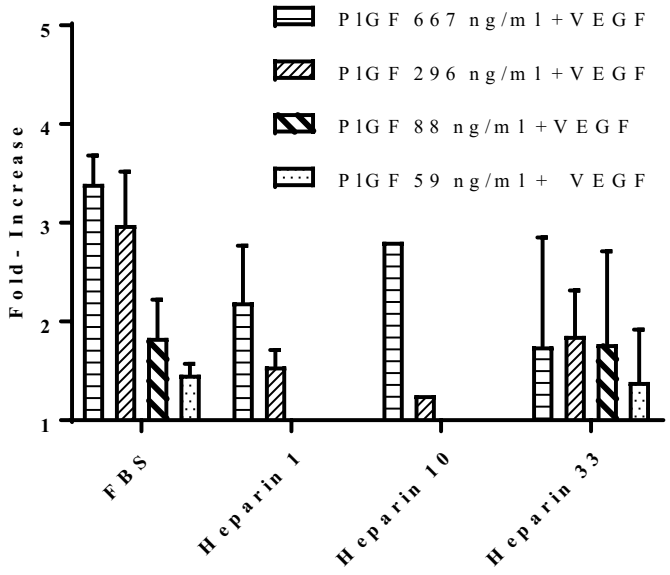

Figure 9: Area Under the Curve for PlGF:VEGF Combinations
The area under the curve was calculated for normalized cell index TCRP values in the presence of 2 ng/ml VEGF with PlGF at 296 ng/ml, 88 ng/ml or 59 ng/ml. The VEGF control AUC was set to 1. Heparin was tested with the combinations at concentrations from 1 - 33 µg/ml.

VEGF and/or PlGF Inhibitor Characteristics

Single and multi-target VEGFR-1 ligand inhibitors were found to reduce PLGF-2: $VEGF_{165}$-induced HUVEC proliferation. Both VEGF and PlGF ligands bind to VEGFR-1. To evaluate effectiveness of VEGFR-1 ligand inhibitors, HUVECs were stimulated

with PlGF-2:$VEGF_{165}$ or non-heparin-binding isoforms PlGF:$VEGF_{121}$. As in proliferation studies 2 ng/ml $VEGF_{165}$ was selected for the combination to allow for observation of synergistic PlGF mitogenic effects. A concentration of 125 ng/ml PlGF-2 was selected for evaluating inhibition at all heparin concentrations as it was near or above the EC_{50} dose without falling outside the linear range of dose response curves.

VEGF-Trap, and anti-VEGFA, or anti-PlGF antibodies were tested for inhibition of HUVEC proliferation. VEGF-Trap binds both $VEGF_{165}$ and $PlGF_{-2}$. In addition, it has also been reported to bind heparin (MacDonald, et al., 2016). The selected anti-VEGF antibody and anti-PlGF antibody have been previously shown to have neutralizing activity (Brave, Eberlein, Shibuya, Wedge, & Barry, 2010; Lowe et al., 2007). In order to assess their effectiveness, inhibitors were tested at concentrations ranging from 11 fM to 6.6 pM based on titration studies in PlGF-2:$VEGF_{165}$ stimulated HUVECs (Figure 10). Plateaus at high and low ends of the curve were observed for all three inhibitors using this range.

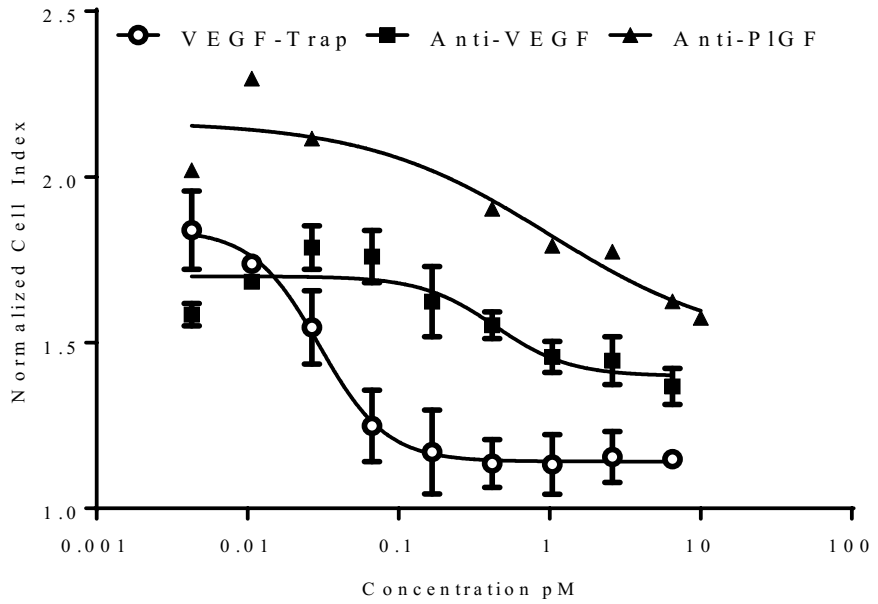

Figure 10: Representative 4-parameter Inhibition Curve in PlGF2:$VEGF_{165}$ Stimulated HUVECs
HUVEC cells were stimulated with PlGF2:VEG_{165} grown in the absence of exogenous heparin. VEGF-Trap, anti-VEGF and Anti-PlGF inhibitors were titrated from 6.5 pM to 11 fM. Resulting 4-parameter curve plots for each inhibitor are shown with mean and SEM.

Determination of Inhibitor IC_{50} Values in the Absence of Heparin

To compare the relative efficacy of inhibitors, the IC_{50} values of each inhibitor was calculated in PlGF-2:$VEGF_{165}$-treated HUVECs (Figure 11). The CI_{max} reflects the peak cell number. CI_{max} was determined for each concentration of inhibitor. VEGF-Trap is the only inhibitor expected to neutralize both VEGF and PlGF activity. As such, VEGF-Trap was expected to be the most potent inhibitor of PlGF-2:$VEGF_{165}$ -stimulated proliferation in the absence of exogenous heparin. The IC_{50} value was determined to be 0.014 pM for VEGF-Trap without heparin. Anti-VEGF antibody was significantly less

effective (p= 0.03) than VEGF-Trap with IC_{50} value of 0.35 pM. Anti-PlGF was the least effective and required significantly higher inhibitor concentrations (p = 0.03) than anti-VEGF antibody with an IC_{50} value of 1.7 pM.

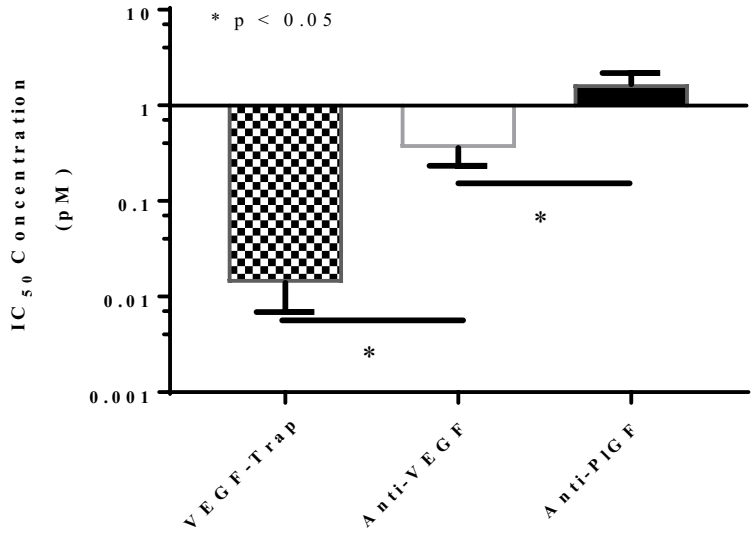

Figure 11: Comparison of Inhibitors in HUVEC without Heparin
HUVECs were stimulated with PlGF:VEGF without exogenous heparin. VEGF-Trap and antibodies targeting VEGF or PlGF were titrated to determine IC_{50} concentration. A 4-parameter curve fit was used to determine IC_{50} of each inhibitor was by plotting the maximum CI measurements of each treatment over the tested concentration range. The mean and SEM are shown from n=3 experiments.

Real-Time Cell Analysis of VEGF and/or PlGF Inhibitor Characteristics

RTCA measurement reveals incomplete inhibition by single target inhibitors. TCRP profiles for the maximum concentration of inhibitor were examined to compare effectiveness of inhibition over extended periods of time in PlGF-2:$VEGF_{165}$-stimulated HUVECs (Figure 11). Examination of TCRP profiles showed that anti-PlGF antibody is the least effective at preventing proliferation. Anti-PlGF antibody allowed HUVECs to reach higher CI_{max} values than both anti-VEGF and VEGF-Trap. In contrast, VEGF-Trap

shows almost complete inhibition of proliferation with CI_{max} values close to those with FBS alone. In FBS-treated cells, the cell index begins to decrease below baseline. This indicates deterioration of cell health and cell death. VEGF-Trap seems to inhibit both anti-apoptotic effects and survival signals from PlGF-2:$VEGF_{165}$ stimulation. Anti-VEGF antibody treatment also allowed for some proliferation above baseline that was detectable at early time points.

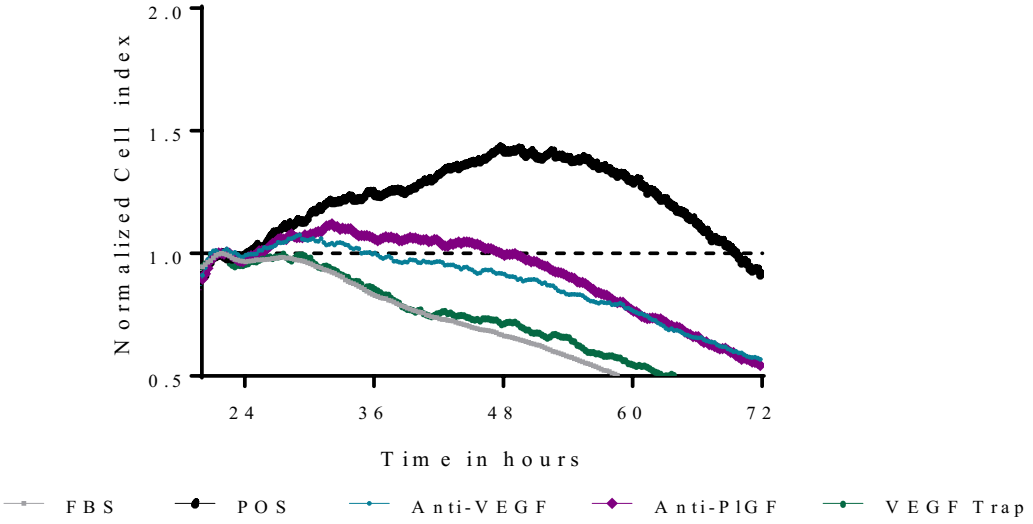

Figure 12: Growth Factor TCRP Inhibition Profile without Heparin
Representative TCRP profiles are shown from a single experiment. Inhibition profiles for VEGF-Trap (green), anti-VEGF (light blue) and anti-PlGF (purple) are shown at 6.5 pM concentration. TCRP for cells grown without stimulation are shown in grey. Uninhibited stimulation with PlGF-2:$VEGF_{165}$ is shown in black.

The effectiveness of the inhibitors examined by IC_{50} and TCRP is consistent with the capacity of each growth factor to induce proliferation. Although PlGF-2 enhances $VEGF_{165}$-induced proliferation, the majority of proliferative activity is due to $VEGF_{165}$ activity (Figure 4). Inhibitors with anti-VEGF activity were observed to require lower concentrations for effectiveness than the anti-PlGF inhibitor. This is further

demonstrated by comparing the maximum percent inhibitory activity observed for each class of inhibitor.

Maximum percent inhibition was calculated based on the area under the curve (AUC). The percent inhibition of the total AUC above normalized baseline was calculated. AUC calculation provides mathematical representation of the TCRP and represents the magnitude and duration of the signal above baseline. The AUC for each inhibitor was calculated and compared to the PlGF-2:$VEGF_{165}$-stimulated AUC to determine percent inhibition. By analysis of percent AUC inhibition, the maximum effect of inhibition for VEGF-Trap was observed at 1 pM (Figure 13). Anti-VEGF and anti-PlGF antibodies at 6.5 times the concentration were not able to achieve the same level of inhibition as VEGF-Trap. VEGF-Trap maximum percent inhibition was 95% compared to 75% for anti-VEGF and 45% for anti-PlGF antibodies.

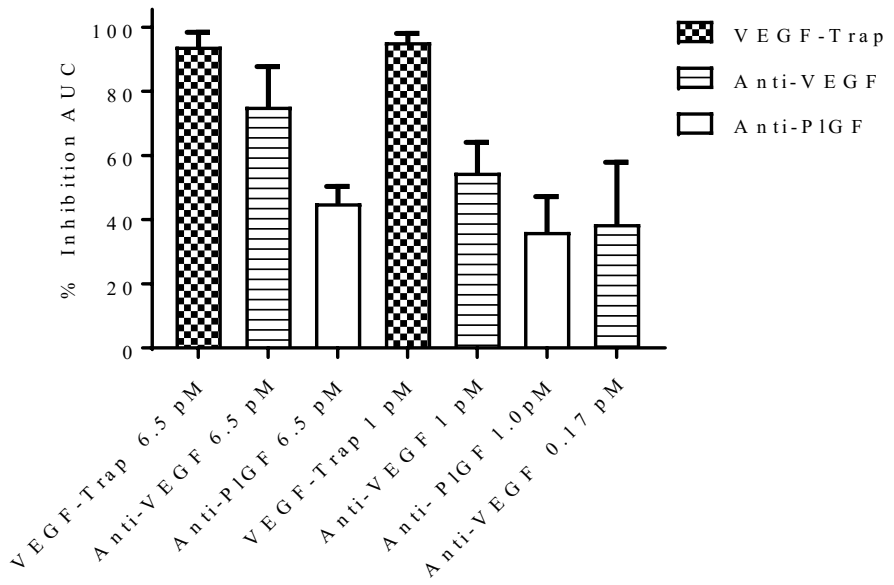

Figure 13: Percent Inhibition of PlGF:VEGF Proliferation by AUC Comparison
The percent inhibition of PlGF:VEGF AUC was determined for each experiment. AUC baseline was set to the normalized cell index of 1. The mean and SEM are plotted for each of the inhibitors from n=3 experiments.

Because differences in T_{max} were previously observed to be correlated to VEGF concentration whether there was a correlation of T_{max} to inhibitor concentration was also determined. Linear fit of the T_{max} versus inhibitor concentration produced significantly non-zero slopes for all three inhibitors (Figure 14). VEGF-Trap and anti-VEGF antibody produced steeper slopes at -5.0 h/pM and -5.1 h/pM respectively than the anti-PlGF antibody which produced a slope of -1.4 h/pM. These data are consistent with proliferation studies that show that alteration in PlGF-2 concentration has less of an impact on T_{max} than changes in VEGF concentration.

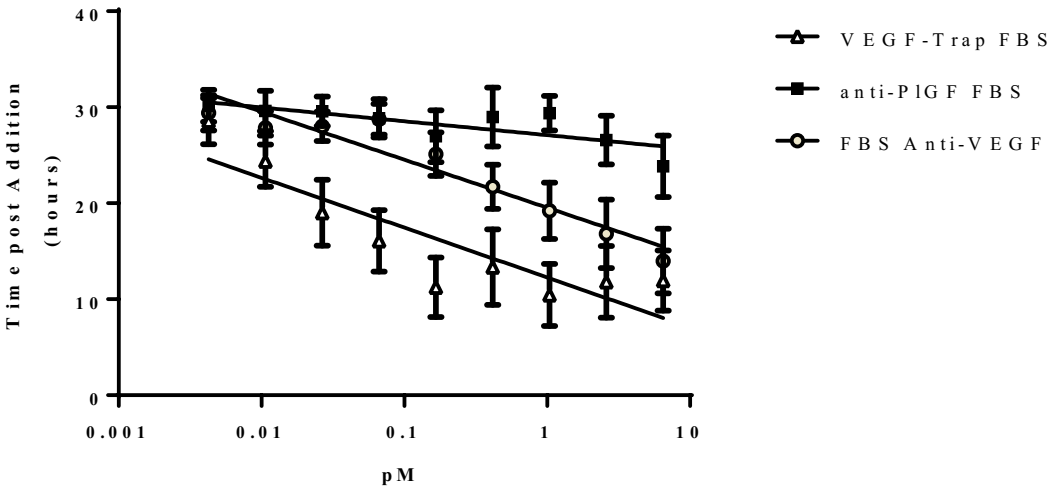

Figure 14: T_{max} of PlGF:VEGF Inhibition by Concentration
The T_{max} for each inhibitor concentration was determined in PlGF:VEGF-stimulated HUVECs in the absence of exogenous heparin. The T_{max} was plotted versus the log concentration and fitted with a y-intercept.

Heparin Effects on Inhibitor Efficacy Measured by IC_{50}

As both PlGF-2 and $VEGF_{165}$ bind heparin, the addition of exogenous heparin bound to each growth factor could alter inhibitor binding and therefore, neutralization of growth factor activity. The addition of 33 µg/ml heparin did not produce significant differences in IC_{50} values for either anti-VEGF or anti-PlGF antibodies compared to FBS control (Figure 15). VEGF-Trap's IC_{50} increased more than 10-fold when 33 µg/ml heparin was added to PlGF-2:$VEGF_{165}$ combination. VEGF-Trap efficacy may be sensitive to high concentrations of soluble heparin which can interact with the inhibitor in the absence of ligand through its VEGFR-1 domain.

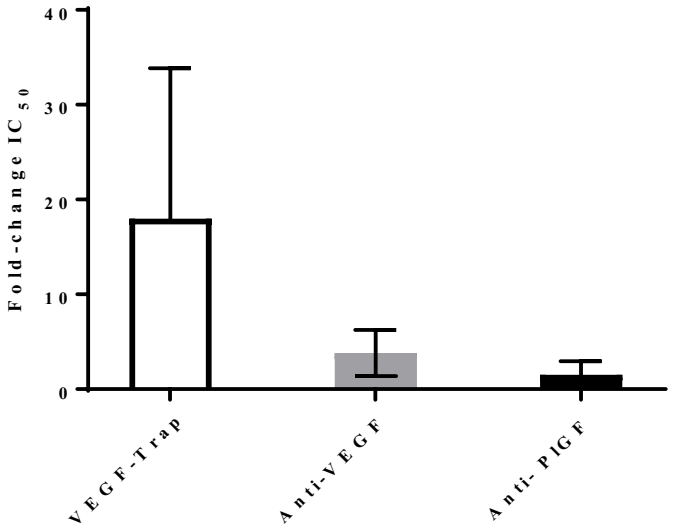

Figure 15: Changes in Inhibitor IC_{50} values in the Presence of 33 µg/ml Heparin
HUVECs were stimulated with PlGF-2:$VEGF_{165}$ with and without the addition of 33 µg/ml heparin. Inhibitors were titrated from 6.5 pM to 11 fM and the IC50 calculated from a 4-parameter curve fit. The fold-change in IC_{50} was calculated relative to the no heparin FBS control for each of three experiments. The mean fold-change with SEM is plotted for each inhibitor in the presence of 33 µg/ml heparin.

Heparin Effects on Time-dependent Inhibitor Efficacy

Heparin induced alterations in the PlGF-2:$VEGF_{165}$- induced proliferation of HUVECs. TCRP profiles for the 6.5 pM inhibitor in the presence of increasing concentrations of exogenous heparin are shown below (Figure 16). As shown in early proliferation data heparin alters the CI_{max} of PlGF-2:$VEGF_{165}$-induced proliferation. A trend was observed that the AUC also increased for PlGF-2:$VEGF_{165}$ control with the addition of soluble heparin compared to FBS control; however, these differences were not statistically significant.

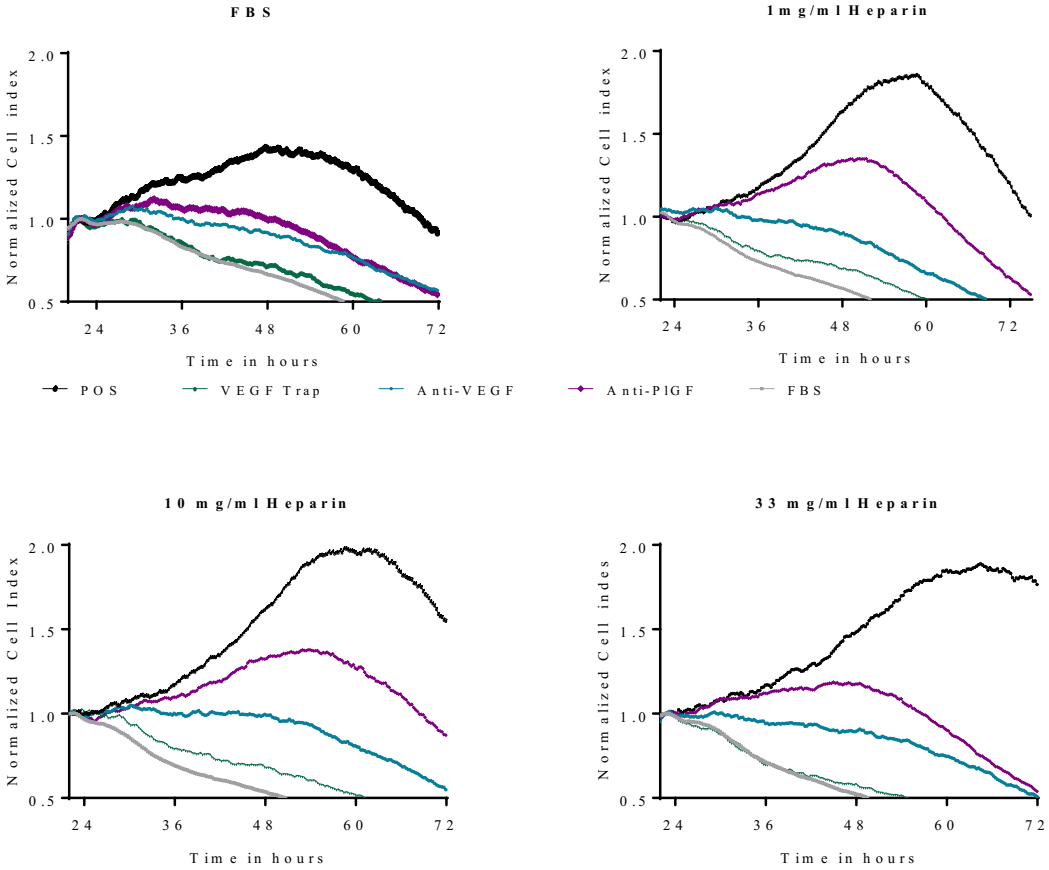

Figure 16: TCRP of anti-VEGF and anti-PlGF Inhibitors in HUVECs
Representative TCRP profiles are shown from a single experiment. Inhibition profiles for VEGF-Trap (green), anti-VEGF (teal) and anti-PlGF (purple) are shown at 6.5 pM concentration. TCRPs for cells grown without stimulation are shown in grey. TCRPs for uninhibited responses (PlGF-2:$VEGF_{165}$) are shown in black.

Heparin alters VEGF-Trap Maximum Percent Inhibition and T_{max} at Low VEGF-Trap Concentrations. Percent inhibition by AUC was not significantly altered by the presence of exogenous heparin for anti-PlGF and anti-VEGF antibodies (Figure 17).

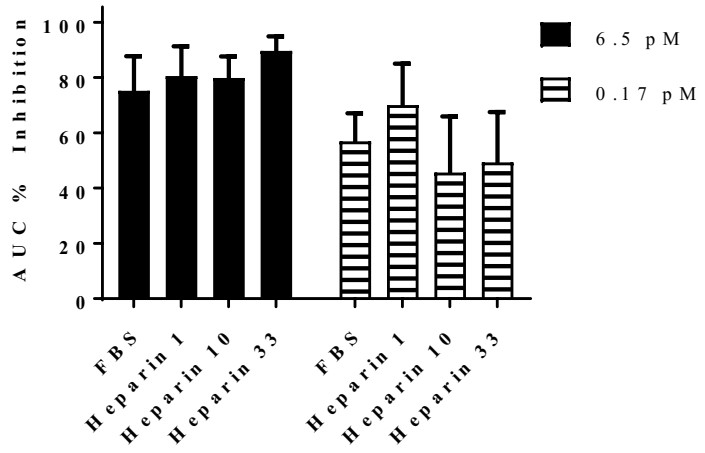

Figure 17: Percent Inhibition of PlGF:VEGF Induced Proliferation by Single Target Inhibitors
HUVECs were stimulated with a combination of PlGF:VEGF. The percent AUC inhibition by anti-VEGF or anti-PlGF was determined from three experiments. Anti-VEGF and anti-PlGF antibodies were tested in the presence of three different heparin concentrations: 1 µg/ml, 10 µg/ml and 33 µg/ml. a) Percent inhibition of anti-VEGF at 6.5 pm and 0.17pM is shown. b) Percent inhibition of anti-PlGF antibody is shown at 6.5pM and 1.0pM. The mean and SEM are plotted for each of the inhibitors from n=3 experiments.

However, heparin altered the percent inhibition of VEGF-Trap at lower concentrations (Figure 18). At 0.027 pM of VEGF-Trap in the absence of heparin 84% of inhibition was observed. The percent inhibition observed in the presence of 1 µg/ml heparin was reduced to 45%. In the presence of 10 µg/ml and 33 µg/ml heparin a statistically significant reduction in effectiveness was observed. At 10 µg/ml only 37% inhibition was observed ($p=0.02$) and at 33 µg/ml only 23% inhibition was observed ($p = 0.003$). VEGF-Trap is a composed of both VEGFR-1 and VEGFR-2 binding domains. VEGFR-1 has been shown to bind to heparin which may explain the influence of soluble heparin on inhibitor binding (Teran & Nugent, 2015).

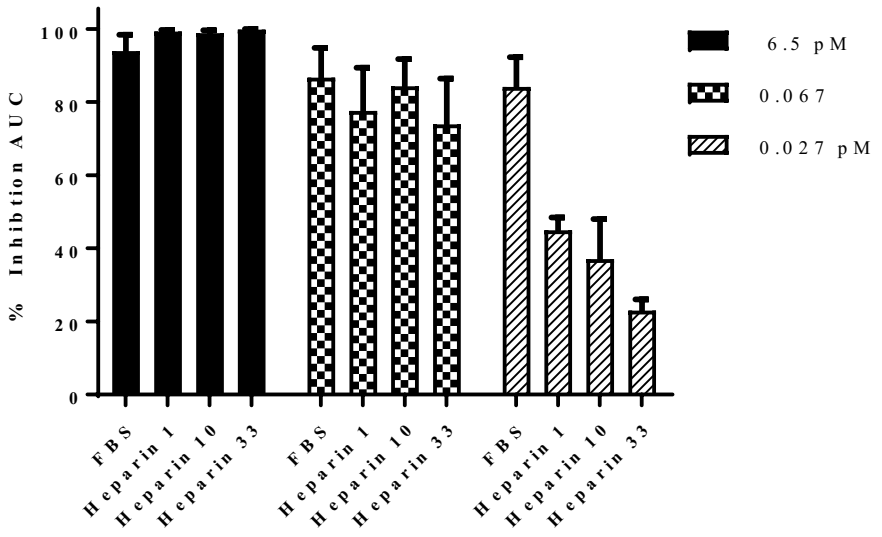

Figure 18: Percent AUC Inhibition of VEGF-Trap in Presence of Heparin
HUVECs were stimulated with a combination of PlGF:VEGF. The mean percent AUC inhibition by VEGF-Trap was determined from three experiment. VEGF-Trap was tested in the presence of three different heparin concentrations: 1 µg/ml, 10 µg/ml and 33 µg/ml. Percent inhibition by VEGF-Trap at 6.5 pm, 0.067 pM and 0.027pM is shown. The mean and SEM are plotted.

Similar trends for reduction of VEGF-Trap activity were observed in HUVECs stimulated with non-heparin-binding isoforms (Figure 19). In PlGF:$VEGF_{121}$ stimulated cells, an increase in required IC_{50} concentration and a trend for reduction in maximum percent inhibition were observed for VEGF-Trap in the presence 33 µg/ml soluble heparin. Further suggesting binding of heparin may reduce VEGF-Trap efficacy. The addition of 33 µg/ml heparin with PlGF:$VEGF_{121}$ did not alter the inhibition activities of either anti-PlGF or anti-VEGF.

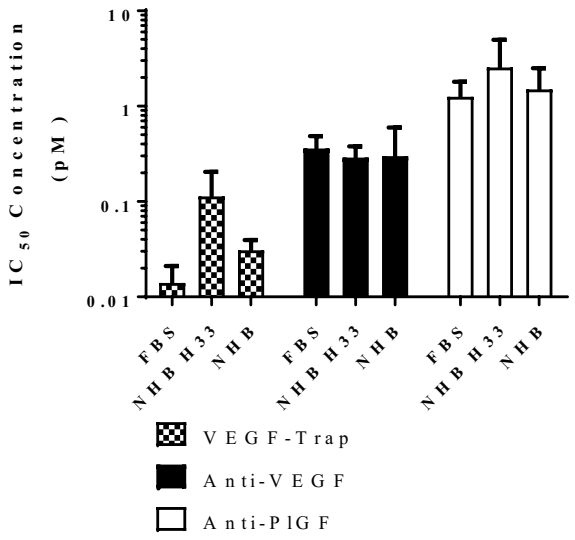

Figure 19: Efficacy of Inhibitors for Non-Heparin-binding isoforms
HUVEC cells were stimulated non-heparin-binding isoforms PlGF-1:$VEGF_{121}$ without heparin (NHB) or PlGF-1:$VEGF_{121}$ in the presence of 33µg/ml heparin (NHB H33). VEGF-Trap, anti-VEGF and anti-PlGF were tested for the ability to inhibit the combination of non-heparin-binding isoforms with or without heparin. IC_{50} results for each inhibitor were compared to the heparin-binding PlGF-2:$VEGF_{165}$ stimulated cells without heparin (FBS). The maximum percent inhibition for VEGF-Trap tested at 6.5 pM is shown for NHB and NHB H33 stimulated HUVECs compared to heparin binding FBS control.

The presence of 33 µg/ml heparin induced changes in VEGF-Trap inhibition that were not observed when anti-VEGF or anti-PlGF were used as inhibitors (Figure 20). Anti-VEGF and anti-PlGF treatments produce lines with similar slopes for T_{max} whether in the presence of FBS or 33 µg/ml heparin. For VEGF-Trap a significant difference in T_{max} slopes is observed in the presence of 33 µg/ml compared to FBS control ($p = 0.0057$).

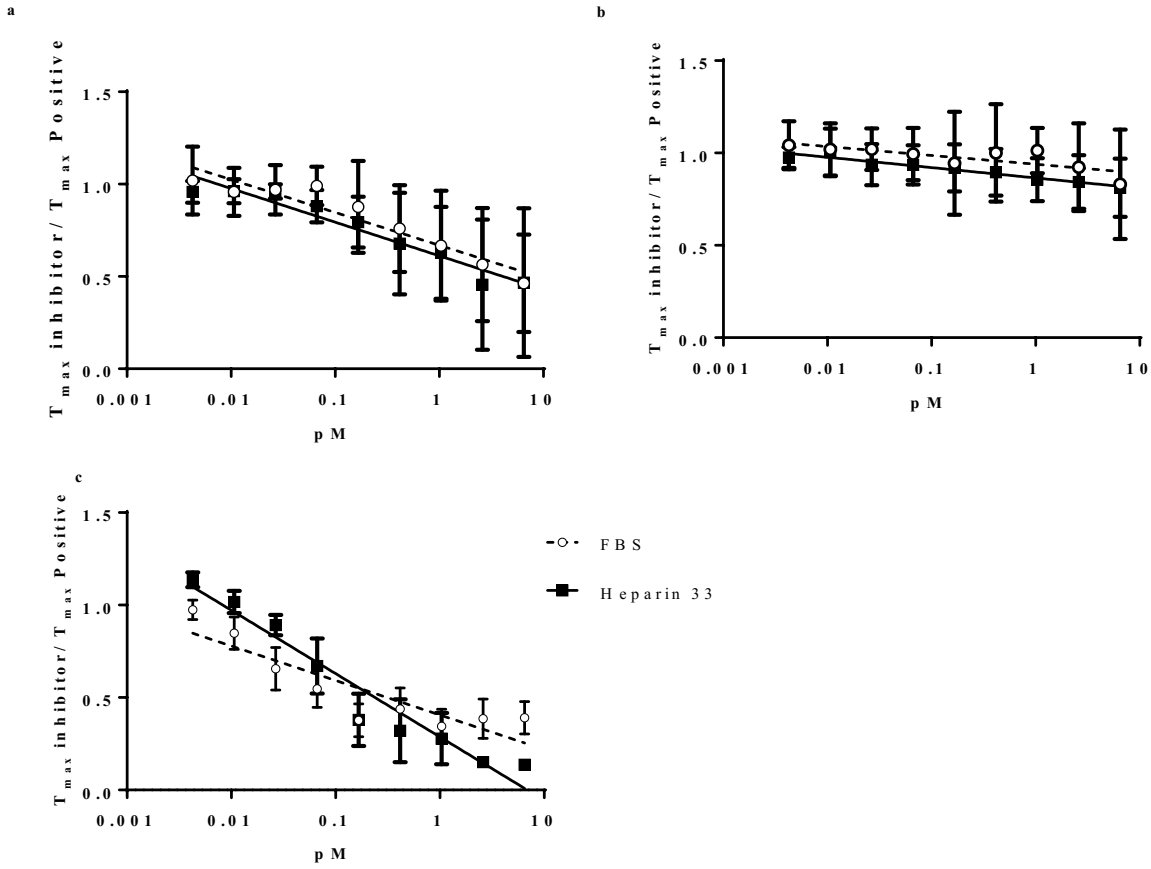

Figure 20: Heparin Effects on Inhibitor T_{max}
HUVECs were stimulated with PlGF-2:$VEGF_{165}$ in the absence of heparin or in the presence of 33 µg/ml heparin. a) Anti-VEGF, b) Anti-PlGF, and c) VEGF-Trap inhibitors were titrated. The inhibitor T_{max} was determined for each concentration and divided by the Tmax of the PlGF:$VEGF_{165}$ control.

BrdU Correlation to Impedance Measurement

Proliferation measurements that are based on nucleoside-analog incorporation during DNA replication like BrdU assays are considered as the standard for proliferation measurement. Once incorporated into DNA BrdU is detected independent of subsequent changes in cell health. As shown (Figure 16) in the TCRP for proliferation section, cell indices can and do decline over time. The peak in CI is expected to represent the

maximum cell number achieved. Correlation of CI_{max} to BrdU incorporation was examined in the same experiment and in the same plate.

Decreasing seeding densities of HUVEC were used to compare impedance and BrdU incorporation measurements. Both impedance and BrdU readouts correlated well to initial seeded cell number. Pearson correlation coefficient for impedance and BrdU measurements relative to cell number were 0.9822 and 0.9914 respectively (Table 5).

Table 5: Proliferation Readout Cell Number Correlation

	Pearson Correlation Coefficient	P-value
BrdU Incorporation	0.9914	0.0001
Maximum Cell Index	0.9822	0.0005

The maximum cell index observed for HUVECs aligned well with BrdU ELISA results to measure proliferation (Figure 21). BrdU ELISA was run using manufacturing recommended conditions. This correlation was observed until high cell densities were used where culture conditions were not favorable to sustain cell growth. This correlative evidence was true in multiple analyses of VEGF and PlGF induced proliferation with various concentrations of heparin.

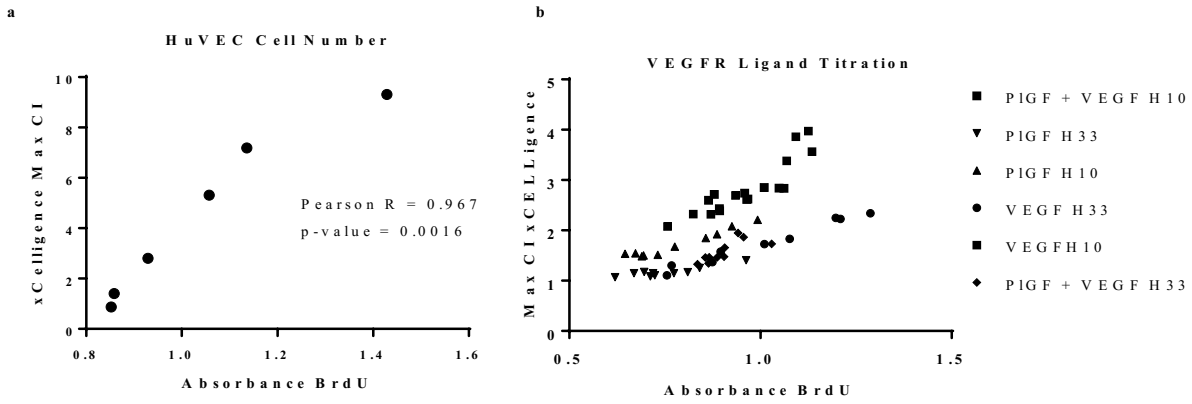

Figure 21: Correlation of RTCA and BrdU Cell Measurements
a) HUVECs were seeded at cell concentrations ranging from 2667 cells/well to 20,250 cells/well then stimulated with VEGF at 4 ng/ml to induce proliferation b) VEGF, PlGF and the combination of VEGF: PlGF were titrated on HUVEC cells and the RTCA and BrdU was measured from the same plate.

RTCA measurements by the xCELLigence were significantly correlated in both the cell number titration and VEGFR ligand titration experiments as indicated by Pearson correlation coefficients and p-values (Table 6).

Table 6: Correlation of Proliferation Readouts for VEGFR Ligand Stimulation in HUVECs

Treatment	Pearson Correlation Coefficient	P-value
VEGF+ 10µg/ml Heparin	0.9681	<0.0001
VEGF + 33 µg/ml Heparin	0.9876	<0.0001
PlGF+ 10µg/ml Heparin	0.9734	<0.0001
PlGF + 33 µg/ml Heparin	0.8975	0.0004
VEGF + PlGF + 10µg/ml Heparin	0.7496	0.0126
VEGF + PlGF + 33µg/ml Heparin	0.7709	0.0090

For measurement of growth factor induced proliferation for some conditions where highest levels of proliferation were observed the BrdU reagent concentration was insufficient resulting in plateaus in measurements that were not present in impedance measurements. Cell index measurements begin to decline over time due to deterioration in cell health and/or cell detachment. The use of CI measurements at a given time point may be misleading especially if the time of maximum observed activity varies significantly as was observed with $VEGF_{165}$.

Chapter IV

DISCUSSION

RTCA verified previous finding when CI_{max} and effective concentrations were examined. New characteristics of VEGF and PlGF were identified using time-dependent measurement. These new time-dependent characteristics are discussed with respect to how they might reveal mechanistic differences in proliferation responses.

Role of Heparin in Altering Proliferation

The use of a real-time cell analyzer (RTCA) was expected to elucidate if heparin binding properties of $VEGF_{165}$ and PlGF-2 altered the timing VEGF-induced proliferation in the presence of exogenous heparin. RTCA enables the assessment of multiple attributes of the proliferation response including: EC_{50}, changes in maximum cell number (CI_{max}), time of peak response (T_{max}), and area under the curve (AUC) calculations which simultaneously evaluates duration and magnitude of response. EC_{50} and cell number changes can be evaluated by traditional endpoint readouts at a given time; however, T_{max} and AUC responses would be difficult to characterize using endpoint proliferation readouts.

Assessment of EC_{50} and CI_{max} using real-time analysis impedance measurements confirmed previous findings. Comparison of RTCA-measured EC_{50} values for $VEGF_{165}$

and PlGF-2 confirmed previous reports that PlGF-2 is a significantly weaker mitogen than $VEGFA_{165}$ (Park, et al., 1994). As expected, synergistic enhancement of cell proliferation was observed by both the addition of PlGF-2 and heparin to $VEGF_{165}$ (Ashikari-Hada, et al., 2005; Park, et al., 1994). Both PlGF-2 and $VEGF_{165}$ had reduced EC_{50} values in the presence of low concentrations of heparin; however, this effect was less significant than the increase in observed cell number. In this study, the EC_{50} of PlGF-2 was reduced in the combination with $VEGF_{165}$ and cell numbers increased compared to $VEGF_{165}$ only stimulated cells.

The use of RTCA allowed further study of time-dependent effects. Although it has been shown that heparin alters VEGF diffusibility (Robinson & Stringer, 2001; Ruiz de Almodovar, Lambrechts, Mazzone, & Carmeliet, 2009), soluble heparin did not induce significant time-dependent changes in VEGF or PlGF-2:$VEGF_{165}$-induced HUVEC proliferation. The absence of time-dependent changes in the presence of soluble heparin, indicates that heparin's main effect is not to sequester or control release of heparin binding growth factors PlGF-2 and $VEGF_{165}$.

Instead this data suggests that soluble heparin is enhancing signaling that leads to proliferation. Heparin has been shown *in vitro* to stabilize the binding of $VEGF_{165}$ to VEGFR-2 (Teran & Nugent, 2015). VEGFA binds VEGFR-1 with a higher affinity than VEGFR-2, but its signaling through VEGFR-2 is essential for endothelial cell proliferation while little evidence exists for a significant role for VEGFR-1 signaling (Gille, et al., 2001; Koch, Tugues, Li, Gualandi, & Claesson-Welsh, 2011). The increase in proliferation as measured by increased CI at lower heparin concentrations would be consistent with the stabilization of $VEGF_{165}$ binding to VEGFR-2.

Concentration and PlGF-Induced Effects on T_{max}

VEGF T_{max} was found to be concentration dependent. The use of real-time cell analysis enabled detailed analysis of timing differences for $VEGF_{165}$-induced proliferation responses. The differences in T_{max} observed in this study support models that suggest more intricate dynamics of signaling for VEGF-induced HUVEC proliferation. Both heparin-binding and nonbinding isoforms of VEGF displayed a significant increase in T_{max} with increasing VEGF concentration. These concentration-dependent T_{max} effects on VEGF-induced proliferation and the shift in time-dependent effects were unanticipated. However, this data supports recent models of VEGFR-2 signaling that expand beyond cell surface receptor/ ligand interactions. Recent evidence suggests that the duration of VEGF signaling is in part regulated by the rate of movement through the endosomal pathway during which signaling is still possible (Lampugnani, Orsenigo, Gagliani, Tacchetti, & Dejana, 2006; Nakayama et al., 2013; Simons, Gordon, & Claesson-Welsh, 2016). VEGF binding at high concentration to VEGFR-2 stimulates receptor recycling to the surface (Simons, 2012). The delayed timing of peak proliferation is consistent with receptor recycling resulting in prolonged signaling in the presence of high concentrations of VEGF.

Heparin may also function to enhance intracellular signaling by promoting endosome formation and directing VEGFR2 trafficking preferentially through functional endosomal compartments (Zhang, Lanahan, & Simons, 2013). The T_{max} of non-heparin binding (NHB) VEGF isoform, $VEGF_{121}$, also increases with increased concentration suggesting that heparin is not required for prolonging T_{max}. However, $VEGF_{121}$ and

$VEGF_{165}$ differ in their slopes of T_{max} proliferation and responses in the presence of heparin. Largest differences are observed the high concentrations. Therefore, heparin may still have a role in time-dependent characteristics of VEGF signaling. These differences in the isoforms may be due to the absence of stabilization of binding to the receptor rather than availability of free VEGF. It may also be that heparin's interaction with other cell surface receptors increases the efficiency of endosome formation for $VEGF_{165}$, but not $VEGF_{121}$.

PlGF-2 T_{max}

These studies suggest that PlGF-2's effects on proliferation operate through different mechanisms than $VEGF_{165}$. The T_{max} observed for increasing concentration of PlGF-2 produced a narrower range than that for VEGF. This suggests that PlGF-2 signaling is driven more by ligand interaction with cell surface receptors than VEGF signaling. Even though PlGF has been shown to be mitogenic the phosphorylation of its receptor VEGFR1 is only weakly observed in in endothelial cells in response to PlGF-2 alone and in the presence of both PlGF-2:$VEGF_{165}$ (Cao, 2009; Cudmore et al., 2012; Gille, et al., 2001). In this study PlGF-2 was observed to have weak mitogenic effect which is consistent with weak VEGFR-1 activation. Considering that HUVECs express low levels of PlGF-2's signaling receptor VEGFR-1 on their surface, the required concentration to observe the effect was surprisingly high. PlGF-2 has also been found to be bound with NRP-1 on HUVECs; however, PlGF-2 binding to NRP-1 does not result in proliferation (Migdal, et al., 1998). Migdal et al. reported that addition of heparin reduced binding to NRP-1. In this study, the addition of heparin reduced the amount of PlGF-2 required to induce proliferation which would be consistent with the increase of available

PlGF-2 for binding to VEGFR-1. Binding of VEGFR-1 could increase proliferation either by direct phosphorylation of VEGFR-1 or by blocking binding to VEGFR-1 which would then increase available VEGF for activation of VEGFR-2.

Synergistic Effects of VEGF and PlGF

The synergistic proliferation observed by combining the growth factors also produced a shift in T_{max}. As T_{max} increases with $VEGF_{165}$ concentration, PlGF-2 extension of $T_{max\ for}$ PlGF-2:$VEGF_{165}$-induced proliferation may indicate an increase in available $VEGF_{165}$ concentration produced by blocking of VEGFA binding to VEGFR-1.

The effective concentration of PlGF-2 was lowered in combination with $VEGF_{165}$. If PlGF-2 is operating mainly through blocking VEGF binding to VEGFR-1 then the reduced concentration required for PlGF-2 could indicate some competition for non-signaling receptors by VEGF. For example if VEGF is competitively binding to NRP-1 and increasing PlGF-2 binding to VEGFR-1. This observed reduction in effective PlGF-2 concentration may also be related to changes in VEGFR-1 localization in the presence of VEGF. The majority of VEGFR-1 in unstimulated HUVECs has been found to be expressed intracellularly associated with the Golgi (Mittar et al., 2009). In response to calcium signaling which can be induced by VEGF, VEGFR-1 has been reported to translocate to the plasma membrane. Because VEGF has a higher affinity for VEGFR-1, VEGF in the absence of PlGF would be expected to be bound preferentially to VEGFR-1. Again competition of PlGF-2 with VEGF for binding to VEGFR-1 could increase

available VEGF concentration for binding to VEGFR-2 which would produce a longer T_{max}.

This does not rule out the possibility that endocytosis or calcium signaling are increasing PlGF-2 VEGFR-1 signaling as well. If PlGF-2 intracellular localization is required for PlGF-induced VEGFR-1 phosphorylation in HUVECs then high levels of PlGF-2 binding to other plasma membrane proteins such as its non-signaling receptor NRP-1 or co-internalization with $VEGF_{165}$ may be required for induction of endocytosis prior to initiation of PlGF signaling (Gampel et al., 2006). PlGF-2 has not been found to induce calcium-signaling in HUVECs at similar concentrations tested in this study (Cunningham, Tran, Arrate, Bjercke, & Brock, 1999). In combination with VEGF which can induce calcium signaling the necessary translocation of VEGFR-1 to the cell surface might also lower the necessary amount of PlGF-required to induce proliferation.

Real-time Cell Analysis of Inhibitors

Real-time cell analysis enabled the study of VEGF-Trap, VEGF and PlGF inhibitors over an extended period of time. Differences in the inhibition profiles could be observed relevant to the known specificity of the inhibitors. The VEGFR-1-VEGFR2 chimeric fusion, VEGF-Trap, was the most effective at inhibiting PlGF-VEGF-induced proliferation in HUVECs as evidenced by a lower IC_{50} and higher percent achievable maximum inhibition. AUC analysis which incorporates both duration and size of effect demonstrates that VEGF-Trap is capable of inhibiting 95% of all VEGF: PlGF-induced proliferative effects. Using AUC to study inhibition profiles can elucidate whether other

factors are inducing proliferation. Targeting only VEGF resulted in 75% inhibition of PlGF-2:$VEGF_{165}$ proliferative activity and PlGF only 45%. Treatment with growth factors can trigger multiple pathways and trigger up regulation of other growth factors. This compensation has been suggested to be one of the mechanisms for anti-VEGF drug tolerance (Forooghian, et al., 2009). PlGF levels have been found to be correlated to VEGF levels in eye disease (Chen, et al., 2015). Based on percent AUC inhibition data, it did not appear that PlGF-2:$VEGF_{165}$-stimulation induced significant up regulation of non-VEGF family mitogens. This study was performed in low serum (1% FBS) and it would be expected that at higher serum concentrations other growth factors would be present that might compensate for reduced VEGF and PlGF activity. Uninhibited serum growth factors would be expected to result in lower maximum percent inhibition. This type of information would be helpful for predicting inhibitor efficacy and the risk of compensation by other growth factors that could lead to increased tolerance. With AUC analysis it is not necessary to identify each player involved in the process to determine the relative efficacy and completeness of inhibition.

Because VEGF concentration was found to shift T_{max} much more dramatically than PlGF, the T_{max} slope of anti-VEGFA and anti-PlGF antibodies was also measured. Inhibitors targeting the different growth factors reflected the T_{max} effects of reducing $VEGF_{165}$ and PlGF-2 concentrations. The T_{max} slope of inhibitors targeting VEGF were much steeper than the slope of inhibitors targeting only PlGF-2. When screening inhibitors in the absence of known specificity this type of time-dependent information would be helpful for identifying inhibitors with distinct target profiles and

pharmacological effects. Analysis of the TCRP showed reduced efficacy when only a single ligand was targeted versus multiple ligands.

VEGFR-1/VEGFR-2 Chimeric fusion proteins have been shown to bind heparin (MacDonald, et al., 2016). This was supported by *in vitro* studies. Of the tested inhibitors VEGF-Trap was the only inhibitor in which alterations of efficacy were observed in the presence of soluble heparin. Unlike the antibodies tested, the VEGF-Trap has the potential to bind a larger range of molecules. Molecules capable of binding to VEGFR-1 and VEGFR-2 may also bind VEGF-Trap. In some cases this may be advantageous. As in when the binding results in inhibition of ligands involved in angiogenic processes, but this study demonstrates that there may also be reduced efficacy. Recently, several non-ligand molecules including galectins 1 and 3, gremlin-1 and lipoproteins have been identified as molecules that can bind to the VEGF receptors resulting in signaling (Simons, et al., 2016). VEGF-Trap may block the signaling of multiple VEGFR-1 and VEGFR-2 binding proteins; however, if insufficient inhibitor is present binding to less active signaling molecules could result in reduced efficacy. Evidence from this study suggests that this is the case in the presence of excess soluble heparin. Overall VEGF-Trap might be expected to have a higher potential for off-target effects resulting from treatment. This increased binding to less active molecules might result in unintended consequences for processes not involved in pathological angiogenesis.

Correlation of Impedance Proliferation Measurements to BrdU-Incorporation

Use of real-time cell impedance measurements for evaluation of proliferation provides an accurate real-time methodology for evaluating differential mitogenic responses. RTCA measurement correlated well with traditional BrdU-incorporation measurements of proliferation. RTCA provided advantages over BrdU as there is no need for pre-determining timing or magnitude of proliferation. Both methods are capable of measuring asynchronous proliferation, but only real-time analysis can reveal differences in the peak timing for proliferation. Impedance measurement requires a specialized plate and instrumentation, but it can also be combined with traditional endpoint colorimetric, fluorometric or compare methods and data correlated to proliferation times.

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