INTRODUCTION

Cancer is one of the leading causes of disease-associated death in the United States (Woloshin et al., 2008). Lung cancer is one of the most common forms of cancer (Ilhan et al., 2004), accounting for 29% of all cancer deaths and is the second most commonly diagnosed cancer in adults in the United States (Yano et al., 2006; www.lungcancer.org). These statistics reflect primary lung cancers, that is, those which originate in the lung. There are different statistics related to metastatic lung cancer, which is one of the most prevalent locations, along with lymph nodes, for cancer metastasis (www.lungcancer.org). The prognosis for lung cancer patients is usually poor, with many not diagnosed until an advanced stage and less than 10 percent actually being cured (Boring et al., 1994; Yano et al., 2006).

In recent years, research has turned to finding new ways of treating cancer. Research has focused on targeting the specific mechanisms that encourage the growth and progression of cancer. One of the most notable processes is angiogenesis, which is considered a hallmark of cancer development (Hanahan and Weinberg, 2000). Tumor growth is well known to be angiogenesis dependent (Folkman, 1971; Fontanini et al., 1997; Carmeliet and Jain, 2000; Tanaka et al., 2001). Solid tumors cannot grow beyond 1-2 mm^3 (Folkman et al., 1966, 1987) before requiring additional blood supply to provide adequate nutrients and oxygen (Hanahan and Folkman, 1996). Angiogenesis is the formation of new blood vessels from pre-existing blood vessels (Zhang et al., 2003). It involves several steps resulting from the interaction of numerous growth factors and cells. It plays an essential role in normal physiologic processes, such as embryogenesis (Carmeliet et al., 1996), wound healing, cyclic endometrial hyperplasia, and pregnancy (Ferrara et al., 2003; Nguyen and D'Amore, 2001). Moreover, it has been well established as an important process in pathologic conditions, such as solid tumor growth (Fontanini et al., 1997; Carmeliet and Jain, 2000; Tanaka et al., 2001), diabetic ocular neovascularization (Spranger and Pfeiffer, 2001), rheumatoid arthritis (Shibuya, 2006) and pre-eclampsia (Ferrara et al 2003). Additionally, it is important in tumor metastasis (Roberts et al., 2006; Kearney et al., 2004).

Angiogenesis is regulated by vascular endothelial growth factor (VEGF), and its actions are mediated through several tyrosine kinase cell-surface receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR) being the most important (Cao et al., 1998; Ferrara et al., 1999; 2003). These receptors are found primarily on endothelial cells of the blood

vascular system (Barleon et al., 1994); however, they are also expressed on white blood cells (Barleon et al., 1996), platelets (Verheul et al., 1997) and hematopoietic precursor cells (Sunderkotter et al., 1991). Through the binding of VEGF to its receptors on endothelial cells, signaling pathways are activated that stimulate endothelial cell proliferation and migration (Ferrara et al., 1999; 2003). There is an alternatively processed, soluble form of Flt-1, denoted sFlt-1, which acts as an inhibitor of VEGF (Kendall and Thomas, 1993; Kendall et al., 1996; He et al., 1999). Variation in the expression of sFlt-1 compared to Flt-1 has been shown in both physiologic and pathologic conditions (He et al., 1999). However, the mechanism that drives the production of one form over the other remains unclear (Huckle and Roche, 2004).

Numerous studies have shown an overexpression of VEGF and VEGF receptors in various human malignancies, particularly solid tumors (Boocock et al., 1995; Zhang et al., 2003; Dvorak et al., 1995; Harper et al., 1996; Takanami et al., 1997; de Jong et al., 1998; Ferrara et al., 2003). Often the levels of expression, detected in tumor tissue or circulating blood, are associated with metastasis and poor patient prognosis (Takanami et al., 1997; Ferrajoli et al., 2001; Donnem et al., 2007). The ability to detect angiogenic factors has become a pivotal point for studies focused on ways of inhibiting tumor angiogenesis (Folkman, 1985). A few compounds directed at VEGF production and receptor binding have already been developed and are being used in clinical trials (Kendall et al., 1993; Ferrara et al., 2003; Johnson et al., 2004; Herbst et al., 2005). Specifically Avastin, an anti-VEGF monoclonal antibody, is in clinical use for therapy of colorectal cancer (Herbst et al., 2005).Furthermore, the production of a “natural” angiogenic inhibitor, sFlt-1, can be exploited for the benefit of treating and slowing cancer progression (Aliello et al., 1995; Kong et al., 1998; Shiose et al., 2000).

The development of various primarily mouse models of angiogenesis and cancer has allowed investigation into the diverse pathways that mediate angiogenesis (Wang et al., 1995; Yan et al., 2000; Shibuya, 2006) and individually provide targets for antineoplastic therapeutic interventions.

My hypothesis, based on this information, is that there will be variation in the ratio of sFlt-1 to Flt-1 in the presence of solid tumors. I predict based on the results of other studies, that the sFlt-1:Flt-1 ratio will decrease in solid tumors.

The experimental aims that will allow me to test my hypothesis are: 1) To implement a tumor metastasis model that produces vascularized nodules in the mouse

lung and 2) to use the model to evaluate sFlt-1:Flt-1 mRNA expression in tumor-bearing versus control lungs.

LITERATURE REVIEW

Angiogenesis

Angiogenesis is the formation of new blood vessels, beginning as small endothelial buds (McGavin and Zachary, 2007). Intearctions between endotheial cells and growth factors regulate the process, which has been dissiolved into a series of five steps. They include: 1) proteolysis of the extracellular matrix and basement membrane of pre-existing vessels, 2) migration of immature endotheial cells, 3) proliferation of endothelial cells, 4) formation of the capillary lumen and maturation of endothelial cells, and 5) increased permeability by movement through gap junctions (McGavin and Zachary, 2007). The control of angiogenesis occurs through a balance of pro- and anti-angiogenic factors. Angiogenesis, when well regulated, contributes to normal tissue growth and development. However, unregulated angiogenesis leads to the formation of abnormal ineffective vessels that contribute to pathologic conditions such as tumor growth and metastasis (Roberts et al., 2004; Kearney et al., 2004), diabetic retinopathy (Spranger and Pfeiffer, 2001) and ischemic conditions (Carmeliet, 2003).

Angiogenesis is regulated by vascular endothelial growth factor (VEGF) and its actions are mediated through various cell surface receptors (Ferrara et al., 2003). The primary target for VEGF is endothelial cells, which are dependent on VEGF for normal development (Ferrara et al., 2003) and survival, both *in vivo* and *in vitro (*Gerber et al., 1998). In addition to inducing endothelial cell growth, VEGF also promotes vascular leakage, hence the synonym Vascular Permeability Factor, which has shown to be important in inflammation and solid tumor growth (Ferrara et al., 2003, Thomas 1996). Increased endothelial permeability is a result of either increased intravascular pressure and/or endothelial cell fenestrations (Ferrara et al., 2003).

Vascular Endothelial Growth Factor (VEGF)

VEGF belongs to a family of secreted homodimeric glycoproteins, including $VEGF_A$, $VEGF_B$, $VEGF_C$, $VEGF_D$, $VEGF_E$ and PlGF (placental growth factor) (Ferrara et al., 1997, 1999). They regulate not only blood vessel growth but hematopoiesis and lymphatic vessel development (Shalaby et al., 1995; Ferrara et al., 1997). The members of the VEGF family can be divided into three groups based on their receptor binding and angiogenic potential: 1) $VEGF_A$; 2) PlGF and $VEGF_B$; and 3) $VEGF_C$ and $VEGF_D$ (Eriksson et al., 2002).

$VEGF_A$. $VEGF_A$ is the key mediator of both physiologic and pathologic angiogenesis, specifically blood vessel vasculogenesis (Ferrara, 2004; Shibuya et al., 2006). VEGF is detected in several adult tissues including lung, kidney, liver and brain in varying concentrations (Thomas, 1999). VEGF expression must be well-regulated for proper vessel formation (Ruhrberg et al., 2002; Gerhardt et al., 2003; Roberts et al., 2004). $VEGF_A$ has several isoforms, which arise as a result of alternative mRNA processing (Houck et al., 1991; Tischer et al., 1991). These forms vary in their ability to bind various molecules and receptors, causing differing mitogenic activity (Ferrara et al., 2003). $VEGF_A$ mediates its actions by binding to and activating two tyrosine kinase receptors, found primarily on vascular endothelial cells, designated VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) (Ferrara et al., 1999; 2003) (Figure 1).

PlGF and $VEGF_B$. PlGF expression is primarily limited to the placenta in adults (Xu and Jain, 2007), but has been detected in lower levels in the heart, brain, lung, and skeletal muscle (Thomas, 1999). PlGF has three alternatively spliced isoforms, PlGF-1, PlGF-2 and PlGF-3 (Cao et al., 1997; Maglione et al., 1991; 1993; Eriksson et al., 2002). PlGF-1 is an alternatively spliced form that does not induce angiogenesis. It acts as a natural antagonist of VEGF when co-expressed in the same cell (Eriksson et al., 2002). PlGF forms heterodimers with VEGF, depleting VEGF homodimers and diminishing angiogenic signaling (Cao et al., 1996a; Eriksson et al., 2002). It binds to and mediates its actions only through Flt-1 (Xu et al., 2006). PlGF-2 does induce angiogenesis and like VEGF can bind Flt-1 and neuropilin-1 on endothelial cells (Midgal et al., 1998; Soker et al., 1998). PlGF-3 binds to Flt-1 (Maglione et al., 1993; Cao et al., 1997). The molecular mechanism and regulation of PlGF are not completely understood, and there are varying opinions on its role in pathologic angiogenesis (Xu and Jain, 2007). The differing function of PlGF from other members of the VEGF family is similar to the differing actions of the Bcl-2 family (Xu et al., 2007).

$VEGF_B$ binds only Flt-1, and its biological function is unknown (Eriksson et al., 2002). However, it has been shown to form heterodimers with $VEGF_A$ (DiSalvo et al., 1995; Cao et al., 1996a; Olofsson et al., 1996) when produced in the same cell.

$VEGF_C$ and $VEGF_D$. $VEGF_C$ and $VEGF_D$ are involved in proliferation, migration and survival of the lymphatic endothelial cells both during embryonic development and tumor progression (Cao et al., 1998), and play a minor role in blood vessel angiogenesis (Marconcini et al., 1999; Skobe et al., 2001; Staker et al., 2001; Plate et al., 2001; Donnem et al., 2007). Their actions are mediated through both KDR and VEGFR-3 (Cao

et al., 1998; Makinen et al., 2001; Marconcini et al., 1999; Skobe et al., 2001; Staker et al., 2001). (Figure 1)

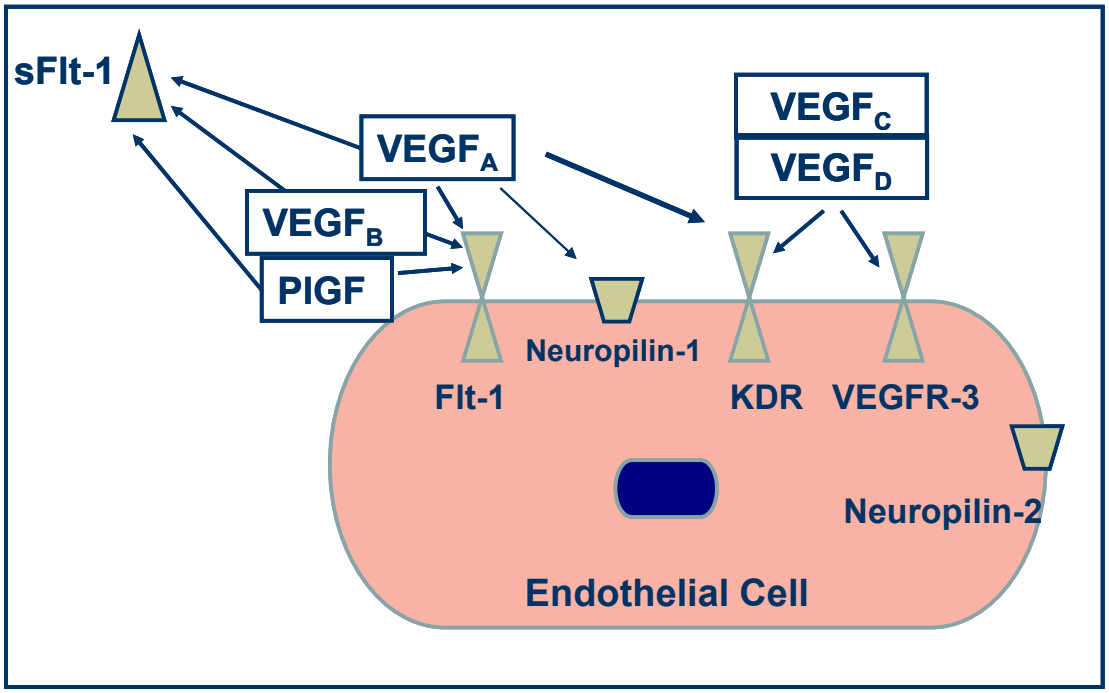

Figure 1. Vascular endothelial growth factor and its receptors. There are three structurally homologous tyrosine kinase receptors that mediate the actions of the members of the VEGF family, VEGFR-1, VEGFR-2 and VEGFR-3. Other molecules that VEGF interacts with include the neuropilins.

VEGF Receptors

There are three structurally homologous tyrosine kinase receptors that mediate the actions of the members of the VEGF family, VEGFR-1 (Flt-1/fms-like tyrosine kinase receptor), VEGFR-2 (KDR/Flk-1/fetal liver kinase) and VEGFR-3 (Cao et al., 1998; Ferrara et al., 1999) (Figure 1). Ligand binding causes receptor dimerization, phosphorylation and signal transduction.

Flt-1. Flt-1 is found primarily on vascular endothelial cells (Barleon et al., 1994), but expression has been shown on monocytes (Barleon et al., 1996), smooth muscle cells (Ishida et al., 2001) and tumor cells (Donnem et al., 2007). Flt-1 is expressed in two forms: 1) a full length form that encodes for a membrane bound receptor with an extracellular domain, a transmembrane domain and a tyrosine kinase domain (Shibuya et al., 1990), and 2) a truncated, secreted form, which lacks the transmembrane and cytosolic domains but retains the ability to bind VEGF (Kendall and Thomas, 1993; Kendall et al., 1996). Flt-1 binds VEGF$_A$, PlGF and VEGF$_B$ (Park et al., 1994). Flt-1 binds VEGF$_A$ with higher affinity than does KDR (De Vries et al., 1992).

The function of Flt-1 has not been completely elucidated, mainly because its biological activity as a mitogenic mediator is considered weak compared to KDR (Shibuya, 2006). Flt-1 has the unique ability to act as both an inhibitor and activator of angiogenesis (Shibuya, 2006) depending on the cell type and stage of development (Ferrara et al., 2003). The inhibitory effects of Flt-1 have been studied mainly in mice during embryonic development (Kearney et al., 2002; Roberts et al., 2004). Flt-1 has been considered a decoy receptor (Landgren et al., 1998; Hiratsuka et al., 2001; Zeng et al., 2001). Studies indicate that binding of $VEGF_A$ to Flt-1 causes a suppressive effect by limiting the availability of VEGF to bind to KDR (Fong et al., 1996; Roberts et al., 2004; Kiratsuka et al., 2005). Conversely, the pro-angiogenic effects of Flt-1 were shown by Luttun et al. (2003), where tumor growth was reduced when Flt-1 was inhibited.

KDR. KDR is a cell surface receptor restricted to endothelial cells and is thought to be more critical than Flt-1 for endothelial cell mitogenesis, and blood vessel growth (Ferrara et al., 2003; Thomas, 1999; Shibuya, 2006). KDR binds $VEGF_A$, $VEGF_C$, $VEGF_D$ and $VEGF_E$ (Thomas 1999; Ferrara et al., 2003) and has greater tyrosine kinase activity than other VEGF receptors (Shibuya, 2006).

VEGFR-3 is similar to Flt-1 and KDR but is primarily found on lymphatic endothelium in adults (Veikkola et al., 2000) and embryonic vasculature early in development (Shibuya et al., 1995; 1999).

Other molecules that VEGF interacts with include the neuropilins (Ferrara et al., 2003), specifically neuropilin 1 (NRP1) which was first identified by Soker et al. in 1998 (Figure 1). Its biological activity is not known, although it appears to have a role as a co-receptor for VEGF in conjunction with KDR (Figure 1).

sFlt-1. Flt-1 has an alternatively processed soluble/secreted form, denoted sFlt-1 (He et al., 1999), which binds both VEGF and PlGF with high affinity (Ferrara et al., 2003; Kendall and Thomas, 1993), but has been shown to inhibit the activity of VEGF (Thomas 1999; He et al., 1999; Kendall et al., 1993). In addition to being a VEGF antagonist by inhibiting binding of VEGF to Flt-1, sFlt-1 also forms heterodimers with KDR (Kendall et al., 1996). He et al. (1999), was one of the first to document the presence of sFlt-1 *in vivo* though Shibuya et al. (1990) had detected it in human placental cDNA. Kendall and Thomas (1993) showed that there was a short mRNA version of Flt-1 that encoded for this soluble form.

The mechanism by which this form of Flt-1 is produced is not well understood. It has been shown however that the difference in the two lies in the genomic structures

(Huckle and Roche, 2004) (Figure 2). There is retention of intron 13 in the secreted form that is a result of alternative processing of the Flt-1 pre-mRNA (Kondo et al., 1998; Huckle and Roche, 2004).

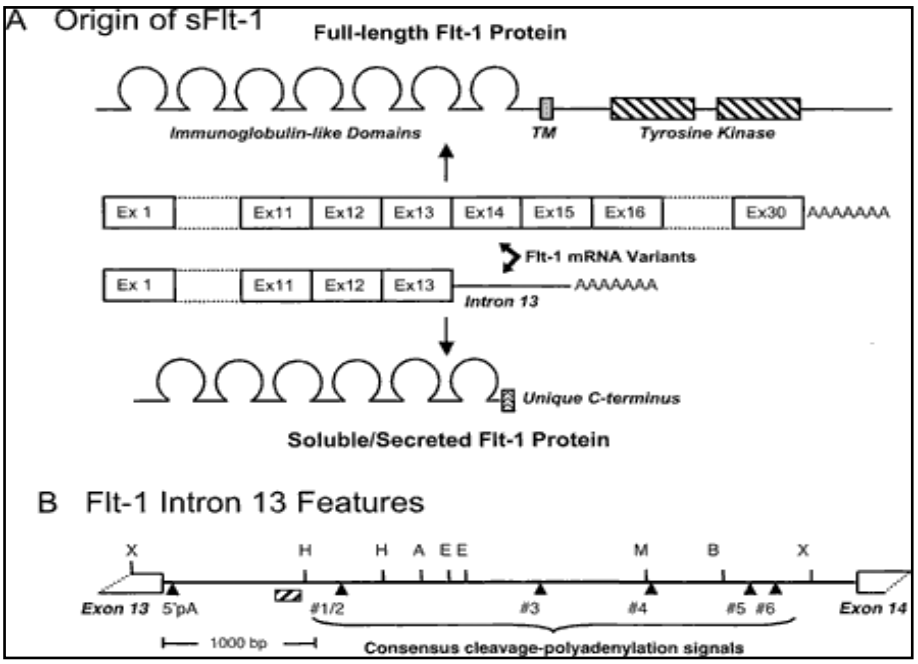

Figure 2. **Flt-1 protein, mRNA and genomic structures**. The mechanism by which sFlt-1 is produced is not well understood; however, It has been shown that the difference in Flt-1 and sFlt-1 is present in the genomic structures. There is retention of intron 13 in the secreted form that is a result of alternative processing of the Flt-1 pre-mRNA. *Post-transcriptional control of expression of sFlt-1, an endogenous inhibitor of vascular endothelial growth factor, WR Huckle and RI Roche, Journal of Cellular Biochemistry. Copyright © (2004, Wiley-Liss, Inc.) Reprinted with permission of Wiley-Liss Inc. a subsidiary of John Wiley & Sons Inc.*

Previous studies have shown that there is variation in the expression of sFlt-1 and Flt-1 mRNA under various physiologic conditions (Clark et al., 1998; He et al., 1999; Krussel et al., 1999) and suggest that a balance of factors that inhibit and induce angiogenesis is important in both pathologic and physiologic angiogenesis (He et al., 1999; Roberts et al., 2004). In studies done with pregnant mice, a variation in the expression of sFlt-1 and Flt-1 in the placenta was dependent upon the stage of pregnancy (He et al., 1999), suggesting that there is a physiologic trigger that initiates the expression of either one or the other receptor. High levels of sFlt-1 have been detected in females with preeclampsia (Koga et al., 2003; Maynard et al., 2003). The high level of sFlt associated with preeclampsia is thought to be caused by a suppression of $VEGF_A$ (Maynard et al., 2003).

Huckle and Roche (2004) showed that a decrease in the sFlt-1 mRNA expression created by mutations in the processing sites caused a subsequent increase

in the Flt-1 mRNA expression. This suggests that there are triggers which control the expression of these receptors, but the mechanism is still to be elucidated (Huckle and Roche, 2004). It is likely that sFlt-1 plays a role in numerous pathologic conditions.

Angiogenesis and Solid Tumors

Angiogenesis is essential for solid tumor growth (Fontanini et al., 1997; Carmeliet and Jain, 2000; Tanaka et al., 2001), and dissemination (Folkman, 1995; Carmeliet and Jain, 2000; Yano et al., 2006). Tumors require additional blood supply for oxygenation and nutrients to sustain their increased metabolic needs (Hanahan and Folkman, 1996; Papetti and Herman, 2002; Yano et al., 2006). Solid tumors are dependent on angiogenesis to grow beyond 1-2 mm^3 (Folkman and Klagsburn, 1987). Angiogenesis in solid tumors occurs by remodeling of pre-existing vessels surrounding the mass (Zhang et al., 2003). The vessel caliber enlarges and sprouts begin to develop from the pre-existing vessels (Yancopoulis et al., 2000; Yano et al., 2006). In the presence of suitable pre-existing capillary beds, some tumors have been shown to grow without the presence of new blood vessels (Pezzella et al., 1997). The balance of pro and anti-angiogenic factors is maintained in the tumor microenvironment (Hanahan and Folkman, 1996).

During tumor development, an angiogenic switch occurs which involves the expression and secretion of growth factors by tumor cells (Donnem et al., 2007). This eventually leads to secretion of growth factors by the surrounding stroma and subsequent activation of endothelial cells (Ferrara et al., 2005). The stroma surrounding and involving tumors has a different composition than normal tissue. There are increased numbers of leukocytes and an increase in the microvessel density (IMVD); many of the vessels are abnormal and more leaky than normal vessels (Carmeliet, 2005). Increased vessel permeability and leukocyte numbers contribute to a growth factor rich environment which enhances cell recruit and regional remodeling (McGavin and Zachary, 2007).

VEGF. VEGF is considered by many to be the initiating step in tumor angiogenesis (Carmeliet and Jain, 2000), and has been shown to influence tumor growth and metastasis (Stacker et al., 2001; Ilhan et al., 2004). VEGF is produced in response to hypoxia, growth factors and genetic alterations (Maxwell et al., 2001). Studies have shown specifically an increased expression of VEGF mRNA in solid tumors (Ferrara et al., 1997; Dvorak et al., 1995; Ilhan et al., 2004).

The overexpression of VEGF and its receptors have been shown in many human malignancies, including ovarian carcinomas (Boocock et al., 1995; Zhang et al., 2003), invasive breast tumors (de Jong et al., 1998), prostatic tumors (Harper et al., 1996), pulmonary adenocarcinomas (Takanami et al., 1997; Ilhan et al., 2004), pancreatic tumors (Itakura et al., 2000) and some brain tumors (Ferrara et al., 2003). Additionally, elevated levels of VEGF have been detected in the serum of dogs with neoplastic disease (Troy et al., 2006).

The important role of VEGF in solid tumor development can be accredited to the production of VEGF by tumor cells (Donnem et al., 2007), stromal cells (Fukumura et al., 1998) and host cells (Yano et al., 2006), as well as the presence of VEGF receptors on these cells (Dziadziuszko et al., 2001; Shukova et al., 2003). VEGF acts in both a paracrine and autocrine fashion (Ferrara et al., 2003). Paracrine refers to the secretion of VEGF by one cell to stimulate a nearby cell. Autocrine refers to the secretion of a substance by one cell to act on itself. This is the case in many tumors, where tumor cells secrete VEGF that activates VEGF receptors on other tumor cells (Donnem et al., 2007; Yano et al., 2006). This mode of regulation allows the cell independence from the control of other molecules, hence an advantage for proliferating tumor cells (Figure 3).

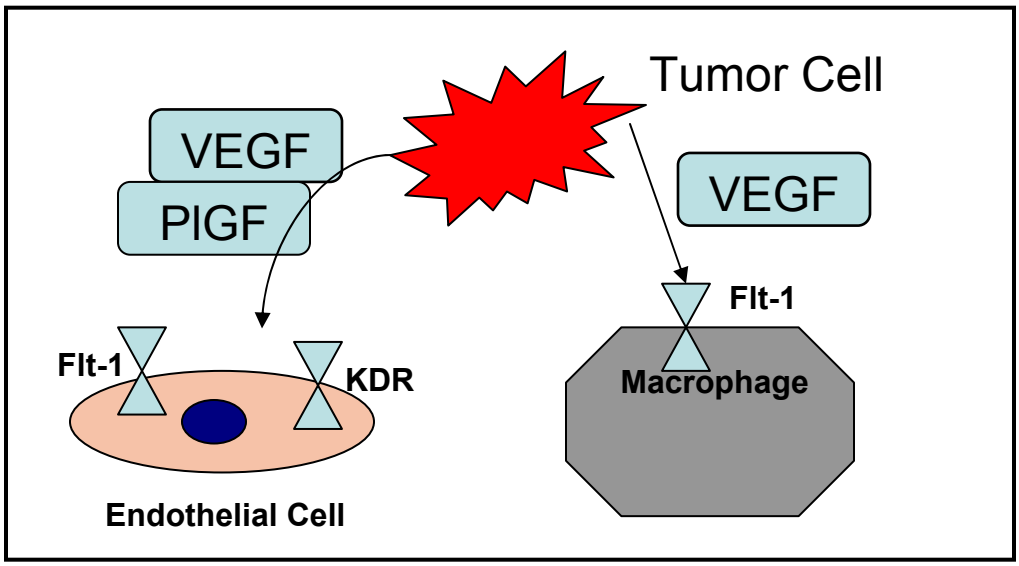

Figure 3. Tumor cells produce VEGF and PIGF. The important role of VEGF and PIGF in solid tumor development can be accredited their production by tumor cells, as well as the presence of VEGF receptors on these cells.

Several studies have correlated the expression of VEGF and VEGF receptors on cancer cells and stromal cells to a poor patient prognosis (Takanami et al., 1997; Ferrajoli et al., 2001; Donnem et al., 2004). Moreover, studies have shown that tumor development can be inhibited by suppression of VEGF and VEGF receptors (Ferrara et al., 2003).

VEGF receptors, specifically Flt-1, sFlt-1 and KDR have been found in human tumor tissue and tumor cells (Ilhan et al., 2004; Horning et al., 1999). Studies have shown that sFlt-1 is important in the complex process of tumor angiogenesis (Hornig et al., 1999; 2000; Kendall et al., 1996). sFlt-1 acts as a dominant negative inhibitor of angiogenesis by binding VEGF and preventing it from activating Flt-1 (Thomas, 1996; Kendall et al., 1996). Mae et al. (2005) showed that in the presence of tumor cells secreting excess VEGF, this inhibitory effect can be overwhelmed. Additionally, they showed the importance of sFlt-1 in tumor metastasis and progression *in vivo*, by suppressing the growth of existing and developing lung metastasis with administration of an adenovirus vector carrying sFlt-1 (Mae et al., 2005).

PlGF. The regulation and molecular mechanisms of PlGF are not completely understood and studies have reported varying results. Some studies show that tumor angiogenesis is enhanced by PlGF because it facilitates crosstalk between Flt-1 and KDR (Carmeliet et al., 2001; Luttun et al., 2002; Autiero et al., 2003). Furthermore, Ferrara et al. (2003) showed a synergistic relationship exists between VEGF and PlGF, binding to Flt-1, in pathologic conditions such as tumor development. Additionally, two studies showed that administration of PlGF-1 can enhance VEGF induced angiogenesis *in vitro* and *in vivo*, suggesting competition between PlGF-1 and VEGF for Flt-1 and subsequent increased VEGF binding to KDR (Park et al., 1994; Carmeliet et al., 2001). Moreover, Taylor and Goldenberg (2007) showed PlGF enhanced motility and invasiveness of breast cancer cells forming spontaneous pulmonary metastases.

Conversely, other studies show that PlGF inhibits tumor growth and angiogenesis by forming inactive PlGF/VEGF heterodimers which can not bind KDR and initiate an angiogenic event (Eriksson et al., 2002; Bjorndhal et al., 2004). Furthermore, Xu et al. (2006), using a mouse model of tumor growth and metastasis, showed overexpression of PlGF in tumor cells inhibited tumor growth, angiogenesis and lung metastasis of human lung, colorectal and brain tumors *in vivo*. They suggested a similar mechanism as Eriksson et al. (2006) of PlGF/VEGF heterodimer formation and VEGF homodimer depletion (Xu et al., 2006). DiSalvo et al. demonstrated in 1995, that

VEGF/PlGF heterodimers naturally occur in tissue when both factors were produced by the same cell. Additionally, PlGF expression has shown to be down regulated or low in various cancer cell lines (Takahashi et al., 1994; Viglietto et al., 1995; 1996; Hatva et al., 1996; Xu and Jain, 2007). Xu and Jain (2007) showed this was a result of hypermethylation of the PlGF promoter, a similar mechanism shown to regulate tumor suppressor genes in other human cancers (Zochbauer-Muller et al., 2001; Lee et al., 2004). The genes include those that control the cell cycle, angiogenesis, DNA repair, signal transduction, and others associated with tumor metastasis and invasion (Zochbauer-Muller et al., Lee et al., 2004).

Eriksson et al. (2002) has tried to clarify the mechanisms of PlGF, by explaining it as a dual functioning factor. Through their work, they were able to show that when PlGF-1 and VEGF are co-expressed by tumor cells, angiogenesis is diminished by the formation of inactive PlGF/VEGF heterodimers (Eriksson et al., 2002). Conversely, when PlGF and VEGF were expressed by two different cell populations, angiogenesis was enhanced by the increased availability of VEGF to bind KDR (Eriksson et al., 2002). In this case, PlGF prevented VEGF binding by competitively binding Flt-1 (Eriksson et al., 2002). (Figure 4)

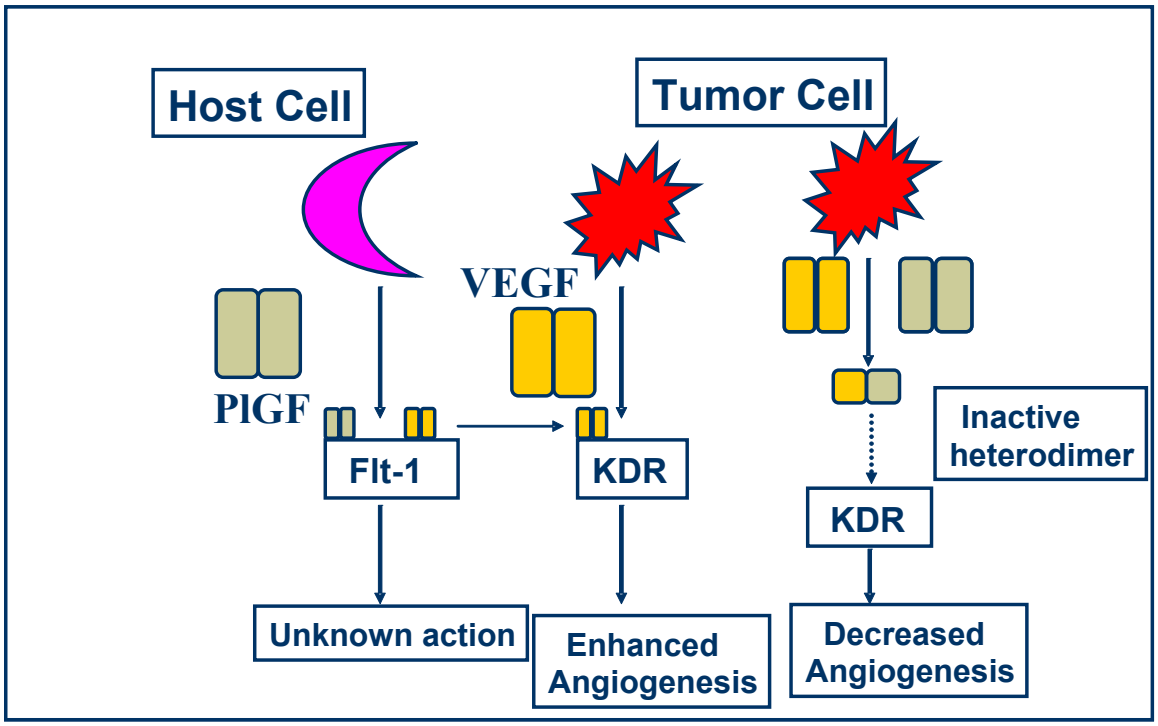

Figure 4. The dual role of PlGF in pathologic angiogenesis. When produced by the same cell as VEGF, inactive heterodimers are formed. When produced alone, homodimers bind Flt-1 and prevent VEGF binding, allowing more VEGF to bind KDR.

In addition to its actions in tumor angiogenesis, PlGF has been shown to enhance atherosclerotic lesions by activating monocytes and increasing macrophage infiltration into vessels (Selvaraj et al., 2003; Khurana et al., 2005).

Hypoxia-inducible factor (HIF). Hypoxia, low oxygen tension, is a major initiator of angiogenesis (Fong, 2008). Growth beyond 1-2 mm^3 makes solid tumors subject to hypoxia (Matepe et al., 1998; Zhang et al., 2003; Fong, 2008). In response to hypoxia, various factors cause up-regulation of VEGF (Dor et al., 2001). These factors include epidermal growth factor, TGF-α, TGF-β, insulin-like growth factor, platelet derived growth factor (Ferrara et al., 1997) and hypoxia-inducible factor (HIF). HIF-1α and HIF-2α (Semenza et al., 1998; Elson et al., 2000; Lee et al., 2000; Kelly et al., 2003) are transcription factors that activate numerous angiogenic associated genes, such as $VEGF_A$ and Flt-1 (Fong, 2008). HIFs interact with hypoxia response elements (HRE) on these genes to activate transcription (Fong, 2008). Both $VEGF_A$ and Flt-1 have been shown to contain these elements, but similar elements have not been shown on the PlGF gene (Fong, 2008). HIFs have other methods of upregulating angiogenic factors that do not involve transcription (Fong, 2008). The induction of these HIFs varies amongst cells and tissues (Fong, 2008). HIF is expressed in numerous pathologic conditions, including diabetic wound healing, myocardial ischemia (Lee et al., 2000), atherosclerosis, retinal neovascularization (Ozaki et al., 1999), rheumatoid arthritis (Hitchon et al., 2002; Peters et al., 2004) and solid tumor development (Vaupel et al., 1989; 1990; Zhong et al., 1999; Talks et al., 2000). Additionally, Ozawa et al. (2001), showed that secretion of $VEGF_A$ is more efficient in a hypoxic environment due to a 150 kDa oxygen-regulated protein (ORP150), an intracellular protein induced by hypoxia to transport $VEGF_A$ to the Golgi apparatus.

Other contributors. Other cells have been shown to contribute to the effects of VEGF on tumor angiogenesis. Verheul et al. (1997) determined that platelets act as a mode of transportation for VEGF in the circulation. Once platelets arrive at their site of action and become activated, they release VEGF into the microenvironment (Mohle et al., 1997). In tumors, the adhesion of platelets to vessels concentrates more VEGF and promotes angiogenesis (Ilhan et al., 2004). A study by Ilhan et al. (2004), suggested that

elevation of serum VEGF in lung cancer patients was due to increased leukocyte and platelet numbers in the circulating blood and tumor tissue.

Additionally, macrophages play a role in solid tumor growth and metastasis by expressing various growth factors (Lewis et al., 2005). Monocytes are recruited into hypoxic tissue by chemoattractants such as $VEGF_A$ (Murdoch et al., 2004; Ferrara et al., 2004) and PlGF (Luttun et al., 2002; Fischer et al., 2007). The expression of Flt-1 on the surface of monocytes facilitates interaction with $VEGF_A$ and PlGF (Hiratsuka et al., 2002; Khurana et al., 2005). Once in the tissue, monocytes become macrophages and promote angiogenesis by secreting more $VEGF_A$ (Sunderkotter et al., 1991) and matrix metalloproteases (MMP) (Gordan et al., 1995). In a study by Xu et al. (2006), the presence of macrophages contributed to variation in blood vessel morphology and density but their presence had minimal effects on the growth of tumor cells expressing PlGF.

Angiogenesis and the Biology of Metastasis

Hematogenous spread of solid tumors is facilitated by the vascular system. Tumors can stimulate the formation of new blood vessels, for required nutrients, as well to gain access to the vascular system. This process is complex and must be coordinated for metastasis to occur (McGavin and Zachary, 2007). The first step involves detachment of the tumor cell from the solid tumor and replication to form a clonal cell population (McGavin and Zachary, 2007). The cells acquire the ability to invade the surrounding extracellular matrix and basement membrane and gain access to the blood vessel lumen (McGavin and Zachary, 2007). Once in the lumen, the cells form clusters or emboli which make them more efficient travelers and more likely to survive within the blood vascular system (McGavin and Zachary, 2007). The embolus travels to a site where its growth is favored and attaches to the blood vessel wall. Then the cells migrate through the basement membrane and invade the extracellular matrix to form a metastatic site (McGavin and Zachary, 2007). The metastatic site must provide an adequate environment for cells to grow. Therefore, formation of new blood vessels at the metastatic site is important to provide the cells required nutrients (McGavin and Zachary, 2007).

Angiogenesis and Lung Tumors

The lung is one of the most vascular organs in the body, making it a ”hot spot” for cancer metastasis and likewise primary lung cancer. Lung cancer is the second leading cause of cancer related death (www.lungcancer.org; Yano et al., 2006) among men and women in the United States and other industrialized nations. In most cases, there is recurrence of disease even after treatment due to the presence of undetectable micrometastases at the time of treatment (Brattstrom et al., 2002). Lung cancer can be considered primary, originating from cells in the lung, or secondary, usually as a metastasis from another organ. Primary lung cancer can be divided into two groups, non-small cell (NSCLC) and small cell (SCLC) lung cancer (www.lungcancer.org; Yano et al., 2006). Most cases, 80% or more, fall into the non-small cell category (Yano et al., 2006), which includes squamous cell carcinoma, adenocarcinoma, bronchioalveolar carcinoma and large cell undifferentiated (www.lungcancer.org). The prognoses for both NSCLC and SCLC are poor, though NSCLC tends to have a worse prognosis (Woo et al., 2004). Patient outcome is largely dependent on the stage of the cancer (Yano et al., 2006), which accounts for the presence of tumor metastasis, which is quite common in lung cancer (Herbst et al., 2000). The most common sites for metastasis are bone, lung, liver and brain (Yano et al., 2006).

VEGF is expressed on normal epithelial cells of the bronchioles and on alveolar macrophages (Ilhan et al., 2004) and stimulates surfactant production by acting on Type II pneumocytes (Compernolle et al, 2002). Though VEGF plays a role in normal maintenance of the pulmonary vasculature, increased expression is associated with tumor progression and metastasis (Ilhan et al., 2004). Merrick et al. (2005) and others have shown that angiogenesis is present early in the development of lung cancer and is associated with elevation of VEGF. Moreover, the expression of VEGF and angiogenesis are correlated with poor prognosis in lung cancer patients (Koukourakis et al., 2000). Additionally, PlGF is ubiquitously expressed by normal bronchial epithelium, pneumocytes and alveolar macrophages (Zhang et al., 2005; Xu and Jain, 2007).

Yano et al. (2006) stated that most of the lung cancer related deaths are actually related to metastatic cancer. Using a mouse model of metastasis, Mae et al (2005) were able to demonstrate that lungs enter a pro-angiogenic state after pneumonectomy, mimicking the process that occurs in pulmonary metastasis (Mae et al., 2005). Years of research have shown that after partial or complete removal of a lung, the remaining lung undergoes regeneration (Kodama et al., 1992). Angiogenesis has been shown to play a pivotal role in this process (Kodoma et al., 1992; Mae et al., 2005). Surgical removal of

tumors in patients with NSCLC could initiate development of micrometastases into larger metastases due to compensatory lung growth (Mae et al., 2005; Kodama et al., 1992). Modulation through the VEGF receptor, Flt-1, has been associated with the facilitation of pulmonary metastasis (Hiratsuak et al., 2002).

The profound presence of angiogenesis in NSCLC has led many to consider it necessary for lung cancer progression (Fontanni et al., 1997; Tanaka et al., 2001). Fontanini et al. (1997) showed increased tumor size and vascularity are associated with a worse prognosis and reduced overall survival in NSCLC patients. This suggests that the larger tumors, which are more prone to hypoxia, have a greater angiogenic potential and likely could be more prone to metastasis. Furthermore, Herbst et al. (2000) state that metastasis is the most common cause of death in NSCLC patients.

Expression of VEGF, PlGF and their receptors has been studied extensively in lung cancer, mostly NSCLC. In a recent study by Yilmaztepe et al. (2007), Flt-1 was detected in 83% of tissue samples from patients with lung carcinomas. Over-expression of $VEGF_A$ and KDR has been associated with higher intratumoral microvessel density (MVD), shorter survival (Yuan et al., 2001; Han et al., 2001; Meert et al., 2002) and poor prognosis (Donnem et al., 2007) in NSCLC patients. Conversely, Donnem et al. (2007) found a positive correlation between increased expression of $VEGF_A$, $VEGF_C$, $VEGF_D$ and Flt-1 and KDR by stromal cells and a good prognosis. These findings appear contradictory but highlight the interplay between tumor cells and stromal cells in tumor development and angiogenesis. In two additional studies by Karjita et al. (2001) and Arinaga et al. (2003), a correlation was made between $VEGF_c$ expression and poor prognosis and lymphatic invasion and metastasis in patients with NSCLC. Additionally, high levels of VEGF have been detected in pleural effusion (Yanagawa et al., 1999) and circulating blood (Poon et al., 2001) in cancer patients with advanced disease.

Ilhan et al. (2004) found that high levels of sFlt-1 and VEGF are associated with advanced disease and poor prognosis in various lung cancers. Conversely, Yilmaztepe et al. (2007), found lower levels of sFlt-1 in patients with advanced disease before and after treatment with chemotherapy. However, studies have shown that expression of sFlt-1 may not be a good prognostic tool because sFlt-1 expression is reduced in smokers, which represent a large population of lung cancer patients (Belgore et al., 2000). The levels of sFlt-1 detected in smokers in the study by Belgore et al. (2000) suggest differences in the regulation of angiogenesis and endothelial cells in smokers.

PlGF expression has been associated with a poor clinical prognosis, increased microvascular density, tumor size and tumor stage in NSCLC (Zhang et al, 2005). On the other hand, a recent study by Wei et al. (2005) showed that PlGF expression was inversely related to progression and patient survival in NSCLC. As mentioned before, Xu and Jain (2007) showed that low expression of PlGF in some human tumors was a result of hypermethylation of the PlGF promoter. A similar mechanism has been shown to regulate tumor suppressor genes in human NSCLC and colon carcinomas (Zochbauer-Muller et al., 2001; Lee et al., 2004), suggesting various regulatory mechanisms are involved in angiogenesis and NSCLC progression.

Compared to NSCLC, SCLC tends to disseminate earlier and more rapidly (Tanno et al., 2004; Woo et al., 2004). Tanno et al. (2004) was able to show the simultaneous expression of VEGF, KDR and VEGFR-3 in various SCLC cells. They concluded that the rapid metastasis of SCLC i*s* most likely due to increased VEGF expression and the autocrine stimulation that occurs between VEGF and its receptors in the tumor microenvironment (Tanno et al., 2004). In a study by Woo et al. (2004), expression of PlGF was localized to endothelial cells of small vessels in and around tumors, but not found in tumor cells suggesting a paracrine mode of receptor activation (Woo et al., 2004). Woo et al. (2004) showed that there is a greater expression of PlGF in SCLC cells and tissue compared to NSCLC, and this difference could explain the difference in progression and metastasis between the two cancer types.

Mouse Models

Angiogenesis. Various mouse models have been developed to study angiogenesis. A number of these models have been modified to study cancer associated angiogenesis and metastasis. One such model is the mouse corneal angiogenesis model. This model was developed to evaluate the *in vivo* effects of various growth factors on angiogenesis. The cornea, normally avascular, provides a convenient location for administration of substances and visual evaluation of effects. A pocket is made in the cornea and treatments are applied (Cao et al., 1998; Eriksson et al., 2002). Cao et al. (1998) evaluated the specific effects of the dimeric forms of VEGF on angiogenesis using this model. With the injection of VEGF homodimers (VEGF/VEGF), an elaborate bed of immature microvessels was formed (Eriksson et al., 2002). With an injection of the same amount of PlGF-1 homodimers (PlGF/PlGF) or PlGF-1/VEGF heterodimers, no new vessel formation was seen (Eriksson et al., 2002). Additionally, with co-implantation of

VEGF homodimers and PlGF homodimers, there was no interference with VEGF induced neovascularization (Eriksson et al., 2002). These results show that PlGF inhibits angiogenesis at the protein level, by inhibiting VEGF binding in two ways (Eriksson et al., 2002). This was an important discovery in the mechanism of PlGF.

Metastasis. The lungs serve as the primary organ of interest in models of metastasis. The model used in our study was developed by Wang et al. in 1995. A lethal clone of murine colon adenocarcinoma cells (CT26.CL25) were derived and when injected intravenously form pulmonary metastases (Wang et al., 1995). Mae et al. (2005) used this model and created a pro-angiogenic environment by pneumonectomizing mice, to study the effects of metastasis and document the inhibition of angiogenesis by sFlt-1. With the administration of an adenoviral vector containing sFlt-1, they were able to suppress the growth of pulmonary metastases in these mice (Mae et al., 2005).

Yano et al. (2000) has developed various nude mouse models to observe the mechanisms of lung cancer metastasis. Metastasis to the brain proves to be the most detrimental to lung cancer patients. In this model, a direct correlation was made between the potential for NSCLC cells to produce brain metastasis to the level of VEGF produced by the cell (Yano et al., 2000). It was also noted that metastases developed rapidly when mice were injected with cells producing higher levels of VEGF (Yano et al., 2000). Yano et al. (2000) concluded that the presence of VEGF promotes angiogenesis and a greater potential for lung cancer patients to develop brain metastases.

Studies by Kaplan et al. (2005) and Hiratsuka et al. (2006) have shown that malignant tumor cells develop within secondary organs, such as lung, from clusters of bone-marrow derived endothelial progenitor cells. These primed regions within secondary organs are called “premetastatic niches” (Kaplan et al., 2005). Gao et al. (2008) used the Lewis lung cancer mouse model to track bone-marrow derived cells and determined their presence within micro and macrometases within the lung. Moreover, KDR, among other cell surface markers, was identifyied (Gao et al., 2008). In some cases, the infiltration of these cells was enough to initiate the “angiogenic switch” and promote development of macrometastases (Holmgren et al., 1995; Naumov et al., 2006) The angiogenic switch is a phenomenon that occurs during tumor development, where there is a rapid growth of new blood vessels (Rafii and Lyden, 2008) and the secretion of angiogenic factors by tumor cells (Donnem et al., 2007). This switch transforms a tumor from a more dormant/inactive state to a more aggressive state (Rafii and Lyden, 2008;

Townson and Chambers, 2006). Gao et al. (2008) were able to document the angiogenic switch occurring in a spontaneous model of metastasis. After transplantation of breast cancer cells into a mouse model, new vessels were detected within metastatic foci as early as week 16 using CD31 immunohistochemistry (Gao et al., 2008). The bone-marrow derived endothelial cells were recruited to the metastatic foci and contributed to vessel formation and tumor expansion (Gao et al., 2008).

Additionally, Shibuya (2006) evaluated the effects of macrophages and metalloproteases on tumor progression in a mouse VEGFR-1 (-/-) model. He found that the presence of lung metastasis was lower in these mice than in wild type mice, though the rate of tumor growth was comparable. Shibuya (2006) also determined that there were fewer macrophages and reduced matrix metalloproteinase 9 (MM9) expression in the lungs of VEGFR-1 (-/-) mice compared to wild type, and this likely contributed to the differences.

Lung Cancer. Cao et al. (2006) used a lung cancer xenograft model, created from human lung adenocarcinoma cells, to investigate the success of a KDR inhibitor (AZD2171) combined with radiotherapy in inhibiting tumor growth. Growth of the xenograft was delayed and tumor vascular density was diminished after treatment with the inhibitor and radiation (Cao et al., 2006). They concluded that AZD2171 sensitizes tumoral endothelial cells to radiation and that administration time is critical to catch the tumor during the normalizing phase where the balance between pro and anti-angiogenic factors can be disrupted.

A specific model was developed to evaluate pleural effusion and brain edema, a common sequela seen in lung cancer patients with advanced disease (Yano et al., 1997; 2000; Heiss et al., 1996). After injection with human lung adenocarcinoma cells or a metastatic variant of the cells, high levels of VEGF were detected in the pleural effusion of mice (Yano et al., 1997; 2000). Additionally, a study by Heiss et al. (1996) showed that brain edema in rats was associated with increased vascular permeability, induced by VEGF.

The use of mouse models is advantageous, allowing *in vivo* manipulation of biological systems. However, they cannot always mimic spontaneous disease. In the case of cancer, other than chemical and radiation induction of cancer, most models are manipulated by injection with an already confirmed cancer-causing cell line. Often these

cells are human cells. However, there are numerous models that use murine derived cells.

The use of mouse models in angiogenesis have expanded the field by leaps and bounds, especially giving researchers the ability to evaluate tumor angiogenesis. Additionally, models allow development and evaluation of therapies targeted at cancer cells and factors associated with angiogenesis. There is no doubt that mouse models have contributed to the development of numerous therapeutic advances; however, the difference in human and mouse biology may prove a challenge once drugs are used in clinical trials.

Therapeutics

Angiogenesis is an important component of tumor development and metastasis (Folkman, 1971; Fontanini et al., 1997; Carmeliet and Jain, 2000; Tanaka et al., 2001). Therefore, targeting angiogenesis as a means to treat cancer is a wise therapeutic strategy. Folkman mentioned this approach as early as 1985. Members of the VEGF family and its receptors provide good targets for inhibiting angiogenesis and suppressing the growth of lung cancer in human patients (antiangiogenic agents). Several have been developed and used *in vitro* and *in vivo*. Overall, Yano et al. (2006) recommended combined therapies to inhibit tumor growth.

Targeting VEGF. Anti-VEGF antibody, bevacizumab (Avastin) has been investigated the most (Herbst et al., 2005). In phase III clinical trials, bevacizumab in conjunction with chemotherapy, increased survival with minimal toxic effects in patients with nonsquamous NSCLC (Johnson et al., 2004). In one particular study, the growth of solid tumors in nude mice injected with various human tumor cell lines was inhibited by the use of anti-VEGF antibodies (Kim et al., 1993; Ferrara et al., 2003). Furthermore, a clinical trial using a monoclonal humanized antibody against KDR (rhuMab), has resulted in longer survival time and slower tumor progression, in conjunction with chemotherapy in human metastatic colorectal carcinomas (Kabbinavar et al., 2003). Symptoms similar to preeclampsia have been seen in patients treated with antibodies to block the effects of $VEGF_A$ (Shibuya, 2006). Additionally, elevations of PlGF have been seen in the blood of patients receiving treatment with anti-VEGF antibody and inhibitors (Willett et al., 2005; Motzer et al., 2006).

Targeting VEGF receptors. There are several tyrosine kinase inhibitors. An inhibitor of KDR is ideal because of the receptor's selective expression on endothelial cells, its stronger mitogenic activity and its proven mediation of VEGF-induced angiogenesis (Millauer et al., 1993; Quinn et al., 1993). Administration of an oral inhibitor of both Flt-1 and KDR showed inhibition of tumor growth and possibly restriction of metastasis in various tumor types (Yano et al., 2006). Additionally, Yano et al. (2000) showed inhibition of vascular permeability. Another inhibitor of KDR has been shown to inhibit signaling by VEGF through this receptor, as well as decrease the progression of metastatic tumors produced by NSCLC and SCLC cell lines (Matsumori et al., 2006; Yano et al., 2005). Additionally, combination therapies with KDR inhibitors have inhibited tumor growth in a lung cancer xenograft model (Cao et al., 2006).

Studies have shown that natural inhibitors of angiogenesis such as sFlt-1 can be administered exogenously and inhibit angiogenesis (Aliello et al., 1995; Ferrara et al., 1998; He et al., 1999). In studies by Aliello et al. (1995), suppression of neovascularization was achieved in a mouse model of ischemic retinopathy with local administration of sFlt-1. Additionally, retinal neovascularization in pediatric patients after oxygen treatment has been reduced by suppression of $VEGF_A$ by sFlt-1 (Aliello et al., 1995). Furthermore, treatment of mature rats with exogenous sFlt-1 suppressed formation of ovarian corpus luteum, which is an angiogenesis dependent event during endometrial maturation, leading to infertility (Ferrara et al., 1998). Kong et al. (1998) and Shiose et al. (2000) showed that administration of sFlt-1 in mice can largely inhibit the growth of tumor xenografts.

Targeting Tumor Vessels. Endothelial cells of newly formed vessels within solid tumors are dependent on VEGF (Benjamin et al., 1999; Yuan et al., 1996). Gato et al. (2002; 2004) have shown in several studies a potential use for a tubulin inhibitor that targets endothelial cells of tumor vessels. In one study, injection of the substance induced necrosis of tumors within 24 hours. Similarly, Gato et al. (2002) were able to inhibit lung metatastasis by decreasing angiogenesis in various human NSCLC cell lines.

Other Strategies. There are several natural inhibitors of angiogenesis, including endostatin, angiostatin, interleukin 12 (IL-12) and thrombospondin. In particular, endostatin, works by blocking endothelial cell migration and proliferation (Troy et al., 2006). Endostatin inhibits tumor growth of numerous neoplastic cell lines in various

mouse models (Verheul et al., 2006) and is correlated with type, stage and aggressiveness of various human tumors (Richard et al., 1999; Feldman et al., 2000; 2001). In other studies, elevated serum endostatin has been associated with cancer and increased age in dogs (Verheul et al., 2000; Troy et al., 2006).

There have been numerous studies evaluating the role of angiogenesis in solid tumor growth and development, as well as the identification of angiogenic factors within tumor tissue. Moreover, in recent years studies have shifted to determining the role expression of these factors play both individually and together in the biological behavior of these tumors. Using this information has allowed physicians to more effectively diagnose and treat cancer patients.

Variation in the expression of VEGF, VEGF receptors and other members of the VEGF family occur in numerous solid tumors. The mechanisms by which these variations occur are not completely understood. In my studies I plan to evaluate the expression of these factors in solid tumors, focusing mostly on the role of sFlt-1 in the development and progression of solid tumors, because of its inhibitory actions.

I hypothesize that, in the presence of solid tumors in the lung, there will be a variation in the ratio of sFlt-1 to Flt-1 that is compatible with a pro-angiogenic state.

My aims to test my hypothesis are:

1) **To implement a tumor metastasis model that produces vascularized nodules in the mouse lung and**
2) **To use the model to evaluate sflt-1:flt-1 mRNA expression in tumor-bearing versus control lungs.**

MATERIALS AND METHODS

Part I. Cell Culture and Analysis of Cell Characteristics

Cell Culture

The murine derived colon carcinoma cell line, CT26.CL25 was obtained from the American Type Culture Collection (ATCC). The cells were maintained at 37˚C, 5% CO_2 in a T75 flask (Falcon). Media (Appendix 1) was changed every 2-3 days, and cells were passaged every 5-6 days or when cells grew to an 80-90% confluent monolayer. Passaging was accomplished by washing cells once with DPBS, harvesting using trypsin-EDTA and resuspending (1:10) in complete media.

CT26.CL25 cells are Balb/c mice-derived cells transfected with a plasmid containing the lacZ gene, which encodes for β-galactosidase (Wang et al., 1995). The morphology of the cell resembles that of fibroblasts with a central body and elongated tapering cytoplasmic tails. An aliquot of cells was sent to Charles River for Infectious Disease Screening. Using a viral PCR panel, cells were negative for all common murine viruses and *Mycoplasma* species.

***β*-galactosidase expression**. To detect the *β*-galactosidase activity in the cells, an in-situ *β*-galactosidase Expression stain (Specialty Media/Millipore) was used. Cells were grown to ~80% confluence in a 24 well plate, washed with DPBS and fixed for 5 minutes with a gluteraldehyde/formaldehyde fixative (1ml/well). Cells were washed twice more and DPBS was replaced into three wells (1ml) to be used as controls. Complete *β*-gal Stain Solution (1:40) was placed in the remaining three wells (0.5 ml/well). The dish was sealed with Parafilm to prevent evaporation and incubated in the dark at 37°C. Cells were examined at 2, 4 and 24 hours. After 24 hours, cells were washed twice with DPBS and stored at 4°C in 1 ml of DPBS. Cells were examined again at 60 hours.

VEGF ELISA. Culture media were collected from two T75 flasks of cells after 3 and 5 days of growth at near-confluence. The conditioned media were pooled, centrifuged at 150 x g, and the supernatant was collected. Using the Quantikine M Mouse VEGF Immunoassay (R&D Systems), VEGF concentrations were obtained. Dilutions of the media supernatants and standard (recombinant murine VEGF) were prepared using the Calibrator Diluent, and run in duplicate along with a positive (undiluted kit control) and negative (diluent) control. After an initial 2 hour incubation and

color generation using peroxidase-conjugated secondary antibody, the optical densities (OD) were determined at 450 and 540 nm using Soft Max Pro Program and Spectrometer. VEGF concentrations in unknowns were estimated by interpolation onto a log-log standard curve.

Part II. Implementation of Mouse Metastasis Model

Pilot Study

Tumor Development

All animal studies were conducted using protocols approved by the Virginia Tech Animal Care and Use Committee. CT26.CL25 cells were chosen based on their ability to form metastatic lung nodules when injected IV (Wang et al., 1995). Twenty, 6-7 week old female Balb/c mice were purchased from Charles River Laboratories and monitored for two weeks prior to injections. Mice were injected into the lateral tail veins using a 27 gauge, 0.5 inch needle. Cells (passage 18 in our possession) were harvested using trypsin-EDTA within an hour prior to injection, washed twice with DPBS, and resuspended at a concentration of $5x10^5$ cells/ml in DPBS. The cell suspension was mixed frequently during the procedure to minimize cell clumping. Five mice were injected with 0.25 ml of sterile DPBS, and 15 mice were injected with 10^5 cells in 0.2 mls of sterile DPBS. Mice were designated as Control (PBS injected), Good/Tumor (0.2 mls of cells) or No Good (<0.2 mls of cells) based on the type and success of injection. They were separated into groups of 3-5 and placed into five cages: Cage 1,2,3 - Good/Tumor (11 mice), Cage 4 -Control (5 mice) and Cage 5 -No Good (4 mice). Mice were monitored daily and weighed every two days.

Harvesting Lung Tumors

Mice chosen at random were euthanized on day 3, 9, 14 and 16 post-injection using CO_2 inhalation. A complete necropsy was performed on all animals and tissue was fixed in either 10% Neutral Buffered Formalin (Fisher Scientific) or *β*-galactosidase Tissue Fixative (Specialty Media/Millipore) (Figure 5).

Establishment of Tumor Burden

Tissue *β*-galactosidase Activity.

On day 3, half of each lung from three mice (Good/Tumor group) was rinsed in DPBS and placed in 5 mls of cold *β*-galactosidase Tissue Fixative for 1.5 hours. Tissue was then rinsed with buffer solution for 35 minutes and placed in 1ml of Complete X-gal solution containing 100 ul X-gal stain solution and 4000 ul of Stain Base Solution (1:40). Tissue was incubated in the dark at 37°C and examined at 1, 5 and 24 hours. After 24 hours, tissue was rinsed with DPBS and placed back in DPBS for storage at 4°C.

Histopathology.

Tissue was fixed at a 1:10 tissue to formalin ratio in 10 % Neutral Buffered Formalin for several days to weeks and then embedded in paraffin. Five (5 um) sections were cut and mounted for histopathologic examination. All sections were blindly reviewed by veterinary pathologist, Dr.Tanya LeRoith.

PRIMARY STUDY

Tumor Development

CT26.CL25 cells were retrieved from a frozen stock (Passage 3) and cultured for 7 weeks prior to injections. Forty, 8-9 week old female Balb/c mice were injected IV using a 27 gauge, 0.5 inch needle into the lateral tail vein. Fifteen were injected with 0.25 ml of sterile DPBS and 25 were injected with 2×10^5 cells in 0.25 mls DPBS. Cells were harvested within an hour prior to injection and re-suspended often during the procedure. Mice were separated into groups of 5 and placed into eight cages: Control (PBS) – Cages 1,7,8 (15 mice); Good/Tumor (0.2 mls of cells) – Cages 2,3,5,6 (20 mice) and No good (<0.2 mls of cells) – Cage 4 (5 mice). Mice were weighed every other day and individuals tracked through the use of colored markings placed on the dorsal surface of the tail (blue, red, green and black).

Harvesting Lung Tumors

On day 14, all animals were weighed and then euthanized individually using CO_2 inhalation. A routine necropsy was performed on all animals using a ventral midline incision to gain quick access to the lungs. Visible nodules were counted and recorded. All tissues, except some sections of lung, were fixed in 10% neutral buffered formalin. Lung tissue was preserved in one of four ways: 11 in either *B*-galactosidase fixative or 10% Neutral Buffered Formalin, 27 in RNAlater (Ambion) and 2 in DPBS (inflated first).

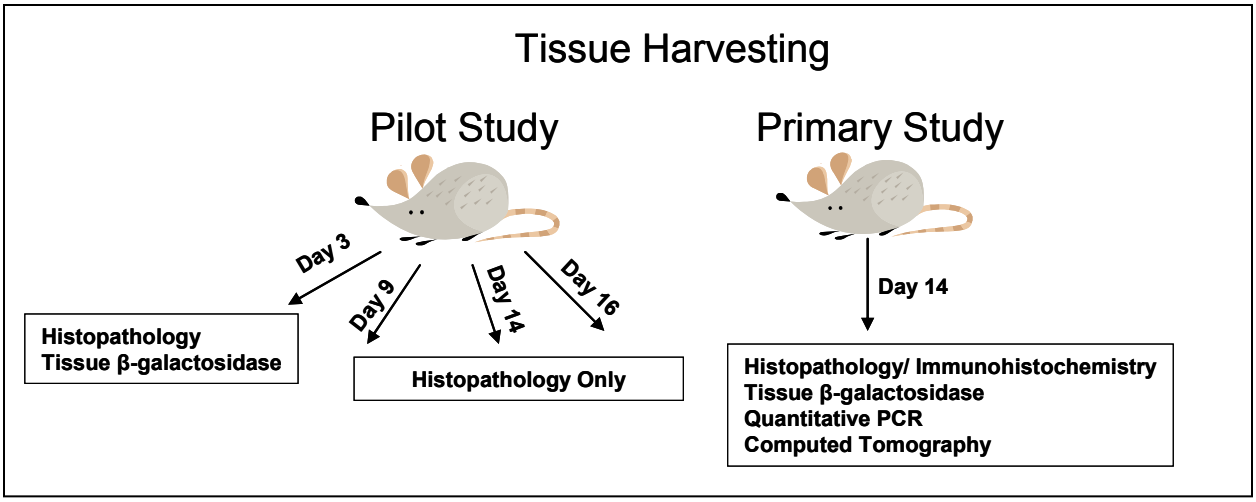

Figure 5. Tissue harvesting and procedures. Performed with lung tissue in pilot and primary study.

Establishment of Tumor Burden

***B*-galactosidase Activity.**

Lung tissue was rinsed in cold DPBS and fixed in 5 mls of cold β-galactosidase Tissue Fixative for 45 to 90 minutes and subsequently incubated in the dark at 37˚C in complete X-gal solution. Lungs were observed at 1 and 24 hours. After 24 hours, lungs were moved to DPBS and stored at 4˚C. Six days later, three lungs were cleared using a graded series of ethanol (30%, 50%, 75%, 95%, 100% and 100%) for 45 to 60 minutes each and 99 % Methyl Salicylate (Oil of Wintergreen – Fisher Scientific) overnight.

Histopathology and Immunohistochemistry.

Lung tissues from three mice (2 Tumor, 1 Control) were fixed for 24 hours in formalin and then placed in 70% alcohol overnight before being processed and embedded in paraffin. Five 5-µm sections were mounted and either stained with Hematoxylin and Eosin for histopathologic examination or left unstained for immunohistochemical staining. As in the pilot study, all sections were reviewed by veterinary pathologist, Dr. Tanya LeRoith.

Immunohistochemistry using a monoclonal mouse Anti-*B*-galactosidase Antibody (Promega) and a Polyclonal Goat Anti-Mouse PECAM-1 antibody (Platelet Endothelial Cell Adhesion Molecule, CD31; Santa Cruz) was done on tissue from 2 tumor-bearing (2W, 3W) and 1 control (7 Blk) mouse. Dr. Eva Schmelz provided the lab space and antibodies for the PECAM-1 immunohistochemical staining.

The Anti-*B*-galactosidase antibody was selected to identify the presence of CT26.CL25 cells within pulmonary nodules. The primary antibody was diluted 1:1000. Sections were deparaffinized and rehydrated before beginning the Vectastain ELITE ABC Kit (Vector) protocol. Endogenous antigens were blocked with provided mouse serum and a horse derived biotinylated secondary antibody was used. The sections were incubated for 2 minutes with DAB before a dark brown color began to develop. The sections were counterstained with Gill's Hematoxylin, dehydrated and mounted.

The PECAM-1 antibody was selected to highlight the vascularity of the lung nodules. New unstained paraffin embedded sections were used. Slides were dried for 20 minutes at 56ºC before deparaffinizing and rehydration. Antigen unmasking was done using the low pH Vector Unmasking Solution (Vector) at 1:100 dilution. The solution was warmed and slides incubated for 20 minutes in a steamer, after which the slides were allowed to cool to room temperature. Endogenous peroxidase activity was quenched with 2 drops of Peroxo-Block Solution (Zymed). Endogenous antibodies were blocked with a 1% Fetal Bovine Serum solution for 1 hour. The primary antibody (1:300) was applied to the slides, cover-slipped and incubated at 4ºC overnight. No primary antibody was applied to one slide to serve as a negative control. After washing, a secondary Broad Spectrum Biotinylated Antibody (Zymed Lab Histostain Bulk Kit Lab-SA Detection System) was applied (100 ul) and incubated for 15 minutes.

After washing, slides were incubated with Enzyme Conjugate (Zymed Histo Bulk Kit) for 10 minutes. Again after rinsing, DAB chromagen (DAKO Chromagen System) was applied and allowed to incubate until color change was noted. No color change was noted so several steps were repeated. The slides were rinsed in tap water. Then a solution composed of Blocking Solution (Zymed) and Goat IgG Secondary Antibody (Vectastain Elite ABC Kit; Vector) was applied to slides and incubated for 30 minutes. Subsequently, Vectastain ABC Reagent was applied and incubated for 30 minutes. Lastly, the DAB Chromagen Solution was applied and color change began to develop within 1 minute. The slides were then counterstained with Hematoxylin, rehydrated and mounted.

Computed Tomography.

The lungs of two mice (1 control, 1 Tumor) were inflated with air using a 27 gauge needle and placed in DPBS overnight at 4ºC. The following two days, they were scanned by Dr. Megan Oest in the Dr. Jeryl Jones' lab using the vivaCT 40 MicroCT machine. To stabilize the lung and prevent movement artifacts, the lung was wrapped in a DPBS soaked sponge and placed in a 50 ml Falcon tube filled with DPBS.

Part III. Analyzing the Expression of VEGF Receptors, Flt-1 and sFlt-1.

RNA Isolation.

*In Vitro Cell lysate***.** CT26.CL25 cells were grown to an 80-90% confluent monolayer in a 6 cm culture dish. Cells were lysed with 600 ul of RLT Buffer with mercaptoethanol (Qiagen, RNeasy Mini Kit) and loosened from the dish with the aid of a cell scraper (Costar). The lysate was pipetted directly into a QIAshredder spin column (Qiagen) and centrifuged for 2 min. The homogenized lysate was collected and stored at -20˚C prior to RNA isolation.

Lung tissue. Whole lung was rinse in ice-cold DPBS and placed in 10 ml of cold RNAlater (Ambion) at the time of necropsy and stored at 4˚C for 10-21 days. On the day of homogenization, all visible adipose and excess tissue was trimmed before lungs were weighed. Tissue was then cut into small (1-4 mm) pieces and placed in 5 ml of cold RLT Buffer with mercaptoethanol (Qiagen RNase –Free DNase Set) and homogenized for 15-20 seconds with a hand held tissue homogenizer until liquid was a clear light to dark amber color. Homogenates were stored at -20˚C prior to RNA isolation.

RNA isolation from lung homogenates was done in groups of 6-8 and the CT26.CL25 lysate was processed individually. Seven hundred microliters of each thawed homogenate was clarified by centrifugation (14000 rpm), and the supernatant was used for RNA isolation. An equal volume of 70% Ethanol (600 ul) was added to 600 ul of the sample (cell lysate or lung homogenate). The mixture was applied to the RNeasy Minicolumn, in two equal batches, and centrifuged. Next, the spin column was washed with 350 ul of Buffer RW1. Then, 80 ul of DNase 1 incubation mix (10 ul of stock

DNase 1 solution, 70 ul of Buffer RDD) was added directly to the spin column membrane and incubated at room temperature for 15 minutes. The column was washed again with 350 ul of RW1 buffer. The flow through and collection tube were discarded and the column was placed in a new 2 ml collection tube. Next the column was washed two times (15 sec, 2 minutes respectively) with 500 ul of Buffer RPE (diluted in Ethanol) and dried by centrifugation. The flow through and tube were discarded. Lastly, 40 ul of RNase Free Water was added directly to the spin column membrane and centrifuged for 1 minute to elute the RNA. This step was repeated once and eluates pooled. The concentration of the elution (Total RNA) was determined by measuring absorbance at 260 and 280 nm. Dilutions of total RNA were made to serve as Non-Reverse Transcribed (NoRT) controls for PCR, and all RNAs were stored at -20ºC prior to cDNA synthesis.

cDNA Synthesis.

Samples were thawed and cDNA synthesized in groups of 13 or 14 (BIO-RAD iScript Select cDNA Synthesis Kit). To begin, the master mix was prepared with three components, added in this order to a 0.2 ml PCR tube. First, 2 ul of provided Random Primer, next Nuclease Free Water and lastly total RNA from lung or cultured cells (1 ug) to a total volume of 15 ul. The volumes of water and total RNA were determined based on the Total RNA concentration of each sample. The mix was incubated for 5 minutes at 65ºC and then snap chilled for 60 seconds on ice. Next 5 ul of a separately prepared master mix was added to each tube making a total volume of 20 ul. The master mix consisted of 5x iScript Select Reaction mix (4 ul per sample) and iScript Reverse Transcriptase (1 ul per sample). The tubes were places into the thermocycler (HYBAID, PCR Sprint) for 5 minutes at 25 ºC, followed by 30 minutes at 42ºC and ending with a 5 minute incubation at 85ºC to inactivate the reverse transcriptase. The samples were allowed to cool and then dilutions were made by adding 30 ul of DNase Free Water (20 ng eq/ul). The cDNA was stored at –20ºC.

PCR was used to screen the newly synthesized cDNA for murine Flt-1, which has a unique Exon 13/ Exon 14 junction, using primers that generate a product of 186 bp (Huckle and Roche 2004) (Figure 3). Samples included a tumor- bearing and control lung, cultured CT26.CL26 cells and each of their corresponding NoRT samples. A positive control was obtained from a frozen stock of cDNA from a HEK293 Flt-1 minigene transfectant. cDNA (1 ul) was added to 19 µl of master mix containing 2X

Taqman® PCR mix (Qiagen), DNase Free water and 10μM primers BH205 and BH206 (Table 1), then separated on a 2% agarose gel. The gel was run at 100 V for 45 minutes until the dye front had moved approximately 2/3 the length of the gel. The gel was stained with 1 ug/ml Ethidium Bromide for 15 minutes and destained in water for 5 minutes.

Quantitative Real Time PCR (QPCR).

In addition to Flt-1 and sFlt-1, six other cDNA targets were selected: KDR, $VEGF_A$, Angiotensin Converting Enzyme (ACE), the phosphotransferase that confers neomycin resistance (neo^R), 18S ribosomal RNA, and Placental Growth Factor (PlGF). These targets were selected based on their presence within lung tissue and/or the lung nodules derived from cultured CT26.CL25 cells. A separate 96 well plate (Applied Biosystems) was used for each target. Each sample was run in triplicate, using a 20 ul reaction volume. The cDNA samples included material derived from 27 lungs (14 tumor, 10 control, 3 “No Good”), cultured CT26.CL25 cells, and 3 NoRT (tumor, control, cultured CT26.CL25 cells).

$VEGF_A$,ACE and 18S ribosomal RNA. Pre-designed and validated primer/6FAM-labeled probe sets (Applied Biosystems) were purchased to target murine $VEGF_A$ and murine ACE across an exon-exon junction. The primer/probe master mix was made with 100x (18uM) primer/probe mix and DNase Free water. For each sample, 2 μl of sample (cDNA, NoRT or No template) were added to prediluted Taq Master Mix and primer/probe master mix. For the 18S rRNA control, the primer/probe mix was prepared by combining forward and reverse primers with the VIC-labeled probe. For each unknown, 1 ul of sample (cDNA, NoRT or No template) was added to prediluted Taq Master Mix and primer/probe mix.

Flt-1, sFlt-1, neo^R, PlGF, KDR. Working dilutions of primers/probes were prepared to yield final concentrations of 0.3 uM and 0.2 uM, respectively, in PCR reactions (Table 1). For each sample, 2 μl of sample (cDNA, NoRT or No template) were added to prediluted Taq Master Mix and primer/probe mix. Conditions and amplification efficiency had previously been determined (Huckle and Roche 2004; Matt Rittler, unpublished).

For each plate, a $\Delta\Delta$Ct Real Quantitative Plate Assay (7300 BioApplied Biosystem software) was run in three stages: Stage 1, 1 repetition at 50ºC for 2 minutes; Stage 2, 1 repetition at 95ºC for 10 minutes and Stage 3, 40 repetitions at 95ºC for 15

seconds each and then held at 60ºC for 1 minute or until removed. At the end of the run, threshold cycle (Ct) was determined for each well. Triplicate Ct estimates were averaged to obtain the target- and sample-specific values used for relative gene expression analysis.

Table 1. Primer and Probe Sequences

Name	Target Location	Sequence (5' →3')
BH205	Exon 13 (ss)	AGAAGACTCGGGCACCTATG
BH206	Exon 14 (as)	GGCGCGGGGACACCTCTA
BH211	mus sFlt-1 (exon 13/ In 13) (s)	GGGAAGACATCCTTCGGAAGA
BH212	mus sFlt-1 (exon 13/ In 13) (as)	TCCGAGAGAAAATGGCCTTTT
BHTP1	mus sFlt-1 (exon 13/ In 13) probe	6FAM-CCGCAGTGCTCACCTCTAACGAGAACTTCT-TAMRA
BH228	mus Flt-1 (Exon 13/ Exon 14) (s)	TTCGGAAGACAGAAGTTCTCGTT
BH229	mus Flt-1 (Exon 13/ Exon 14) (as)	GACCTCGTAGTCACTGAGGTTTTG
BHTP3	mus Flt-1 (Exon 13/ Exon 14) probe	6FAM-AGATTCGGAAGCGCCACACCTGCT-TAMRA
BH296	Neo^R (s) in pcDNA	GCGCCCGGTTCTTTTTGT
BH297	Neo^R (as) in pcDNA	GCCTCGTCCTGCAGTTCATT
BHTP5	Neo^R probe	6FAM-AAGACCGACCTGTCCGGTGCCCT-TAMRA
BH322	mus PlGF (s)	CCCTGTCTGCTGGGAACAA
BH323	mus PlGF (as)	GCTGCGACCCCACACTTC
BHTP11	mus PlGF probe	6FAM-TTGAAAGGCACCACTTCCACTTCTGTTGA-TAMRA
BH324	Flk-1/KDR (s)	GGGACCTGGACTGGCTTTG
BH325	Flk-1/KDR (as)	CCGCATTCAGTCACGAATACC
BHTP12	Flk-1/KDR probe	6FAM-TTTCCTCAGAATCACGCTGAGCATTGG-TAMRA

RESULTS

Cell culture characteristics

In Situ β-galactosidase Activity Stain. To demonstrate β-galactosidase activity, cells were stained with a solution containing X-gal, which is a substrate for β-galactosidase. After 2 hours incubation, the cytoplasm of approximately 60% of the cells was blue-green. After 4 hours, 75% of the cells were blue-green and after 24 hours there was localization of the blue-green color within 95% of the cells (Figure 6). At 60 hours post staining (36 hours in PBS) at 4ºC, approximately 90-95% cells still retained the blue-green color.

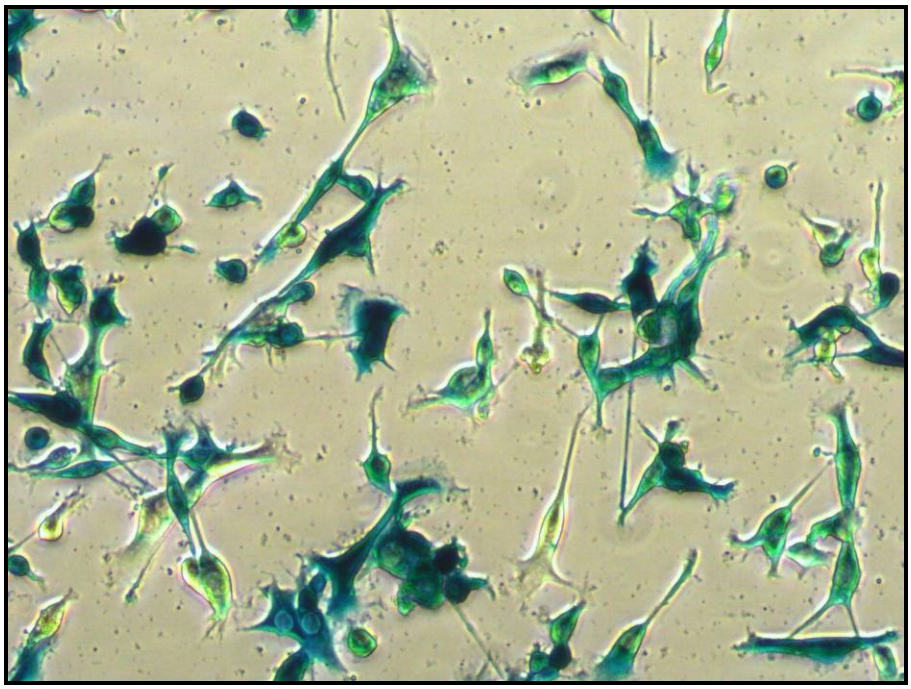

Figure 6. β-galactosidase In Situ Stain. After a 24 hour incubation there was localization of the blue-green color within the cytoplasm of 95% of the CT26.CL25 cells, demonstrating β-galactosidase activity.

VEGF ELISA. The concentration of VEGF in the medium was approximately 8.07 ng/ml (average of adjusted concentration of the 1:32 and 1:64 dilution samples; 8.00 and 8.14 ng/ml, respectively). This demonstrates that the cells are producing VEGF, comparable to that associated with endothelial responses to the growth factor (0.1-25 ng/ml).

Mouse Metastasis Model

Pilot Study

Neither the control nor the tumor cell-injected mice showed signs of distress throughout the duration of the study. There was no significant change in the body weight of control or tumor-bearing animals (Figure 7). There were nodules in the lungs of 9/15 mice injected with CT26.CL25 cells (8 Good/Tumor, 1 No Good).

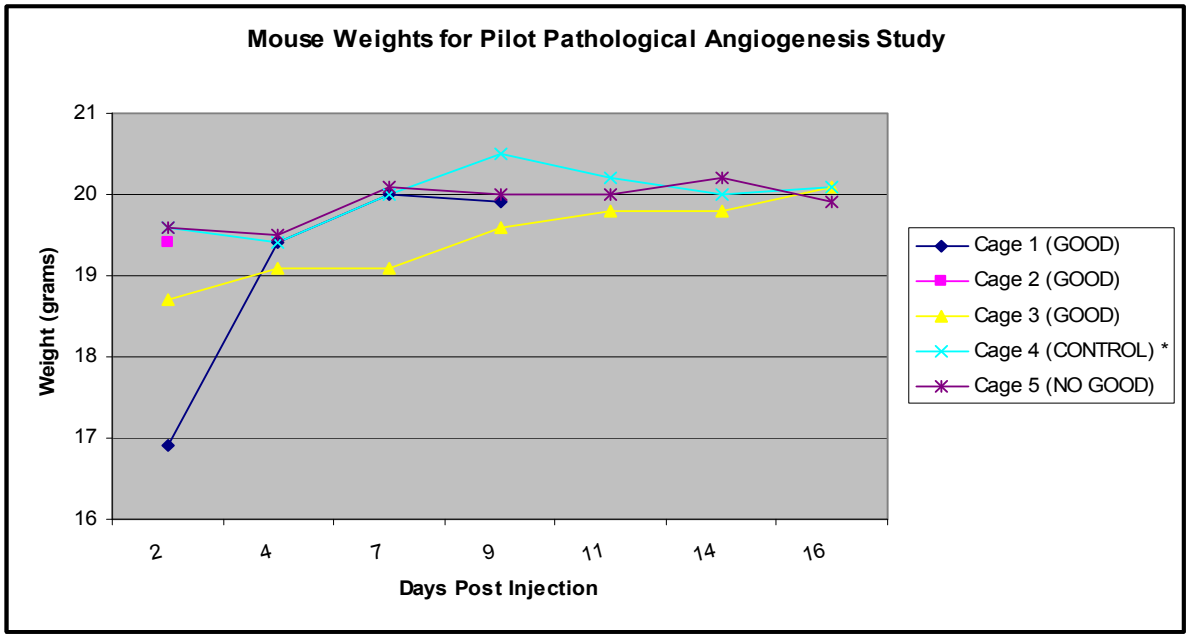

Figure 7. Pilot study: Mouse weights. There was no significant change in the body weight of control or tumor-bearing animals

ß-galactosidase Activity tissue stain. To demonstrate the presence of tumor cells within lung nodules, lung tissue from mice on day 3 post-injection was stained with a solution containing X-gal. No grossly visible nodules were present and no staining was noted after 1, 5 and 25 hour incubation.

Gross and histological findings. There were no significant gross or histologic lesions in any animals on day 3.

On day 9, there were no grossly visible nodules. However, histologically, small pleural and parenchymal nodules (60-100 µm) were noted in treatment “Good” animals (Figure 8).

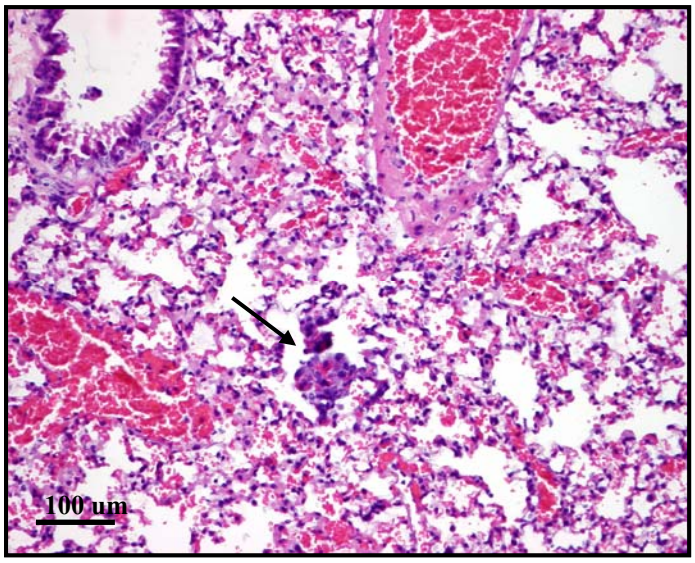

Figure 8. **Pilot Study, Day 9 tumor-bearing lung.** The arrow points to a cluster of neoplastic polygonal cells with round hyperchromatic nuclei, a single nucleolus and eosinophilic cytoplasm with indistinct cell borders. H&E

The nodules were composed of polygonal cells with round hyperchromatic nuclei, a single nucleolus and eosinophilic cytoplasm with indistinct cell borders. There was moderate anisocytosis and anisokaryosis and a rare mitotic figure was noted (metastatic carcinoma). Additionally, there was mild to moderate pulmonary hemorrhage and edema in all animals and segmental suppurative endometritis in one animal. No significant lesions were noted in the liver, heart, brain, kidney, spleen, ovary, stomach or intestine of animals.

On day 14, there were 4-5, 1 mm pink/tan raised nodules on the pleural surface of the lung of one of the “Good” animals. Histologically, there were pleural and subpleural nodules in the lungs of both “Good” animals (Figure 9). The nodules were composed of pleomorphic neoplastic cells, with marked anisocytosis and anisokaryosis and an average of 3 mitotic figures per 400x magnification. The nodules were located near blood vessels and compressed surrounding alveoli and bronchioles. There was mild to marked pulmonary edema and hemorrhage in all animals, including control animals and mild extramedullary hematopoesis (EMH) in 3 of 6 animals. There was moderate suppurative endometritis in one mouse and mild unilateral conjunctivitis in both control mice. There were no other significant lesions in other organs.

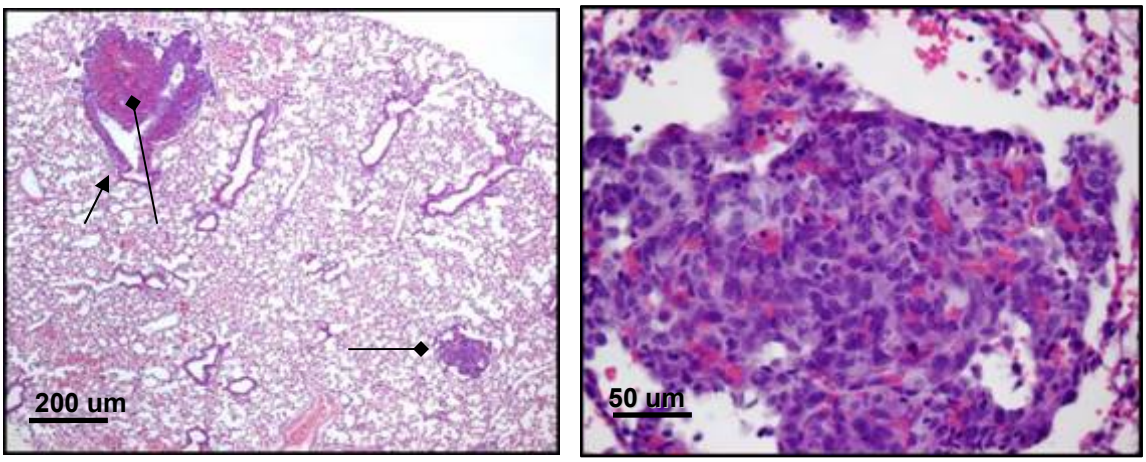

Figure 9. **Pilot Study, Day 14 tumor-bearing lungs.** Tumor nodules are present adjacent blood vessels and compress surrounding alveoli and bronchioles. Diamond headed arrow (——◆) points to nodules and regular arrow points to blood vessel.

On day 16, 2 of 3 treatment "Good" mice had grossly visible pleural nodules. Histologically, pleural and subpleural nodules, similar to those seen in the other mice, were noted in all three treatment "Good" animals and a single nodule was noted in one treatment "No Good" mouse (1/2) (Figure 10).

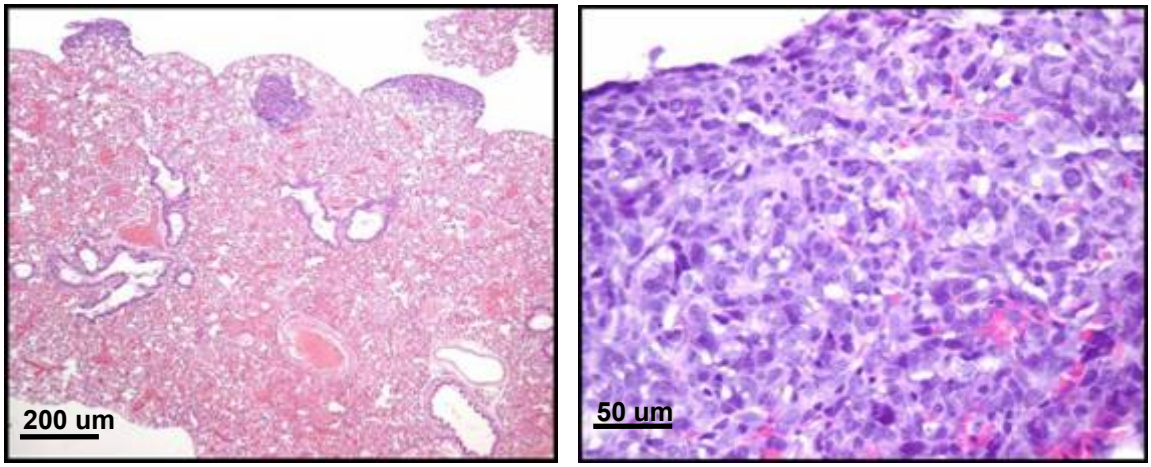

Figure 10. Pilot Study, Day 16 tumor-bearing lungs. There are grossly visible pleural nodules. Histologically, pleural and subpleural nodules similar to those seen in the other mice, were noted in all three treatment "Good" animals and a single nodule was noted in one treatment "No Good" mouse. H&E.

There was moderate to marked anisocytosis and anisokaryosis with an average of 3 mitotic figures per 400x magnification, nuclear folding and elongation of cells. In some nodules, cells formed discernable tubules (metastatic carcinoma). There was suppurative inflammation noted, but no necrosis. In addition, there was mild to moderate atelectasis and all animals had mild to marked pulmonary edema. Other lesions

included suppurative endometritis and vaginitis in 2 mice, enlarged uteri in 3 mice and EMH in 1 mouse. There were no other significant histological lesions in other organs.

Primary Study

As in the pilot study, mice showed no observable signs of distress throughout the study. There were mild changes in body weight but no discernable trends (Table 2) There were grossly visible nodules in the lungs of all 20 Tumor mice and one of the five "No Good" mice injected with CT26.CL26 cells.

Table 2. Primary study: Mouse weights

Cage	Mouse	10/17	10/20	10/23	10/25	10/27	10/30
1	Blue	19.2	20.2	20.4	20.7	20.4	19.9
1	Red	18.7	18.8	18.7	19.1	19	19.1
1	Green	18.1	18.6	18.4	19.0	19.1	19.1
1	Black	18.3	19.1	18.7	18.6	18.8	18.8
1	White	17.8	17.9	17.9	18.6	18.5	18
2	Blue	19.7	19.8	20.2	20.6	20.9	20.9
2	Red	19.7	20.3	20.6	20.7	20.7	20.8
2	Green	18.6	19.0	18.9	19.5	19.3	19.6
2	Black	18.3	19.0	19.4	19.3	19.2	19.4
2	White	18.4	18.8	18.7	19.2	18.9	19.1
3	Blue	18.5	18.7	19.3	19.5	19.7	20.3
3	Red	18.5	18.7	18.7	19.0	19.6	19.1
3	Green	18.2	19.1	18.4	18.7	18.7	19.1
3	Black	18.3	18.8	18.6	19.3	19.4	19.9
3	White	18.5	19.2	18.4	19.2	19.2	19.4
4	Blue	18.8	19.1	19.5	19.4	19.5	19.3
4	Red	18.1	18.0	18.1	18.5	18.2	18.1
4	Green	17.9	18.3	18.7	18.7	18.4	18.4
4	Black	17.6	18.1	18.0	18.7	18.4	18.1
4	White	18.2	18.7	18.6	19.0	18.8	18.5
5	Blue	18.4	19.0	18.7	19.2	19	18.1
5	Red	18.5	18.6	18.9	19.2	19.6	20
5	Green	19.6	19.6	19.9	19.9	20.1	20.4
5	Black	18.4	18.5	18.4	18.9	18.4	18.9
5	White	20.2	20.6	20.6	21.3	21.1	21.4
6	Blue	20.1	20.6	20.7	20.6	21	20.9
6	Red	17.2	17.6	17.4	17.8	18.2	18.6
6	Green	19.2	19.6	19.6	20.1	20.1	20.5
6	Black	17.6	18.0	17.6	17.6	17.8	18
6	White	18.5	18.7	18.7	19.5	19.3	19.1
7	Blue	18.1	18.0	17.8	18.3	18.5	18.4
7	Red	19.5	19.6	19.1	20.1	20	19.9
7	Green	19.4	19.4	19.4	19.5	20.3	19.4
7	Black	19.0	20.0	19.3	19.9	19.8	19.9
7	White	17.7	18.7	18.1	18.5	18.8	18.4
8	Blue	18.2	18.5	18.5	19.0	19.0	18.7

8	Red	19.0	19.5	19.7	20.3	20.1	19.8
8	Green	17.6	18.1	17.7	18.3	18	18.6
8	Black	19.5	20.0	19.8	20.5	20.8	20.2
8	White	18.7	19.5	19.5	20.0	19.7	19.2

Gross and Histopathologic Lesions. Grossly, there were raised pink/tan, 1-2 mm nodules present on the pleural surface of 100% of the tumor treatment animals (20/20) and only one of the No Good treatment animals (1/5). The nodules ranged from five to being too numerous to count. Histologically, there were multiple pulmonary nodules, the largest measuring 200µm, compressing the surrounding alveoli and elevating the pleura. The nodules were composed of polygonal cells forming indistinct glands within a collagenous stroma (metastatic carcinoma). The cells had an oval vesicular nucleus, multiple prominent nucleoli and pale basophilic cytoplasm with indistinct cell borders. There was marked anisocytosis and anisokaryosis and up to 5 mitotic figures per 400x magnification. Many of the nodules were centered on blood vessels and there were congested blood spaces within the nodules (Figure 11). There were scattered free clusters of neoplastic cells within alveoli. There were multifocal areas of alveolar compression surrounding nodules. Additionally, there was mild to moderate intra-alveolar hemorrhage present within tumor-bearing and control animals.

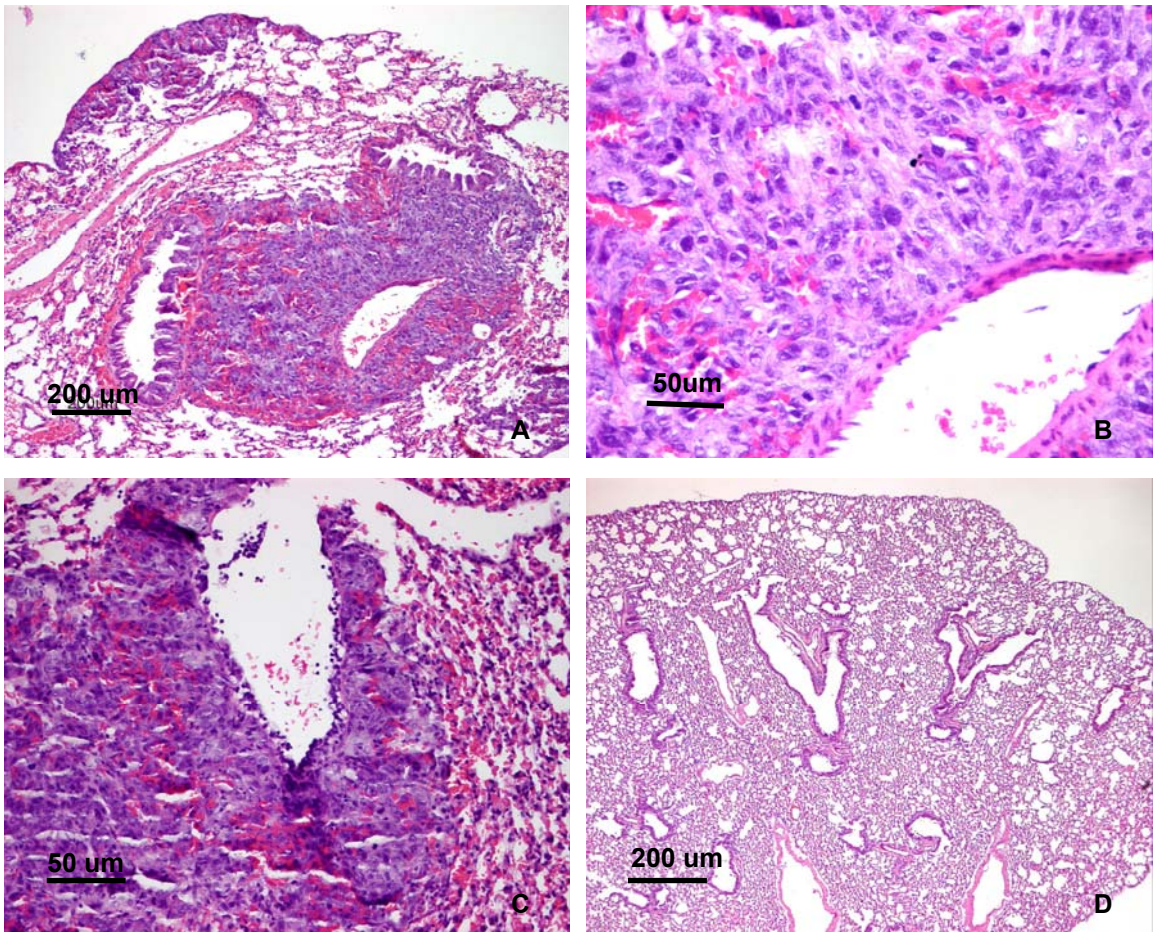

Figure 11. Primary Study, tumor-bearing and control lungs. Tumor-bearing. (A,B, C). There were multiple pulmonary nodules, the largest measuring 200µm, compressing the surrounding alveoli and elevating the pleura. The nodules were composed of polygonal cells forming indistinct glands within a collagenous stroma (metastatic carcinoma). Control (D) mice. H&E

β- galactosidase Activity Tissue Stain. To further demonstrate the presence of tumor cells within lung nodules, lung tissue was stained with a solution containing X-gal. After 1 hour, pleural nodules in tumor-bearing mice showed detectable blue staining. After 24 hours, there was dark blue coloration noted in all the pleural nodules of tumor-bearing animals (Figure 12). There was no color change noted in control mouse lungs. After clearing with graded alcohols and methyl salicylate, the tissue became firm and turned a dark amber color. The pleural nodules were still visible, but appeared darker blue (almost black) and subpleural nodules were visible as a dark blue color.

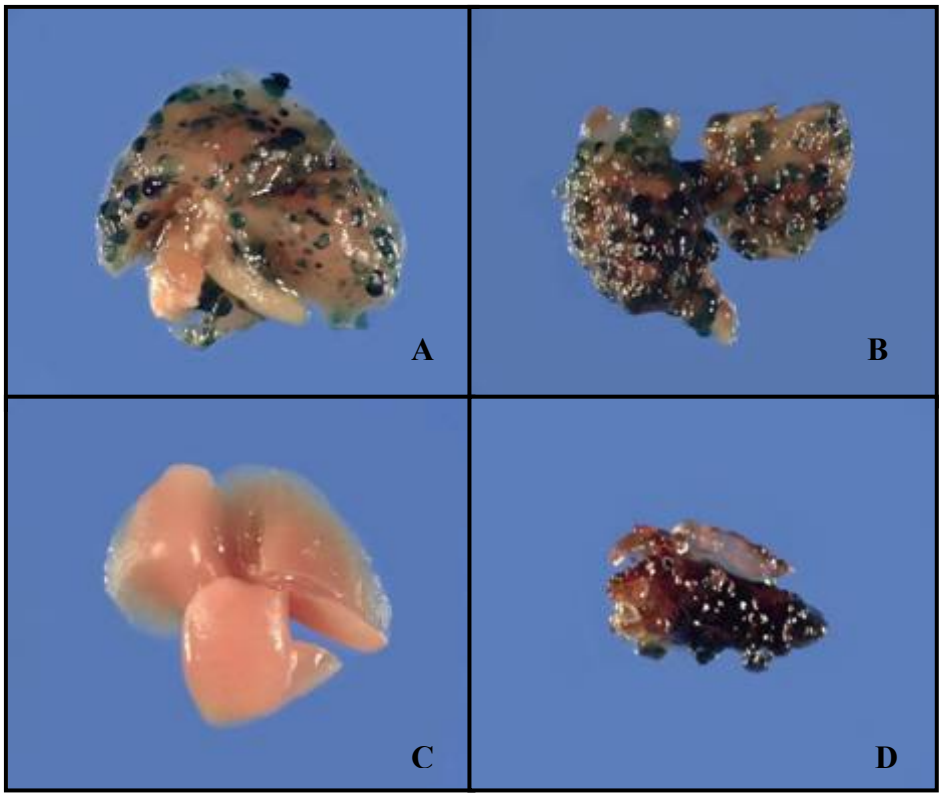

Figure 12. β-galactosidase Activity Tissue Stain. A,B. Lungs from mice injected with 2 x 10^5 CT26.CL25 cells stained with β-galactosidase Tissue Stain. After 24 hours, there was dark blue coloration noted in all the pleural nodules of tumor-bearing animals (A, B). There was no color change noted in control mouse lungs (C). After clearing with graded alcohols and methyl salicylate, the tissue became firm and turned a dark amber color (D). Photos taken by Jerry Baber.

Analysis of mRNA Expression

cDNA Screening PCR. Endpoint PCR was used to screen the synthesized cDNA from control and tumor-bearing lung tissue and CT26.CL25 cells. A band is visible at approximately 186 bp in the lanes corresponding to the tumor-bearing lung and control lung samples (Figure 13). No band is seen in the cultured CT26.CL25 cells lane or either of the NoRT samples from the tumor or control lungs. The positive control produced a band at approximately 186 bp corresponding to the exon 13/exon 14 fragment.

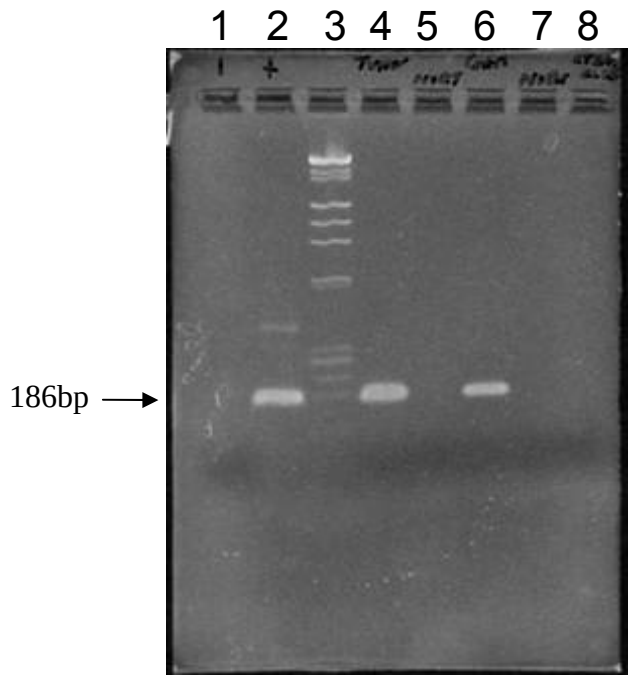

Figure 13. PCR Gel: Flt-1. cDNA Screening, target exon13/exon 14 junction (Flt-1). Lane 1 – negative control (dH_20), Lane 2- Positive control, Lane 3 – Ladder, Lane 4 – Tumor-bearing lung cDNA, Lane 5 – Tumor NoRT, Lane 6- Control lung cDNA, Lane 7 – Control NoRT, Lane 8 – CT26.CL25 cell cDNA. Flt-1 fragment ~ 186 bp noted in Lane 2, 4, 6.

QPCR. Quantitative Real Time PCR was used to evaluate the mRNA expression of various angiogenesis associated growth factors ($VEGF_A$, PlGF) and their receptors (Flt-1, sFlt-1, KDR), a lung associated enzyme (Angiotensin Converting Enzyme [ACE]), a tumor specific marker neomycin phosphotransferase (Neo^R) and a housekeeping gene (18S rRNA). The output is displayed on a logarithmic scale for each individual well, in addition to Ct (Threshold Cycle) values corresponding to the cycle number at which the amount of product reached the threshold level. The average Ct and standard deviation were calculated for each set of triplicate samples. Using the Ct values, ΔCt values (Ct of gene of interest minus Ct for reference gene) were calculated to make comparisons between tumor-bearing and control animals. There were four main comparisons made.

First, to address the primary aim of the study, there is a modest increase in the ratio of sFlt-1:Flt-1 mRNA from control to tumor-bearing animals, manifested as a decrease in the ΔCt (sFlt-1 minus Flt-1) (Figure 14).

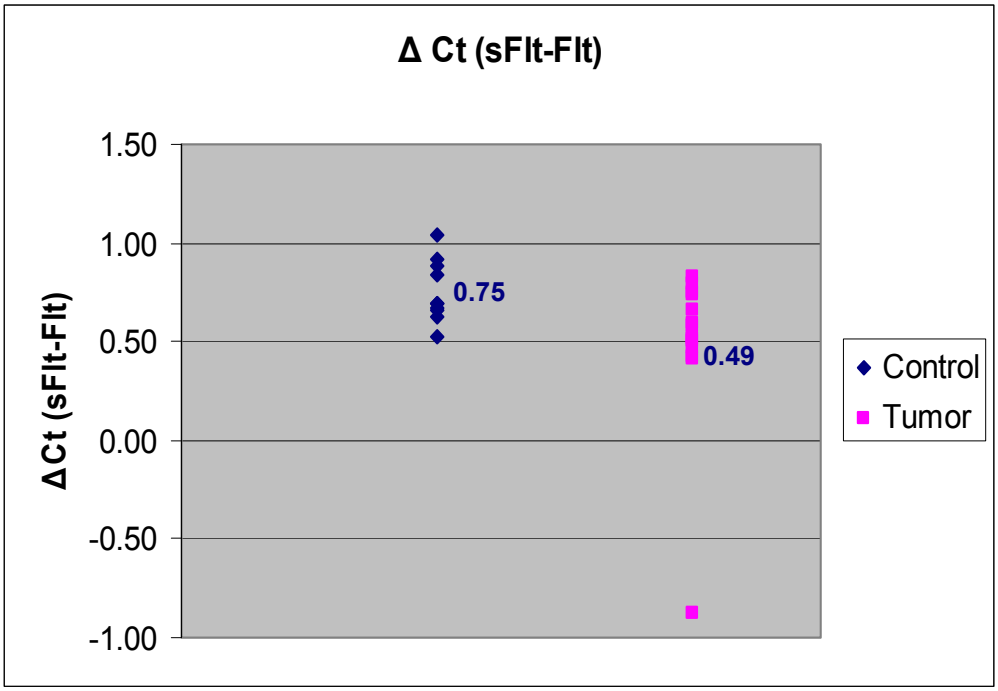

Figure 14. **QPCR: ΔCt (sFlt-Flt) tumor-bearing versus control lung.** There is a modest increase in the ratio of sFlt-1:Flt-1 mRNA from control to tumor-bearing animals, manifested as a decrease in the ΔCt (sFlt-Flt).
There is not a significant difference in the mean ΔCt (sFlt-Flt) for tumor lungs (mean = 0.49 ± 0.42) versus control lungs (0.75 ± 0.16) when all points are included (p=0.0757, 2-sided Student's t-test).
Upon removing the outlier (-0.87), the means become significantly different (p = .0256) (tumor mean = 0.60 ± 0.14; control mean = 0.75 ± 0.16)

Secondly, to determine if neo^R was a good reflection of tumor burden, ΔCt (neo^R – ACE) was calculated. ACE serves as a reference gene based on its localization in lung tissue. In addition, ΔCt (neo^R – ACE) was compared to lung weight. The relationship between neo^R and lung weight is linear (Figure 15). As the lung weight increases, the tumor burden increases, manifested as a decrease in ΔCt (neo^R – ACE).

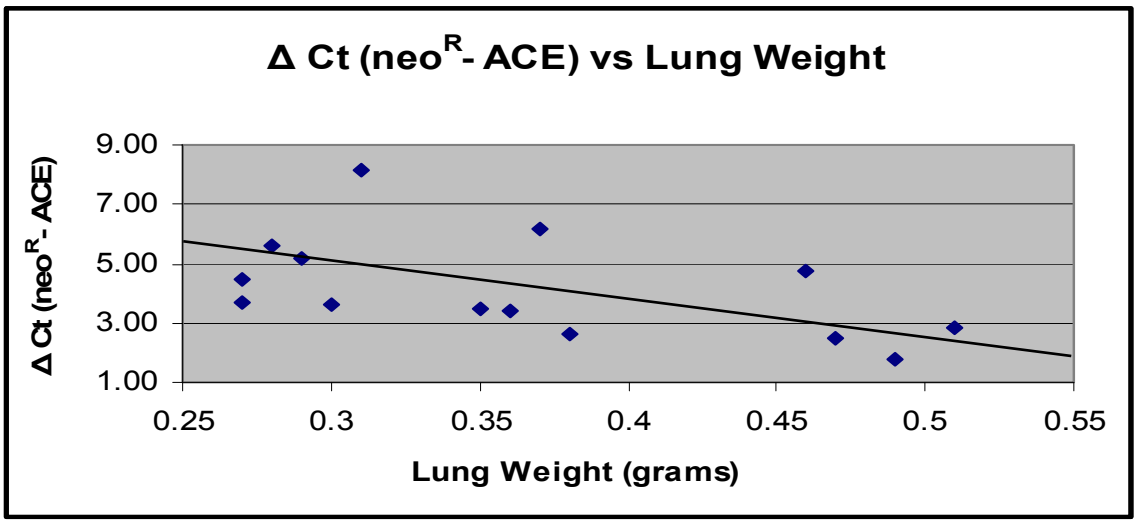

Figure 15. QPCR: ΔCt (neo^R – ACE) versus lung weight. The relationship between neo^R and lung weight is linear (Figure 15). As the lung weight increases, the tumor burden increases, manifested as a decrease in ΔCt (neo^R – ACE). (p=0.0299, one-sided Student's t-test). A line fitted to this data shows a negative slope with an equation: neoR – ACE = 7.87-10.17xweight.

Thirdly, to determine if the expression of sFlt-1, Flt-1, PlGF, KDR and $VEGF_A$ differs between tumor-bearing and control animals, ΔCt (sFlt-ACE), (Flt-ACE), (PlGF-ACE), (KDR-ACE) and (VEGF-ACE) was calculated. ACE reflects the lung's contribution. There was increased mRNA expression of sFlt-1, Flt-1, PlGF and KDR in tumor-bearing versus control animals (manifested as a decreased ΔCt), but not in $VEGF_A$ (Figure 16).

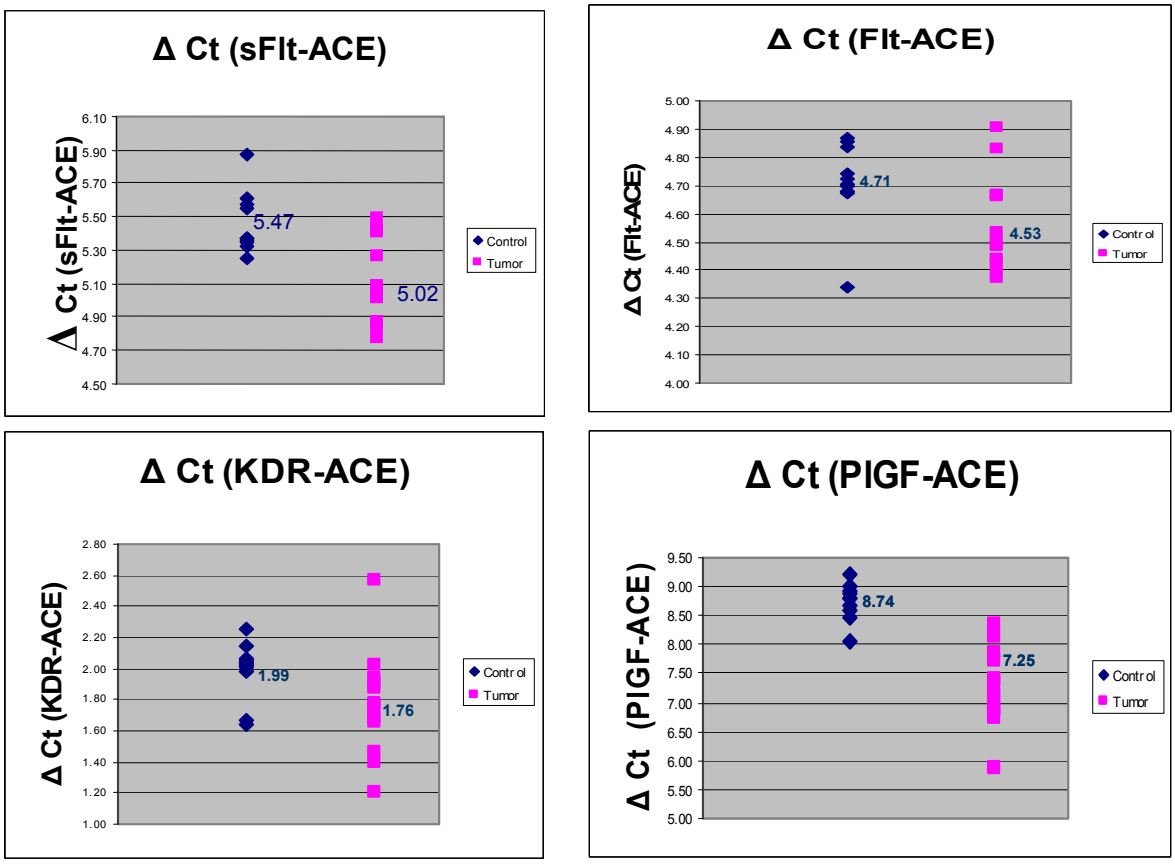

Figure 16. **QPCR: Expression of sFlt-1, Flt-1, KDR and PlGF.** There was increased mRNA expression of sFlt-1, Flt-1, PlGF and KDR in tumor-bearing versus control animals (manifested as a decreased ΔCt). **A.** sFlt (p=0.0111) (tumor mean = 5.02 ± 0.48; control mean = 5.47 ± 0.19), **B**. Flt (p=0.0237) (tumor mean = 4.53 ± 0.22; control mean = 4.71 ± 0.15), **C**. KDR (p=0.0446) (tumor mean = 1.76 ± 0.32; control mean = 1.99 ± 0.19) and **D**. PlGF (p<.0001) (tumor mean = 7.25 ± 0.63; control mean = 8.74 ± 0.32).

There appears to be two distinct populations corresponding to tumor-bearing and control animals when comparing ΔCt (PlGF-ACE) versus lung weight (Figure 17).

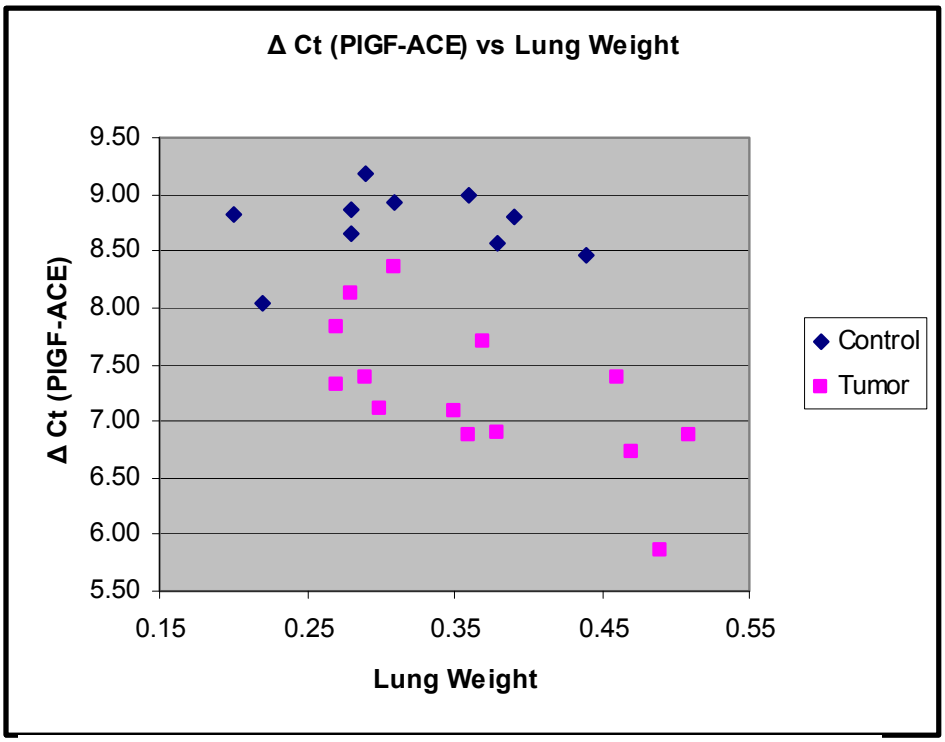

Figure 17. **QPCR: ΔCt (PlGF – ACE) versus lung weight.** There appears to be two distinct populations corresponding to tumor-bearing and control animals when comparing ΔCt (PlGF-ACE) versus lung weight.

This difference from the other targeted genes, suggests a difference in the way PlGF expression changes in the presence of tumors.

Therefore, a fourth comparison was made between PlGF, sFlt and Flt. sFlt and Flt were chosen because they are the genes of primary interest in this study. There is increased PlGF mRNA expression in tumor-bearing versus control animals, manifest as decreased ΔCt (PlGF-sFlt) and ΔCt (PlGF-Flt) (Figure 17). Additionally, PlGF expression appears to increase as tumor burden increases, represented by ΔCt (neo^R minus ACE)

(Figure 18).

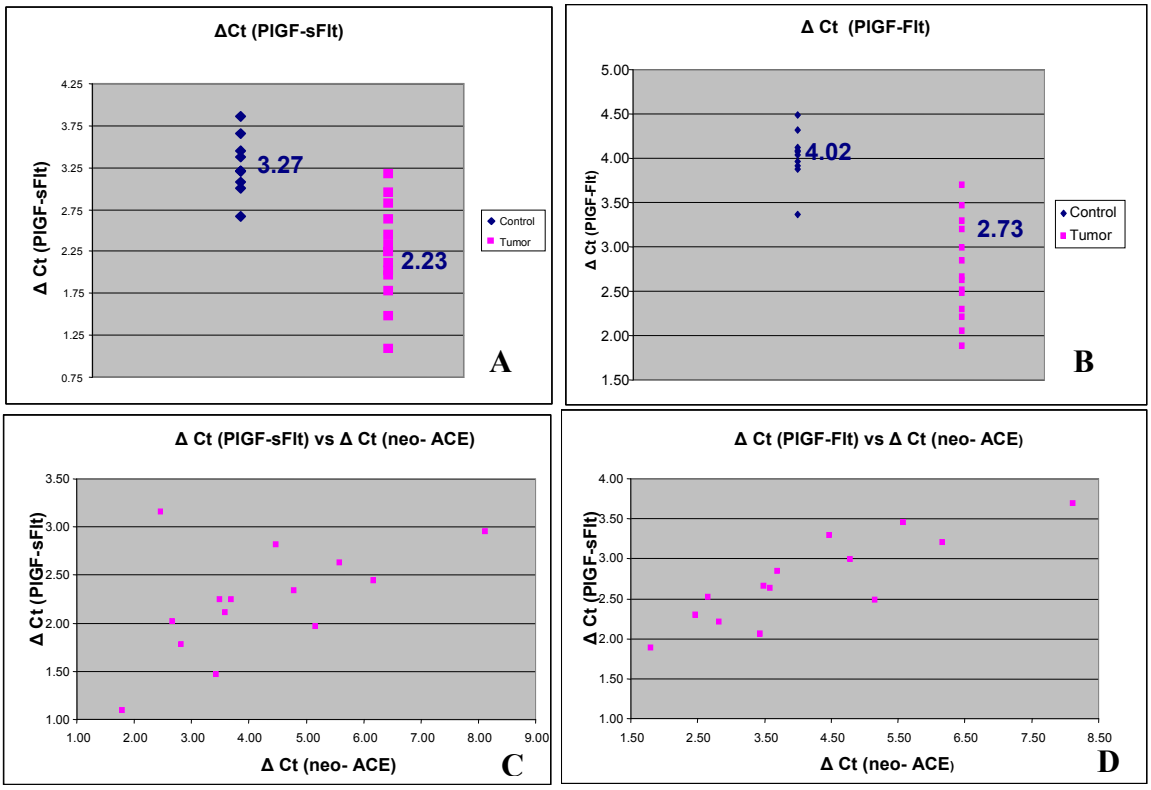

Figure 18. QPCR: Expression of PIGF. PlGF expression appears to increase as tumor burden increases, represented by ΔCt (neo^R minus ACE). There is increased PlGF mRNA expression in tumor-bearing versus control animals when comparing PlGF to sFlt ($p<.0001$) (tumor mean = 2.23 ± 0.56; control mean = 3.27 ± 0.34) (**A**) and Flt ($p<.0001$) (tumor mean = 2.73 ± 0.54; control mean = 4.02 ± 0.30) (**B**). The expression of PlGF increases as tumor burden, ΔCt (neo^R - ACE) increases (**C, D**).

Discussion

Angiogenesis is essential for solid tumor growth and metastasis (Folkman, 1971; Folkman et al., 1987; Ferrara et al., 2003). Vascular endothelial growth factor (VEGF) is the primary angiogenic factor that mediates its actions through Flt-1 and KDR, found on the endothelial cell surface (Barleon et al., 1996; Ferrara et al., 2003). VEGF has been shown to be overexpressed in solid tumors, and to influence tumor growth and metastasis (Stacker et al., 2001; Ilhan et al., 2004). There is a soluble, inhibitory form of Flt-1, denoted sFlt-1, which has been shown to inhibit tumor growth and metastasis by binding VEGF (Kendall and Thomas, 1993; He et al., 1999; Ferrara et al., 2003). Variations in the ratio of sFlt-1:Flt-1 have been shown in tissue undergoing physiologic change (Clark et al., 1998; Krussel et al., 1999). The best example is the placenta where the sFlt-1:Flt-1 ratio increases as pregnancy progresses (He et al., 1999). Additionally, variations exist in pathologic conditions (He et al., 1999; Roberts et al., 2004). The interplay between sFlt-1and Flt-1 in the presence of solid tumors likely contributes to the pro or anti-angiogenic state and the fate of tumor progression. I hypothesize that, in the presence of solid tumors in the lung, there will be a variation in the ratio of sFlt-1 to Flt-1 that is compatible with a pro-angiogenic state.

One of the aims of the study was to implement a mouse metastasis model to study variations in growth factors and receptors, and determine tumor burden. The model was developed by Wang et al. (1995) using a lethal clone of murine derived colon adenocarcinoma cells (CT26.CL25). With intravenous injection these cells form pulmonary metastases. This model has been used by several researchers to study metastasis and angiogenesis. However this is the first time this model has been used to evaluate the mRNA expression of VEGF, VEGF receptors and other VEGF family members in solid tumors. The lung is one of, if not the most common location for tumor metastasis. Its extensive vascularity makes it a prime location for metastatic spread and growth.

The CT26.CL25 cells are transfected with the lacZ gene which encodes for β-galactosidase activity. This allows identification of tumor cells both *in vivo* and *in vitro*, using a *β*-galactosidase activity stain, which provides the cells a substrate (X-gal), and creates a localized colorimetric change in β-galactosidase-expressing cells. In this study, 95% of our cells, *in vitro*, had β-galactosidase activity.

Localization of β -galactosidase activity was demonstrated *in vivo* by using a β -galactosidase tissue stain. In a pilot study, 10^5 cells were intravenously injected and the

presence of lung nodules was evaluated at four time points. Sixty percent of the mice injected with CT26.CL25 cells formed lung nodules. This low metastatic rate could be a result of early temporal evaluation (time point before tumor development) or low metastatic efficiency. Additionally, β -galactosidase activity in lung tissue was not detected in mice on day three, likely due to the same reasons as mentioned above.

In the subsequent study, twice the number of cells was injected resulting in 100% of the mice forming lung nodules. Tumors also grew larger in this study. The increased tumor development and size can be a result of more cells being injected, a longer study time allowing more time for tumor development and/or the inadvertent injection of aggregates of cells. The clumps of cells may have been too large to move through the smaller capillary beds in the lungs, allowing cells to get lodged and grow locally. In this study, demonstration of tumor cells within lung nodules was accomplished by staining with β-galactosidase stain. Lung nodules were visibly blue-green and allowed for evaluation of tumor burden. The ability to evaluate tumor burden and relate it to angiogenic factor expression, could be advantageous for cancer diagnosis.

In addition to β-galactosidase staining allowing gross visualization of tumor burden, other modalities were explored including histopathology, immunohistochemistry, Quantitative PCR (QPCR) and Computed Tomography (CT).

Histopathology is the routine clinical diagnostic tool for evaluating tumors. The presence of micrometastases, which were not noticed grossly, was detected with histopathology. Larger tumors were noted around blood vessels, which is expected since these are metastatic and not primary lung nodules. The use of a polyclonal goat anti-mouse antibody against PECAM-1 (CD31), an endothelial cell marker, to evaluate tumor vascularity did not yield interpretable results owing to high background staining. In addition to repeating this stain, von Willebrand factor, a protein produced by endothelial cells, could also be used to evaluate tumor vascularity.

Localization of β-galactosidase protein within CT26.CL25 cells in lung nodules was attempted by using a mouse monoclonal antibody. There was non-specific staining in tissue. It is possible that the antibody was diluted too much, the temperature and humidity was not right, or the DAB chromagen was not left to incubate long enough. This antibody has been used with success in other labs, but further work is needed to use this antibody for this purpose.

Radiographic imaging is one of the most commonly used diagnostic tools for evaluating tumors, especially lung tumors. MicroCT is being used effectively in clinical

and research settings. Computed tomography is effective when different components of a tissue have contrasting densities. Commonly animals are anesthesized and placed on a ventilator during CT scanning, to retain the lungs normal architecture and to use air contrast to visualize the soft tissue. There was difficulty discriminating the lung nodules from the surrounding soft tissue and trapped fluids with the microCT. There are several reasons, all relating to tissue contrast. In future studies, making the tissue more firm or electron-dense may enhance visualization. Making the tissue more rigid would eliminate the use of a sponge stabilizer. Alternatively, creating more contrast, either by making the nodules more electron-dense with iodine or making the airways or vasculature more electron-dense with Microfill, barium or air, could enhance visualization by microCT.

In this study, there was a modest increase in the ratio of sFlt-1:Flt-1 in the presence of solid vascularized tumors in the lung. This ratio was increased in 13/14 tumor-bearing lung samples. This result was opposite of what was expected. In the solid tumor environment, increased expression of sFlt-1 should contribute to an anti-angiogenic state because of its inhibitory effects on VEGF. Additionally, the fact that these lung tumors are developed in a manner mimicking metastatic progression would suggest dependence on angiogenesis. Increased expression of sFlt-1 would most likely decrease the metastatic potential. An explanation could be the interaction of sFlt-1 with other growth factors such as PlGF or a compensatory change in relative sFlt-1 to Flt-1 expression that was manifest at the time the measurements were made (14 days post-injection).

Furthermore, the expression of Flt-1 has been associated with increased tumor growth and metastasis (Ilhan et al., 2004). In our study, Flt-1 was detected by PCR in tumor and control lung tissue. This is expected because there is expression of Flt-1 on normal cells because of its role in physiologic angiogenesis (i.e. as a receptor for VEGF in normal tissue growth)(Ferrara et al., 2003; Shibuya, 2006). Additionally, there was detectable mRNA expression of Flt-1 in tumor-bearing and control lungs. The absence of measurable Flt-1 cDNA (by end-point RT-PCR) and extremely low Flt-1 mRNA expression (Q RT-PCR) in cultured tumor cells strongly suggests that the presence of Flt-1 in tumor-bearing lungs is associated with endogenous endothelial (stromal) cells. This change in phenotype could occur due to hypoxia or the contribution of surrounding stromal cells (Fukumura et al., 1998; Fong, 2008). There are studies showing that leukocytes, bone-marrow derived cells and platelets can both express Flt-1 and secrete VEGF (Mohle et al., 1997; Ilhan et al., 2004; Lewis et al., 2005). The variation in Flt-1

expression in the present study may be driven by tumor-derived factors or stresses to the tumor microenvironment.

PlGF is another growth factor belonging to the VEGF family of proteins that plays a role in angiogenesis. It also mediates its actions through Flt-1, but can also bind to sFlt-1 (Carmeliet et al., 2001; Luttun et al., 2002). Studies have shown that interactions between VEGF and PlGF in solid tumors play a critical role in angiogenesis (Eriksson et al., 2002). Expression of PlGF was shown to either inhibit or enhance angiogenesis depending on the cell source (DiSalvo et al., 1995; Eriksson et al., 2002).

In our study, there was increased expression of PlGF mRNA (relative to mRNA for ACE, an index of host lung tissue abundance) in tumor-bearing lung tissue. The greater expression of PlGF in tumor-bearing tissue could account for enhanced angiogenesis, manifest as tumor growth and metastasis. This enhancement could be due to increased bioavailability of VEGF to bind to KDR in the presence of competitive binding by PlGF to Flt-1. Additionally, there is increased PlGF expression compared to sFlt-1. Previous studies indicated that increased expression of PlGF may be regulated by the abundance of sFlt-1, shown mostly in females with pre-eclampsia (Shibata et al., 2005). This is related to binding of sFlt-1 by PlGF, displacing VEGF and allowing it to bind to Flt-1 or KDR. Moreover, there appears to be a positive linear relationship between PlGF expression and tumor burden, suggesting that tumor growth is associated with increased expression of PlGF.

QPCR analysis showed that CT26.CL25 tumor cells did not express a detectable amount of PlGF mRNA; conversely the cells did express readily detectable levels of VEGF mRNA, comparable to levels detected in tumor and control lungs. Furthermore, the production of VEGF in concentrations comparable to levels associated with endothelial cell responses to VEGF (8.07 ng/ml, range 0.1-25 ng/ml, respectively) was verified by ELISA. These results are consistent with other studies showing expression of VEGF by tumor cells and in solid tumor tissue. Additionally, VEGF is a growth factor produced by normal pulmonary cells. Therefore expression in control (normal) lung tissue is expected.

There was increased KDR mRNA expression in tumor-bearing lungs. However, there was low mRNA expression in cultured CT26.CL25 cells, suggesting that the increased expression is associated with a pro-angiogenic environment (Ilhan et al., 2004).

The increased expression of phosphotransferase (neo^R) mRNA in tumor-bearing animals was expected, due to the transfected neo^R gene. The expression of neo^R is positively correlated to lung weight, which supports the use of neo^R mRNA measurement as a determinant of tumor burden.

There was pulmonary hemorrhage and edema noted in most animals. This is associated with euthanasia by CO_2 inhalation. There was no evidence of metastases in other organs. Other pathologic changes noted in mice were unrelated to cell injections and more related to age, sexual maturity and housing of multiple animals in one cage. Minor changes in weight were a result of normal growth with increasing age.

Overall, the presence of solid tumors resulted in a modestly increased sFlt-1:Flt-1 ratio. The mechanism that regulates the expression of either is still not completely understood. However, in the presence of solid tumors, lower sFlt-1 expression might logically be predicted, based on the expectation that the microenvironment would be pro-angiogenic. However, overexpression of PlGF mRNA in the presence of increased sFlt-1 mRNA in tumor-bearing lungs does suggest an interaction between the proteins they encode. The displacement of VEGF from sFlt in the presence of increased PlGF could result in higher availability of VEGF; whereas higher expression of sFlt-1 and/or Flt-1 could increase the “VEGF buffering” capacity of such a system.

Future Studies. The presence of metastases within the lung and the over-expression of PlGF, sFlt-1, Flt-1 and KDR suggest their role in tumor growth and metastasis. Tumor growth was only evaluated as present or not present. Therefore, making determinations as to the efficiency of tumor growth cannot be done. However, the expression of these various factors within metastates does shed some light on their involvement and the mechanism of metastatic development. Laser Capture Microdissection could be used to evaluate the mRNA expression of angiogenic factors within individual tumor or host cells. Additionally, determining tumor burden by weighing or measuring tumor tissue could enhance the use of mRNA expression of tumor specific genes. Also, determining the expression of various factors at time points during tumor development may reveal differing variations in angiogenic factors as tumors progress. And lastly, pairing these results with clinical data such as blood chemistries and counts could contribute to understanding the overall effects of tumor growth and metastasis.

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Appendix 1: Materials

1. Media Components

	Final Concentration	Volume
1X RPMI 1640 buffer with L-glutamine		500 mls
Fetal Bovine Serum (lab stock)	10%	47 ml
100X Sodium pyruvate	1Mm	5 ml
100X Non-Essential Amino Acids	0.1 mM	5 ml
1M HEPES	10 mM	5 ml
Gentamicin	10 mg/ml	2.8 ml
20 % solution Glucose	2.5 g/L	7 ml
50mg/ml G418	0.4mg/ml	80 ul

2. Materials

Ambion

RNAlater

Applied Biosystems

Optical 96 well Plate

Optimal Plate Sealing Film

Murine $VEGF_A$ primer/6FAM-labeled probe mix

Murine ACE primer/6FAM-labeled probe mix

18S ribosomal RNA primer

18S ribosomal RNA VIC-labeled probe

BIO-RAD

iScript Select cDNA Synthesis Kit

Costar

Cell scraper

DAKO

DAKO Chromagen System

Falcon

T75 culture flasks

Fisher

10% Neutral Buffered Formalin

99% Methyl Salicylate

MediaTech

RPMI 1649 buffer with L-glutamine

Sodium Pyruvate

Gentamicin

G418

Millipore/Specialty Media

In-situ *B*-galactosidase Expression stain

Tissue *B*-galactosidase Expression stain

PROMEGA

Monoclonal mouse Anti-*B*-galactosidase antibody

QIAGEN

Qiagen RNeasy Mini Kit

- RNA extraction from cells
- Isolation of Total RNA from Animal Cells
- RNase –Free DNase Set

2X Taqman® PCR mix

R&D Systems

Quantikine M Murine VEGF ELISA

Santa Cruz

Polyclonal Goat Anti-Mouse PECAM-1 antibody

SIGMA

1M HEPES

Vector

Vectastain ELITE ABC Kit

Low pH Unmasking Solution

Zymed

Histostain Bulk Kit Lab-SA Detection System

Peroxo-Block Solution