ANGIOPOIETIN-2 INHIBITION BY THE INVESTIGATIONAL ANG-2 ANTIBODY MEDI3617 IN RENAL CELL CARCINOMA INDUCED ANGIOGENESIS: THERAPEUTIC IMPLICATIONS

By

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To my Mom and Dad who brought me into this world and supported me with all my endeavors

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Angiogenesis, the formation of new blood vessels from pre-existing ones, is a hallmark of cancer. Angiogenesis can be separated into two key steps, (a) vascular destabilization mediated by the Angiopoietin/Tie2 axis and (b) endothelial cell activation heavily influenced by the Vascular Endothelial Growth Factor (VEGF) and its receptor VEGFR-2. In the last decade the use of VEGF axis targeted anti-angiogenic agents has become a common therapy in several oncologic settings. However, their clinical efficacy has been limited. Recently, a new class of anti-angiogenic agents targeting Angiopoietin-2 (Ang-2) has emerged in hopes to circumvent lack of response or resistance seen with VEGF targeted agents. There are still many unanswered questions about the biology of the Ang/Tie2 axis and vigorous evaluation of agents targeting this axis is necessary. The presented research focuses on the preclinical evaluation of an investigational fully human monoclonal antibody, MEDI3617, targeting Ang-2 in the renal cell carcinoma setting. We explored the basic principle of Ang-2 dependent vascular destabilization, the loss of endothelial and peri-endothelial cell contacts, and the effect of Ang-2 inhibition. The tumor microenvironment heavily influences tumor

angiogenesis and its effects on the Ang-2/Tie2 axis remain controversial. Key microenvironmental factors and their effect on the Ang-2/Tie2 axis were determined along with the anti-angiogenic effect of Ang-2 inhibition especially on vascular structure. Real time imaging of tumor microvascular response to both VEGF and Ang-2 targeting anti-angiogenic agents was pursued in the murine dorsal skinfold window chamber model that revealed limitations to this model. Additionally, the anti-tumor effects of Ang-2 inhibition were explored both in the primary and metastatic tumor setting. Finally, combinatorial approaches with VEGF targeting agents were evaluated as anti-angiogenic modalities. The presented work shed light on several important considerations when using Ang-2 targeted agents and future directions for the use of these agents are discussed.

CHAPTER 1
INTRODUCTION

Cancer remains the second leading cause of death in the United States affecting millions of lives each year. While advances in diagnostics and treatment have led to a 3.4-fold increased patient survival over the last 40 years, many disease settings remain without any progress, high rate of recurrence or fatality from metastatic disease (de Moor et al., 2012). Increased knowledge of cancer biology has led to the identification of important factors involved in the development of cancer and the discovery of a plethora of potential therapeutic targets. However, with increased knowledge the growing complexity of this disease is unfolded; as one door closes many more open in the fight against cancer. Nevertheless our efforts bring us closer to better management of this disease one step at a time.

Conventional Cancer Therapies

Conventional cancer therapies such as chemotherapy and radiation therapy target rapidly proliferating cells by inducing DNA damage. These therapies are used in several settings; they can be used with a curative intent or palliative treatment to manage the pain associated with advanced and metastatic disease. For curative treatments they can be used either (a) as neoadjuvant, before surgical resection of a tumor to reduce the size or impair the growth of the tumor or as (b) adjuvant treatment with surgery or chemotherapy and radiation therapy combined.

Chemotherapeutic agents target rapidly proliferating cells in a cell cycle specific or independent manner (Siu and Moore, 2005). These therapies can also be highly toxic to normal tissues yielding a narrow therapeutic window. Tumor resistance to these agents stems both from Gompertzian growth of and impaired delivery to the tumor cells

due to irregular tumor vasculature (Cole and Tannock, 2005; Minchinton and Kyle, 2011; Minchinton and Tannock, 2006). Radiation therapy provides another means to treat cancers. Ionizing radiation causes DNA damage resulting in tumor cell death (Bristow and Hill, 2005). This type of therapy also has detrimental effects on normal tissues and the therapeutic outcome can be greatly affected by the tumor microenvironment (Bristow and Hill, 2008; Giaccia and Hall, 2006a). Hypoxic cells are resistant to radiation and pose a real challenge in the treatment of cancer (Bristow and Hill, 2008; Giaccia and Hall, 2006b). Efforts to improve oxygenation in the tumor have shown some improvement in treatment outcome (Overgaard and Horsman, 1996). Targeting the tumor vasculature offers a different approach to combat cancer and combinations with chemotherapy and radiation therapy have shown that these treatments may be complementary (Horsman and Siemann, 2006; Siemann, 2011).

Tumor Vasculature And Angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing ones is a crucial process in normal vascular development and physiological conditions such as wound healing, reproduction and the menstrual cycle (Papetti and Herman, 2002). The importance of angiogenesis in pathological conditions has been well established (Folkman, 2007). Angiogenesis in physiological conditions is tightly regulated by a balance of pro-and anti-angiogenic factors, in disease settings this balance is tipped to favor pro-angiogenic factors (Folkman, 2007; Papetti and Herman, 2002).

A growing tumor cannot sustain its growth without the initiation of angiogenesis (Folkman, 1971; Goldmann, 1908) (Figure 1-1). As the tumor grows, cells existing further than the oxygen diffusion distance (70-100 μm) from blood vessels, become

hypoxic (Hanahan and Folkman, 1996). Hypoxic cells respond to low oxygen levels by upregulating genes involved in metabolism and angiogenesis in order to survive (Betof and Dewhirst, 2011; Dewhirst, 2006). Hypoxia Inducible Factor (HIF) is stabilized under low oxygen conditions and triggers transcription of pro-angiogenic factors such as Vascular Endothelial Growth Factor (VEGF), a key activator of angiogenesis (Charlesworth and Harris, 2008). Fibroblast growth factor (bFGF), Notch and delta-like ligand 4, Interleukin-8 (IL-8), heparanase, and matrix metallo-protease 2 (MMP-2) are also important pro-angiogenic factors in tumor angiogenesis (Ferrara, 2010; Papetti and Herman, 2002). Tumor cells secrete these growth factors and stimulate endothelial cells to form new vasculature towards the tumor (Leung, 1989; Shibuya, 2008). As the tumor grows, angiogenesis is continuously activated to sustain tumor growth and the vasculature expands (Figure 1-1). Tumor vasculature is distinct from normal vasculature. Normal vessels are quiescent with minimal endothelial cell proliferation, tight endothelial-endothelial cell and endothelial-peri-endothelial mural cell (e.g. smooth muscle cells and pericytes) contacts to regulate vessel permeability (McDonald, 2008). Tumor vessels, on the other hand, have highly proliferative endothelial cells with loose to minimal endothelial-endothelial and endothelial-peri-endothelial cell contacts yielding a leaky vascular phenotype (Minchinton and Kyle, 2011; Vaupel, 2006). Yet despite being highly active, the leaky tumor vasculature is unable to provide the tumor with its oxygen and nutritional needs leading to tumor microenvironments that have proven to be problematic in the treatment of cancer (Minchinton and Kyle, 2011; Minchinton and Tannock, 2006).

Anti-Angiogenic Therapy

Anti-angiogenic therapy targets the tumor vasculature, indirectly affecting tumor growth. It was proposed by the late Judah Folkman and Julie Denekamp over 40 years ago, who hypothesized that depriving the tumor of oxygen and nutrients, by targeting the tumor vasculature could have therapeutic benefits (Denekamp, 1982; Denekamp, 1984; Folkman, 1971). Identification of growth factors and signaling pathways important in angiogenesis resulted in the production of inhibitors and in the past decade anti-angiogenic agents have become part of the standard of care in several disease settings (Folkman, 2007; Meadows and Hurwitz, 2012; Young and Reed, 2012). To date, there are thirteen agents with specific or non-specific anti-angiogenic activity (antibody and small molecule inhibitors) that are approved by the FDA for treatment of advanced and metastatic renal cell carcinoma (Bevacizumab, Axitinib, Pazopanib,Sorafenib, Sunitinib, Temsirolimus, Everolimus, Interferon α) , metastatic colorectal cancer (Bevacizumab, Regorafenib), gastrointestinal cancer (Sunitinib), non small cell lung cancer (Bevacizumab), hepatocellular carcinoma (Sorafenib), thyroid cancer (Cabozantinib, Vandetanib), pancreatic neuroendocrine tumors (Sunitinib, Everolimus), glioblastoma (Bevacizumab), multiple myeloma (Thalidomide, Lenalidomide), astrocytoma (Everolimus); leukemia (Interferon α), melanoma (Interferon α) and AIDS-related Kaposi sarcoma (Interferon α). Many other agents are in clinical trials for a wide variety of cancers. Bevacizumab (Avastin), a monoclonal antibody targeting the VEGF ligand, has proven to be most successful thus far and is approved for the treatment of glioblastoma, metastatic colorectal, non small cell lung cancer, renal cell carcinoma and until recently metastatic breast cancer (Hurwitz et al., 2004; Montero et al., 2012; Pollack, 2011).

Although these agents, in general, alone show minimal anti-tumor effects they have been shown to complement conventional cancer therapies in reducing tumor growth (Horsman and Siemann, 2006; Kerbel, 2006; Siemann, 2011). Combination of such treatments resulted in a slightly wider therapeutic window for both chemotherapy and radiation therapy enhancing tumor cell kill. It's been proposed that anti-angiogenic treatment yields a window of vascular normalization that leads to better chemotherapeutic drug delivery to the tumor core and overall reduced hypoxia within the tumor resulting in better cell kill by radiation therapy (Bottsford-Miller et al., 2012; Jain, 2005a; Siemann, 2011). Anti-angiogenic therapy does have side effects, most commonly hypertension and occasional bleeding along with impaired physiological angiogenesis such as wound healing, reproduction and fetal development (Elice and Rodeghiero, 2012; Hayman et al., 2012; Pralhad et al., 2003). These side effects limit the population of patients that can be treated with such therapy as well as affect the scheduling of treatment for approved patients. However, removal of these agents from the patients' therapeutic regiment quickly restores physiological angiogenesis, making it easy to remove or resume treatment before or after major surgeries.

Unfortunately, tumor resistance to these agents can develop and pose difficulties in successful treatment, lack of overall patient survival led to the removal of Bevacizumab for the treatment of metastatic breast cancer (Ebos et al., 2009; Loges et al., 2010; Montero et al., 2012; Pollack, 2011). Tumors can develop acquired resistance to anti-angiogenic therapies by upregulation of pathways in angiogenesis that are not inhibited by the therapy (Bergers and Hanahan, 2008). Even though VEGF is a major driver of angiogenesis there are other growth factors such as basic fibroblast growth

factor (bFGF), interleukin-8 (IL-8), placental growth factor (PlGF), platelet derived growth factor (PDGF), and insulin like growth factor (IGF) that can compensate in the absence of VEGF pathway activation (Bergers and Hanahan, 2008). Due to such resistance and compensatory mechanisms there is a need for superior anti-angiogenic modalities to fulfill the potential of this vascular targeted therapeutic strategy (Loges et al., 2010).

Angiopoietin/Tie2 Axis

Tyrosine Kinase with Ig and EGF homology domains-2 (Tie2) and its ligands Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), Angiopoietin-3 and Angiopoietin-4 (Ang-4) were identified in the last fifteen years (Davis et al., 1996; Partanen et al., 1992; Ribatti et al., 2000; Schnurch and Risau, 1993; Valenzuela et al., 1999). Ang-1 and Ang-2 are the best characterized of the four ligands and their interactions with Tie2 receptor comprise an important endothelial cell-specific receptor tyrosine kinase signaling system in angiogenesis (Augustin et al., 2009; Loughna and Sato, 2001; Thomas and Augustin, 2009). Tie2 is mainly expressed by vascular and lymphatic endothelial cells and is a transmembrane tyrosine kinase receptor with its tyrosine kinase domain in the cytoplasm (Partanen et al., 1992; Schnurch and Risau, 1993). Tie1 is a receptor that is currently considered an orphan receptor, neither angiopoietin ligands are known to directly bind; however its interaction with angiopoietins and Tie2 has been shown to be important in lymphatic vascular development (D'Amico et al., 2010; Song et al., 2012; Yuan et al., 2007). Structurally, Ang-1 and Ang-2 ligands have two domains, the N-terminal coiled-coil domain responsible for homo-oligomerization of the ligands into trimers, tetramers and pentamers and a C-terminal fibrinogen like domain responsible for receptor binding (Davis et al., 2003; Procopio et al., 1999).

Oligomerization of the ligands has been shown to be important for receptor activation but not binding (Procopio et al., 1999). Ang-1 has 498 amino acids and Ang-2 has 496 amino acids, the two ligands share 60% amino acid sequence homology (Davis et al., 1996; Maisonpierre et al., 1997; Procopio et al., 1999).

Ang-1 and Ang-2 are secreted proteins that interact with Tie2 either in a paracrine (Ang-1) or autocrine (Ang-2) manner; Ang-1 is expressed and secreted by peri-endothelial mural cells (smooth muscle cells, pericytes) while Ang-2 is expressed and secreted by endothelial cells (Maisonpierre et al., 1997). Both angiopoietins bind Tie2 with similar affinities and on the same site on the receptor at the IgG-like and EGF-like domains (Barton et al., 2005; Fiedler et al., 2003). These ligands, however, have opposing functions. Ang1-Tie2 signaling controls vessel quiescence, while Ang2-Tie2 association allows for vessel plasticity (Saharinen and Alitalo, 2011) (Figure 1-2A). Ang1-Tie2 presence and interaction are essential for vessel maturation in embryonic vasculogenesis while Ang-2 expression is expendable for normal embryonic development as shown by loss-of-function studies (Gale et al., 2002; Maisonpierre et al., 1997). The absence of both Ang-1 and Tie2 lead to embryonic lethality due to aberrant vessel remodeling and maturation while the over expression of Ang-2 yields similar phenotypes; these genetic studies support the agonistic, non-redundant role of Ang-1 and antagonistic role of Ang-2 with Tie2 receptor interactions (Maisonpierre et al., 1997; Sato et al., 1995; Suri et al., 1996; Thomas and Augustin, 2009).

In the adult, Ang-1 is found in many types of tissues and is constitutively secreted in low levels throughout the body (Maisonpierre et al., 1997). Ang-2 is found in tissues that undergo vessel remodeling and is secreted by endothelial cells at sites of active

angiogenesis (Maisonpierre et al., 1997). Ang1-Tie2 maintains a stable vasculature through mediating the tight interaction of endothelial cells with mural cells while Ang2-Tie2 functions in their destabilization (Augustin et al., 2009; Maisonpierre et al., 1997). It is believed that Ang-1/Tie2 interaction leads to downstream signaling through Rho and mDia inhibiting VEGF/VEGFR2 mediated vascular permeability through src while Ang-2/Tie2 interaction leads to loss of receptor activation and intracellular signaling (Augustin et al., 2009; Gavard et al., 2008; Thomas and Augustin, 2009). However, it has been shown that Ang-2 can act as a Tie2 agonist in a context dependent manner mainly in the lymphatics and with Tie-2 expressing tumor associated macrophages (Gale et al., 2002; Mazzieri et al., 2011; Venneri et al., 2007).

It appears that the context dependent role of Ang-2 as an agonist or antagonist is mediated by the interaction of Tie2 with Tie1 receptor. Even though none of the angiopoietin ligands bind to Tie1 the receptor can heterodimerize with Tie2 mainly affecting the role of Ang-2. Ang-1 always interacts with Tie2 homodimers however Ang-2 can interact with Tie2 homodimers and Tie1/Tie2 heterodimers. Ang-2 binding to Tie2 homodimers leads to agonistic interactions similar to Ang-1/Tie2 interactions; this is mainly seen in lymphatic vasculature where Tie1 receptor is sparsely expressed (Gale et al., 2002; Song et al., 2012). However in blood endothelial cells Tie1 is commonly expressed leading to an increased Tie1/Tie2 heterodimer interaction with Ang-2 resulting in an antagonistic role of Ang-2 and lack of intracellular signaling leading to vessel destabilization (Kim et al., 2006; Seegar et al., 2010). Recently it has been shown that the ability of Ang-2 to interact with both Tie2 homodimers and Tie1/Tie2 heterodimers is a difference of three amino acids (proline, glutamine, arginine) in the

protein sequence outside the receptor binding site compared to Ang-1 (threonine, alanine, glycine) (Yu et al., 2013).

Based on current knowledge of the angiopoietin/Tie2 axis, angiogenesis can be separated into two key steps (a) destabilization of normal vasculature mediated by the Ang-2/Tie2 axis followed by (b) endothelial cell activation by pro-angiogenic factors such as VEGF (Figure 1-2). In the absence of pro-angiogenic factors destabilized vasculature has been shown to regress due to endothelial cell apoptosis (Holash et al., 1999; Korff et al., 2001).

The angiopoietin ligands are also considered important factors in inflammation, Ang-1 as anti-inflammatory and Ang-2 pro-inflammatory (Fiedler and Augustin, 2006; Fiedler et al., 2006). Ang-1 signaling through Tie2 leads to repression of nuclear factor-κB (NF- κB) while Ang-2 interaction with Tie2 activates it (Augustin et al., 2009). Ang-1 dependent phosphorylation of Tie2 decreases the synthesis of Ang-2 in endothelial cells by activating the PI3K/Akt pathway and inactivating the FOXO1 transcription factor (Thomas and Augustin, 2009; Tsigkos et al., 2006). However, Ang-2 is stored in Weibel-Palade Bodies and can be quickly secreted upon microenvironmental changes (Dixit et al., 2008; Fiedler et al., 2004). There are various factors that can lead to Ang-2 secretion from endothelial cells such as histamine and thrombin that play a role in vasodilation and recruitment of inflammatory cells and clotting respectively in wound healing (Fiedler et al., 2004; Huang et al., 2002). Hypoxia is another potent upregulator of Ang-2 levels in endothelial cells and may lead to Ang-2 release mediated through the COX2-PGE2 pathway rather than the HIF-1α dependent route; the inflammatory mediator COX2 is regularly seen in cancerous tissue (Leahy et al., 2000). Finally,

sphingosine-1 phosphate (SP1), secreted by immune cells and platelets, can also lead to Ang-2 release from endothelial cells (Jang et al., 2009). Elevated Ang-2 levels in tumor vasculature have been observed in many cancers and seem to correlate with poor disease prognosis (Ahmad et al., 2001; Bach et al., 2007; Bhaskar et al., 2013; Currie et al., 2002; Detjen et al., 2010; Diaz-Sanchez et al., 2013; Engin et al., 2012; Goede et al., 2010; Helfrich et al., 2009; Imanishi et al., 2007; Lind et al., 2005; Mitsuhashi et al., 2003; Moon et al., 2006; Stratmann et al., 1998; Sugimachi et al., 2003; Tait and Jones, 2004; Tanaka et al., 2002; Tsutsui et al., 2006; Xia et al., 2012; Yamakawa et al., 2004; Zagzag et al., 1999).

Angiopoietin-2 In The Tumor Microenvironment

Elevated Ang-2 levels have been associated with advanced disease, progression and poor prognosis in several disease settings such as astrocytoma, glioblastoma, colon, gastric, colorectal, breast, prostate, kidney, hepatocellular cancers as well as multiple myeloma, melanoma, and neuroendocrine tumors (Ahmad et al., 2001; Bhaskar et al., 2013; Currie et al., 2002; Detjen et al., 2010; Engin et al., 2012; Goede et al., 2010; Helfrich et al., 2009; Lind et al., 2005; Moon et al., 2006; Stratmann et al., 1998; Sugimachi et al., 2003; Tsutsui et al., 2006; Xia et al., 2012; Yamakawa et al., 2004; Zagzag et al., 1999). Ang-2 serum levels significantly increase in patients compared to healthy individuals and patients with more advanced disease show significantly higher levels of Ang-2 compared to patients with earlier stage disease (Detjen et al., 2010; Helfrich et al., 2009; Lind et al., 2005; Park et al., 2007; Sie et al., 2009). The expression of Ang-2 in chronic lymphocytic leukemia has been shown as a pro-survival phenotype (Xia et al., 2012). Several reports also implicate an integrin–Ang-2 interaction in metastatic breast and glioma (Carlson et al., 2001; Hu et al., 2006;

Imanishi et al., 2007). Furthermore, in the presence of Ang-2 tumor associated macrophages (TAMs) express Tie2 and show pro-angiogenic activity (De Palma and Naldini, ; De Palma and Naldini, 2011). Ang-2 is not only a pro-angiogenic but also a pro-inflammatory factor and elevated Ang-2 serum levels correlate with severity of sepsis (systemic inflammatory response to infection) that often leads to multiple organ dysfunction syndrome, an issue commonly found in emergency and trauma patients (David et al., 2012; Gallagher et al., 2008; van der Heijden et al., 2009). The presence and importance of Ang-2 in the tumor microenvironment and its role in angiogenesis has made this ligand an attractive target as an anti-angiogenic modality in cancer therapy.

Angiopoietin/Tie2 Targeting Agents

In the last few years there has been tremendous interest in targeting the Ang-2/Tie2 axis in angiogenesis in hopes to circumvent the lack of response or adaptive resistance that has been seen in the clinic to VEGF targeted agents. Currently there are no small molecule inhibitors that are selective to Tie2 in clinical development, however several agents in clinical trials do have some activity against the Tie2 axis (Gerald et al., 2013). There are two peptide-based agents, CVX-060 and Trebananib (AMG386) that are in phase II and III clinical trials respectively. Trebananib is the furthest in clinical development and targets both Ang-1 and Ang-2, which has raised some questions about the proper way to target the Ang/Tie axis since Ang-1 is considered to be the vascular stabilizer (Coxon et al., 2010; Doi et al., ; Mita et al., 2010; Neal and Wakelee, 2010; Robson and Ghatage, 2011). In general Trebananib has not show any progression free survival (PFS) benefits in renal cell carcinoma or breast cancer, however in ovarian cancer there seem to be positive anti-tumor effects (Rini et al., ;

Robson and Ghatage, 2011). Several antibodies selectively targeting Ang-2 are being evaluated in phase I clinical trials. For more detailed information refer to Table 1-1.

Human Monoclonal Angiopoietin-2 Antibody MEDI3617

MEDI3617 is a fully human IgG1 kappa monoclonal antibody produced via XenoMouse Technology by MedImmune, LLC. MEDI3617 binds Ang-2 at the fibrinogen like domain with high selectivity (KD value = 42 pM) and potently blocks Ang-2 binding to the Tie2 receptor (EC = 0.510 nM) (Figure 1-3). Initial preclinical studies have shown antitumor effects in human colon, ovarian, renal and hepatocellular xenografts (Leow et al., 2012). In preclinical murine models Ang-2 is provided by the mouse endothelium, however mouse and human Ang-2 are 85% homologous in amino acid sequence and the binding site of MEDI3617 to Ang-2 is conserved in the two species therefore can be used both in murine and human treatments (Leow et al., 2012). MEDI3617 is a more potent Ang-2 binding antibody than its precursor 3.19.3 (KD value = 86 pM), which previously had shown vascular and antitumor effects in several tumor models (Brown et al., 2010). Currently this agent is being evaluated in phase I clinical trials in patients with advanced solid malignancies. Throughout the presented work MEDI3617 will be referred to as the Ang-2 inhibitor.

Renal Cell Carcinoma

Renal cell carcinoma accounts for about 3% of adult malignancies and 90% of kidney cancers and is a highly vascularized disease driven by VEGF (Curti et al., 2013; Vanchieri and Williams, 2012). Von-Hippel Lindau (VHL) disease accounts for 40% of renal cell carcinoma in patients, however the VHL gene is commonly mutated, greater than 90%, in nonhereditary tumors as well (Curti et al., 2013; Vanchieri and Williams, 2012). Mutated VHL causes a constitutive activation of the hypoxia inducible factor

(HIF-1α) leading to high VEGF secretion from tumor cells and promotion of angiogenesis even in the absence of hypoxia in the tumor. Patients with renal cell carcinoma show low response to chemotherapeutic agents due to multidrug resistance mediated by *p*-glycoprotein that is highly expressed by cells (Curti et al., 2013). Furthermore these tumors are rarely sensitive to radiation therapy. Anti-angiogenic agents have shown beneficial patient response and are now commonly used as both first and second line treatment in renal cell carcinoma. Currently, eight out of the eleven FDA approved anti-angiogenic agents (Bevacizumab, Sorafenib, Sunitinib, Axitinib, Pazopanib, Temsirolimus, Everolimus and Interferon α) are used in renal cell carcinoma making this disease setting by far the most common for anti-angiogenic agents.

Motivation And Goal Of Research

Anti-angiogenic therapy has become a standard of care in several solid tumors. Even though this therapeutic modality holds promise in patient care, there have been several limitations regarding lack of patient response or occurrence of patient resistance to currently approved agents in the clinic. Our current knowledge of the complex process of angiogenesis and the tumor microenvironment has led to alternative therapeutic strategies. Angiopoietin/Tie2 axis targeted agents mark a new class of anti-angiogenics in hopes to eradicate the observed patient resistance with the currently used VEGF targeted agents as well as complement conventional therapies.

The current research particularly focuses on the monoclonal antibody that is highly specific to Ang-2 (MEDI3617). At the time this dissertation was started there were much fewer Ang-2 targeting agents in development. Mainly Trebananib (AMG386), the Ang-1/Ang-2 peptibody was in phase I clinical trials. The high Ang-2 specificity of

MEDI3617 deemed this antibody superior to Trebananib; based on the basic biology of the Ang/Tie2 axis there were many questions concerning the dual inhibition of Ang-1 and Ang-2. Many, including myself, believe that solely Ang-2 should be targeted in this axis. The general concept is that the tumor microenvironment has high Ang-2 expressions which out-compete Ang-1 for Tie2 binding leading to vascular destabilization. Using an Ang-2 neutralizing antibody we hoped to eliminate Ang-2 from the tumor microenvironment and allow for increased Ang-1/Tie2 binding. This would promote a normal, quiescent vascular phenotype and thus reduce tumor angiogenesis (Figure 1-3). We aimed to explore the therapeutic implications of Ang-2 inhibition in renal cell carcinoma, a highly angiogenic disease where anti-angiogenic agents are used both as first and second line treatments. Over the course of our studies we have found benefits and shortcomings to this anti-angiogenic modality that is discussed throughout this work.

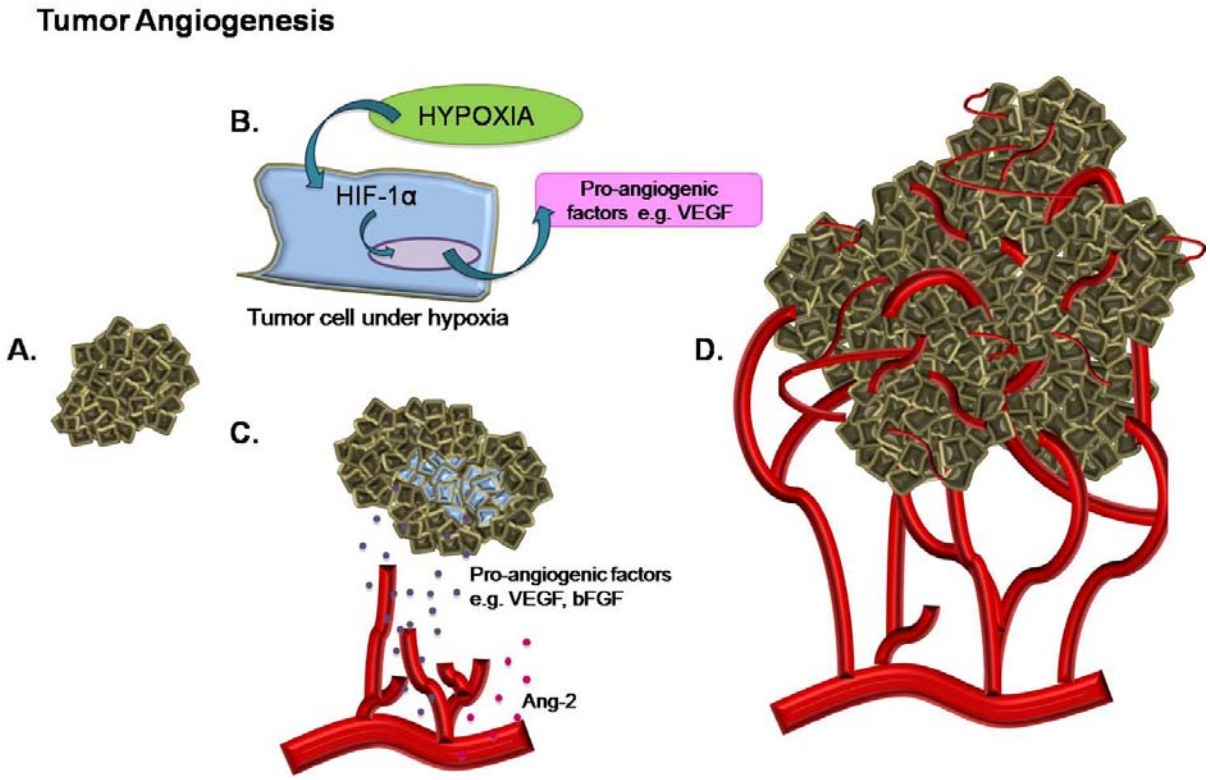

Figure 1-1. Tumor angiogenesis. (A) As a tumor develops it will reach a certain size where cells inside the tumor receive lower levels of oxygen (hypoxia). (B) Hypoxia stabilizes the transcription factor Hypoxia Inducible Factor (HIF-1α) that in turn upregulates genes involved in angiogenesis. (C) In the tumor microenvironment, endothelial cells from nearby vessels secrete Angiopoietin-2 (Ang-2) and tumor cells secrete pro-angiogenic factors to stiumulate the growth of new vessels from nearby normal vasculature. (D) As the tumor develops and grows so does the vasculature with it with the constant stimulation of endothelial cells to proliferate.

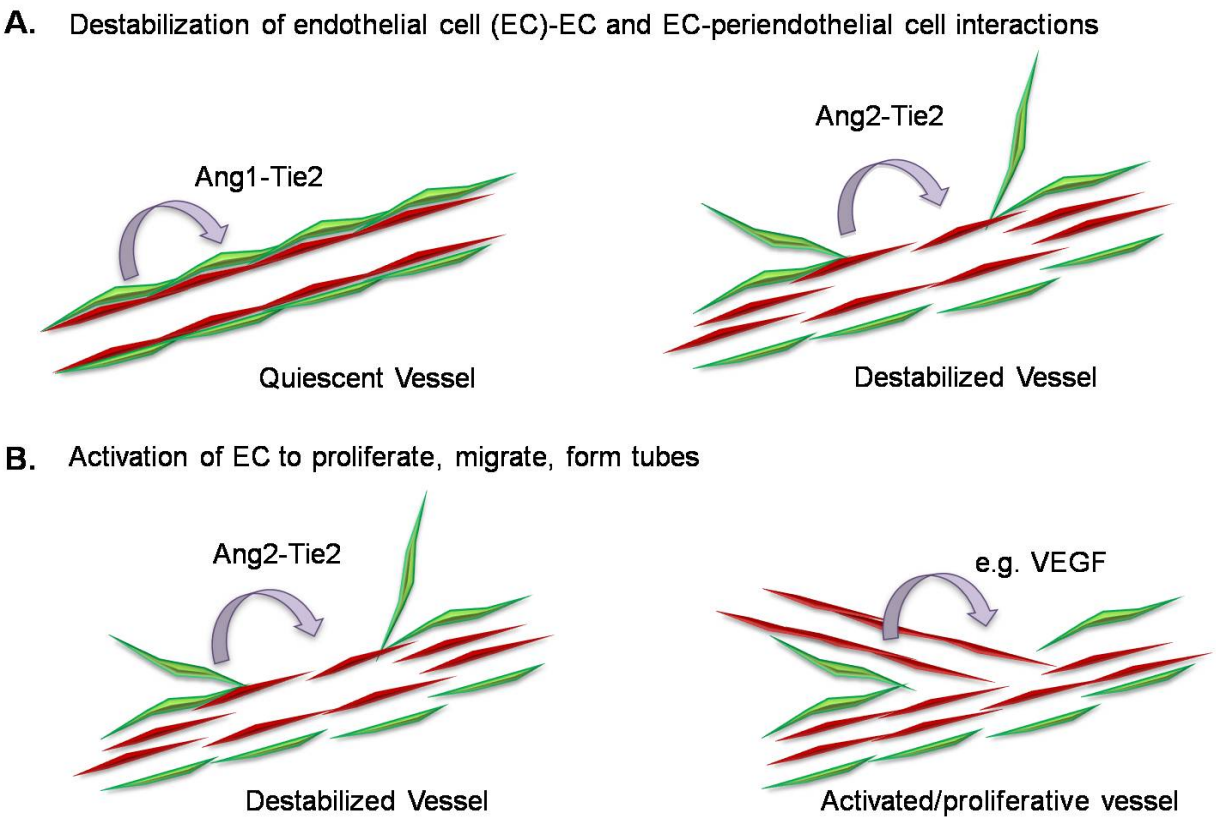

Figure 1-2. Angiogenesis: a two step process. (A) Normal vessels are composed of an endothelial cell layer surrounded by a peri-endothelial cell layer (e.g. smooth muscle cells or pericytes). Quiescent vessels are non-proliferative and cell-cell contacts are maintained by paracrine Angiopoietin-1 (Ang-1) and Tie2 receptor interactions. During angiogenesis Angiopoietin-2 (Ang-2) is secreted from endothelial cells and the autocrine interaction of Ang-2 and Tie2 receptor lead to disruption of cell-cell interactions (vessel destabilization). (B) Endothelial cells are exposed to pro-angiogenic factors (e.g. VEGF, bFGF) in the tumor microenvironment and are activated to proliferate, migrate and from tubes of new vessels.

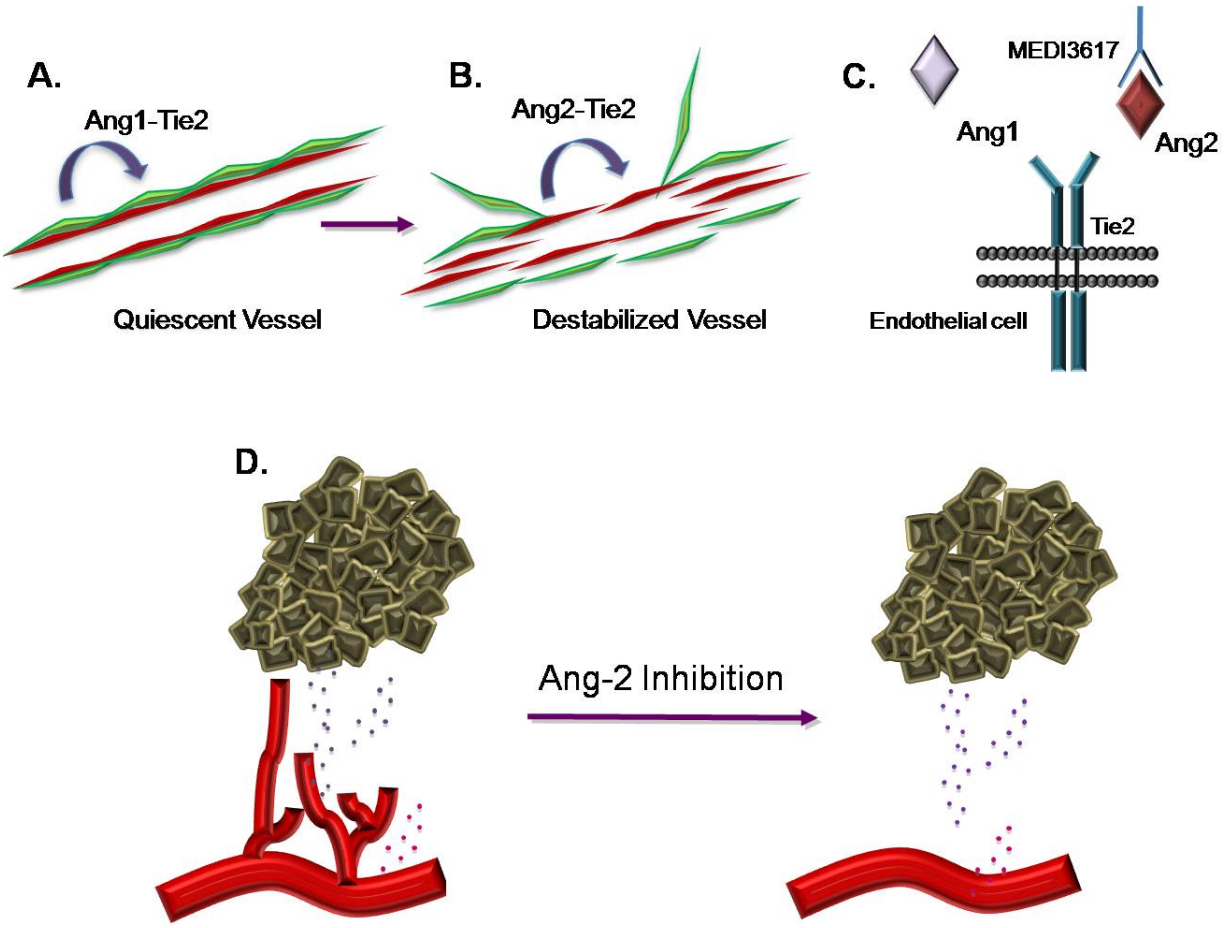

Figure 1-3. Therapeutic benefit of Ang-2 targeting. Angiopoietin ligands and Tie2 receptor are responsible for either maintaining a quiescent vasculature (no endothelial cell proliferation) via Ang-1-Tie2 interaction (A) or destabilizing the vasculature (disrupting cell-cell contacts) via Ang-2-Tie2 interaction (B). Ang-2 is released from endothelial cells during an angiogenic stimulus, Ang-2 inhibitor, MEDI3617, binds to the ligand and inhibits its binding to Tie2 receptor (C). By removing Ang-2 from the microenvironment the active angiogenic phenotype of tumors should be reduced (D).

Table 1-1. Ang-2/Tie2 axis targeting agents and clinical status. There is considerable interest to target the Ang-2/Tie2 axis in tumor angiogenesis.

Agent	Type	Target	Stage of Development	Company
Trebananib (AMG386)	Peptibody	Ang1, Ang2	Phase III	Amgen
Regorafenib (BAY 73-4506)	Small molecule	Tie2, VEGFR1-,2-,3-, Kit, Ret, Raf, PDGFRβ	Phase III	Bayer
CVX-060	Antibody	Ang-2	Phase II	Pfizer
MGCD265	Small molecule	Tie2, c-met, VEGFR1-,2-,3-	Phase I/II	MethylGene Inc.
MEDI3617	Antibody	Ang-2	Phase I	MedImmune LLC.
CEP-11981	Small molecule	Tie2, VEGFR1-, 2-, 3-	Phase I	Cephalon
REGN910	Antibody	Ang-2	Phase I	Regeneron Pharmaceuticals, Inc
AMG780	Antibody	Ang-2	Phase I	Amgen
ARRY-614	Small molecule	Tie2, VEGFR-2, p38, Abl	Phase I	ArrayBioPharma
DX-2240	Antibody	Tie1	Preclinical	Sanofi-Aventis

CHAPTER 2
INHIBITION OF ENDOTHELIAL/SMOOTH MUSCLE CELL CONTACT LOSS BY THE ANGIOPOIETIN-2 ANTIBODY MEDI3617

Chapter 2 explores the use of an endothelial and smooth muscle cell co-culture model to evaluate Ang-2 dependent cell-cell contact loss (destabilization) real time and to evaluate the effect of Ang-2 inhibition using the Ang-2 inhibitor. Studies show the formation and orientation of co-culture spheres, the Ang-2 dependent sphere destabilization in the presence of both exogenous and endogenous Ang-2 and the subsequent inhibition of this cell-cell contact loss in the presence of the inhibitor.

Background

Normal vasculature consists of a single layer of endothelial cells surrounded by peri-endothelial mural cells (smooth muscle cells and pericytes), which form stable vessel networks via tight associations through adhesion molecules such as N- and VE-Cadherin (Armulik et al., 2005; Dejana, 2004). Vascular mural cells are important regulators of vascular development, stabilization, maturation and remodeling; abnormal interactions of endothelial and peri-endothelial cells accounts for several pathological conditions such as tumor angiogenesis, diabetic microangiopathy, ectopic tissue calcification and stroke and dementia syndrome (Armulik et al., 2005). Peri-endothelial cells are a very diverse class of cells that may have different phenotypes in the various vascular beds (e.g. artery, arteriole, vein, venule, or capillary); based on the demands of nearby organs, the extent of peri-endothelial cell coverage of the endothelium changes (Armulik et al., 2005).

The dynamic nature of endothelial cells allows for rapid vascular responses to environmental changes that occur under normal and pathological conditions (Cleaver and Melton, 2003; Rondaij et al., 2006). Angiogenesis requires two distinct events

consisting of the disruption of the vascular structure followed by activation of the endothelium. First, the tight association between endothelial-endothelial and endothelial-peri-endothelial cells is disrupted in order to allow for vessel permeability and exposure of endothelial cells to pro-angiogenic cytokines. Second, pro-angiogenic factors activate the endothelium to proliferate, migrate and establish tubular networks to form new blood vessels.

The angiopoietin/Tie2 system is central to the initial phase of angiogenesis, the disruption of endothelial and peri-endothelial cell interactions (Maisonpierre et al., 1997; Scharpfenecker et al., 2005). Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) are secreted proteins that interact with the Tie2 receptor either in a paracrine (Ang-1) or autocrine (Ang-2) manner; Ang-1 is expressed and secreted by peri-endothelial mural cells while Ang-2 is expressed and secreted by endothelial cells (Augustin et al., 2009). Although both angiopoietins bind Tie2 with similar affinities on the same receptor site, they have opposing functions (Davis et al., 2003; Fiedler et al., 2003); Ang-1 stabilizes and Ang-2 destabilizes the vasculature (Augustin et al., 2009; Davis et al., 1996).

In the adult, Ang-1 is found in tissues throughout the body and continuously secreted at low levels (Davis et al., 1996; Saharinen and Alitalo, 2011). Ang-1-Tie2 association leads to tyrosine kinase phosphorylation of the Tie2 receptor and downstream signaling maintaining tight adhesion molecule interactions in the vascular cellular networks, between endothelial and peri-endothelial cells (Fukuhara et al., 2008; Gavard et al., 2008; Saharinen and Alitalo, 2011). Ang-2 is produced by endothelial cells and stored in Weibel-Palade Bodies (WPB) of endothelial cells (Fiedler et al., 2004). WPBs are rod-shaped organelles that contain several proteins involved in

vascular homeostasis, inflammation, vasodilation, vasoconstriction, and immune cell adhesion and migration (Lowenstein et al., 2005; Rondaij et al., 2006). The most commonly known WPB protein is von-Willebrand Factor (vWF) involved in hemostasis, others include for example p-selectin, interleukin-8 endothelin, CD63; Ang-2 co-localizes with vWF in WPBs however not with any other known WPB proteins (Fiedler et al., 2004; Lowenstein et al., 2005; Rondaij et al., 2006). WPBs are triggered to release their contents via elevated Ca^{2+} (e.g. thrombin, histamine, superoxide anion, VEGF, sphingosine-1 phosphate, ceramide) or cAMP (e.g. serotonin, epinephrine, vasopressin) dependent manner and fuse with the endothelial cell plasma membrane using vesicle SNARE (v-SNARE) on the WPB and target-SNARE (t-SNARE) on the plasma membrane (Lowenstein et al., 2005). Upon stimulation such as inflammation, hypoxia, shear stress at sites of active angiogenesis the WPBs are exocytosed and Ang-2 released from endothelial cells (Lowenstein et al., 2005). Secreted Ang-2 destabilizes the vasculature by autocrine interaction with Tie2, antagonizing Ang-1 signaling, leading to disruption of adhesion molecule interactions between cells and turning the angiogenic switch towards a pro-angiogenic phenotype (Augustin et al., 2009; Scharpfenecker et al., 2005).

The goals of the present investigation were to mimic the interaction between endothelial and peri-endothelial cells using an endothelial-smooth muscle cell co-culture sphere model and directly assess the impact of the Ang-2 inhibitor on the vascular destabilizing effects of Ang-2 using real time imaging of the spheres.

Materials And Methods

Reagents

Phorbol 12-Myristate 13-Acetate (PMA) (Sigma), thrombin (MP Biomedicals, LLC), and human recombinant Angiopoietin-2 (R&D Systems®) were dissolved in DMSO, water and PBS + 0.1% BSA respectively. Human Ang-2 Quantikine ELISA Kits were purchased from R&D Systems® and stored at 4 °C until use. Methylcellulose (viscosity 4000 cP) was obtained from Sigma; stock solutions were dissolved in basal medium (endothelial cell or smooth muscle cell) and ultra centrifuged to a clear solution (3500 rpm, 4 °C, 3 hr) and the top 45% of supernatants stored at 4 °C up to 3 months. CellTracker™ Orange CMRA, CellTrace™ Oregon Green® 488 Carboxylic Acid Diacetate Succinimidyl Ester, and CellTracker™ Violet BMQC (Molecular Probes – Invitrogen) were dissolved in DMSO and stored at -20 °C.

Drug Preparation

Angiopoietin-2 inhibitor, MEDI3617, was kindly provided by MedImmune, LLC. For in vitro investigations 5 mg/ml stock solutions were diluted to 10 μM working solutions in sodium citrate buffer before subsequent dilutions in sterile saline. Stock solutions were kept at -80 °C and working concentrations at 4 °C.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) and human umbilical artery smooth muscle cells (HUASMC) were purchased from Clonetics®. HUVEC were cultured in Nutrient mixture F-12 Ham, Kaighn's supplemented with 0.03 mg/ml Endothelial Cell Growth Supplement (ECGS) and 0.1 mg/ml heparin (Sigma) with 10% fetal bovine serum (FBS). HUASMC were cultured in SmGM-2 (Clonetics®). Cells were kept at 37 °C, 5% CO_2. HUVEC and HUASMC were used between passages 2 and 4.

Endothelial-Smooth Muscle Cell Co-Culture Sphere Formation

Co-culture spheres of HUVEC and HUASMC were generated as previously reported (Korff et al., 2001; Scharpfenecker et al., 2005). Confluent monolayer cultures of HUVEC were traced with 1 µg/ml CellTracker™ Orange CMRA in serum free media for 15 min. Confluent monolayer culture of HUASMC were traced with either 1 µg/ml CellTrace™ Oregon Green® 488 Carboxylic Acid Diacetate Succinimidyl Ester (imaging with Nikon eclipse TS100 microscope) or 1 µg/ml CellTracker™ Violet BMQC (imaging with Leica SP5 confocal microscope) in serum free media for 15 min. A 1:1.5 ratio of HUVEC to HUASMC (1000 cells per spheroid) were suspended in a 1:1 ratio of endothelial and smooth muscle cell complete medium in the presence of 4.2% carboxymethylcellulose into a non-adherent round-bottom 96 well plate (Sigma), 48 hr later the spheres were selected for experiments.

Confocal Imaging Of Endothelial-Smooth Muscle Cell Co-Culture Spheres

Confocal microscopy was used to evaluate co-culture sphere orientation. HUVEC were tagged with CellTracker™ Orange CMRA and HUASMC were tagged with CellTracker™ Violet BMQC prior to sphere formation and imaged with Leica SP5 confocal microscope with an environmental chamber for live imaging at 20x magnification. Spheres were imaged on FluoroDish, FD35-100 (World Precision Instruments, Inc.). 405 Diode laser was used to image HUASMC with CellTracker™ Violet BMQC and HeNe 543 laser was used to visualize HUVEC with CellTracker™ Orange CMRA. LAS AF software was used to obtain confocal images of spheroids. Coronal view of spheroids was obtained from a series of 10 z-stack images at 20.42 µm per step. 3 dimensional images were compiled from a series of 30 z-stack images, at 6.19 µm per step, using LAS AF software and were processed as 3D projections.

Imaging Co-Culture Sphere Formation And Destabilization

Co-culture sphere formation and destabilization, endothelial cell loss, was imaged with a Nikon eclipse TS100 inverted microscope. HUVEC were tagged with CellTracker™ Orange CMRA and HUASMC were tagged with CellTrace™ Oregon Green® 488 Carboxylic Acid Diacetate Succinimidyl Ester prior to sphere formation. HUVEC were fluorescently visualized using TRIC filter and HUASMC were viewed using FITC filter with a Nikon Intensilight C-HGFI lamp. Spheroids were imaged at t=0 and t=4 hr at 10x magnification and NIS Elements D 3.2 software was used to obtain individual and merged images.

Human Angiopoietin-2 ELISA

HUVEC were cultured in 60 mm dishes and grown to confluence. Following media removal and washing 2 ml of media containing either 50 ng/ml PMA or 1 unit/ml thrombin were added. At various times thereafter the media was collected, centrifuged (1000 rpm, 4 °C, 10 min) and 1.5 ml of supernatant collected and stored at -20 °C until analysis. Cells were trypsinized, harvested and counted for each sample. A sandwich ELISA of human Angiopoietin-2 (Quantikine ELISA Kit R&D Systems DY623) was used to analyze Angiopoietin-2 secretion. The manufacturers' protocol was followed; briefly, 96-well plates were coated with the capture antibody and allowed to sit overnight at room temperature. Standards and samples were added to the plate followed by the detection antibody, streptavidin HRP and substrate solution. The concentration of Ang-2 in samples was determined using the optical density at 450 nm. Duplicates of each sample were run and secreted Ang-2 (pg/ml) was normalized to 10^5 cell number and the baseline was set to 0 based on basal level readings.

Stimulation Of Endothelial-Smooth Muscle Cell Co-Culture Spheres

Endothelial and smooth muscle cells were allowed to form spheres for 48 hr and were then transferred to a non-adhesive round bottom 96 well plate (1 sphere per well) and were live imaged (Nikon eclipse TS100 microscope) at t=0 hr prior to any stimulation. Stimulants, human recombinant Ang-2, thrombin or PMA, were then added into the wells and spheres were live imaged at t=4 hr. The number of fluorescent tagged HUVEC that detached from the smooth muscle cell cores was counted in the field of view. Statistical significance between stimulated groups and control (unstimulated) spheres was determined using the Mann-Whitney U test at $p<0.05$. In each experimental group 12 spheres (1 sphere per well) were analyzed.

Results

Endothelial And Smooth Muscle Cells Form Co-Culture Spheres

When combined, endothelial (HUVEC) and smooth muscle cells (HUASMC) form spheroids, which mimic the vascular association of these cell types (Korff et al., 2001; Scharpfenecker et al., 2005). Live imaging of HUVEC and HUASMC with red and green cell trackers respectively (Figure 2-1A), was used to demonstrate the time course of endothelial/muscle cell co-culture spheroid formation (Figure 2-1B). Confocal imaging of these spheroids and its 3 dimensional reconstruction shows an inside-out orientation of spheres with smooth muscle cells found at the core and endothelial cells on the periphery of the sphere (Figure 2-1C).

Ang-2 Inhibitor Impairs Exogenous Ang-2 Dependent Sphere Destabilization

Ang-2 interacts with Tie2 receptor on the endothelial cells and leads to loss of endothelial cell contact with surrounding endothelial and peri-endothelial cells (Augustin et al., 2009; Scharpfenecker et al., 2005). The addition of human recombinant Ang-2

(0.5 ng/ml) to co-culture spheres for 4 hr (Figure 2-2A) led to a 5-fold increase in endothelial cell loss from the spheres ($p<0.0001$). Administration of the Ang-2 inhibitor (0.5 nM) significantly reduced (~ 3.5-fold) the endothelial cell loss as compared to Ang-2 stimulated spheres ($p<0.0001$). Figure 2-2B illustrates the live imaging of co-culture spheroids and demonstrates the endothelial cell loss upon exogenous Ang-2 stimulation and its inhibition in the presence of the Ang-2 inhibitor.

Ang-2 Inhibitor Hinders Endogenous Ang-2 Dependent Sphere Destabilization

Stimulation of HUVEC with PMA or thrombin led to endogenous Ang-2 secretion as measured by ELISA (Figure 2-3). PMA exposure led to detectable Ang-2 secretion at 1 hr and increased to 2.8 and 7.8-fold above control at 6 and 24 hr respectively (Figure 2-3). Ang-2 secretion was not detectable after 1 hr of thrombin stimulation and even at 6 and 24 hr exposure the Ang-2 levels were significantly lower than those observed following PMA treatment (Figure 2-3). Treatment of spheres for 4 hr with 0.5, 5, 50 ng/ml PMA (Figure 2-4A) resulted in 3.4, 6.2, and 6.8-fold increase in endothelial cell loss from spheres respectively ($p<0.0001$). Administration of the Ang-2 inhibitor to PMA (50 ng/ml) treated spheres significantly decreased the number of endothelial cells lost from the spheres (Figure 2-4B). Live imaging of co-culture spheres (Figure 2-4C) illustrates the disruption of endothelial/muscle cell interaction upon PMA stimulation and its inhibition in the presence of the Ang-2 inhibitor.

Discussion

The importance of angiogenesis in the growth of solid tumors has led to the active pursuit of anti-angiogenic anticancer therapies (Folkman, 1971). Although such agents have demonstrated some efficacy in the clinic, concerns about lack of treatment response and the potential for resistance to therapy have been raised (Bergers and

Hanahan, 2008; Jain et al., 2006). Resistance to anti-angiogenic therapy may occur when inhibition of a particular signaling pathway is overcome by the activation of another to sustain tumor blood vessel growth (Bergers and Hanahan, 2008; Jain et al., 2006). One possible approach to overcoming such concerns is to target the angiopoietin/Tie2 axis since it is considered to be the sole pathway responsible for maintaining blood vessel integrity (Augustin et al., 2009; Hu and Cheng, 2009). While Ang-1 binding to Tie2 receptor stabilizes the vasculature, Ang-2 binding primes the vasculature for angiogenesis by freeing endothelial cells from peri-endothelial cells, thus making them sensitive to stimulation by pro-angiogenic factors (Augustin et al., 2009). Minimizing the dissociation of endothelial and peri-endothelial cells by targeting Ang-2 therefore would hinder tumor-induced angiogenesis by blocking the access of pro-angiogenic factors to endothelial cells, thus making this ligand a desirable target for vascular directed anticancer therapeutic approaches.

The present study examined the destabilizing effects of Ang-2 on endothelial/smooth muscle cell contacts in the presence or absence of the Ang-2 inhibitor using a co-culture sphere model and real time imaging (Figure 2-1). The high specificity and neutralizing properties of this inhibitor have been previously confirmed by Leow and colleagues (Leow et al., 2012). The results showed that Ang-2 initiates endothelial-peri-endothelial cell contact loss (Figure 2-2); a finding consistent with the previous report that direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates the response to VEGF (Korff et al., 2001). Although the model is an inside-out orientation of the natural appearance of the vasculature, it nonetheless mimics the interaction between the two cell types essential in the make-up

of blood vessels. Even though smooth muscle cells are usually found on larger arteries while pericytes coat endothelial cells of smaller arterioles, venules, or capillaries, these two cell types share a common lineage and interact with endothelial cells via N-Cadherin, a major adhesion molecule between endothelial and peri-endothelial cells that is broken upon Ang-2-Tie2 association during angiogenesis (Armulik et al., 2005; Dejana, 2004).

Consistent with previous reports on the endothelial cell/peri-endothelial cell interactions (Scharpfenecker et al., 2005), exogenously applied Ang-2 was found to result in a loss of endothelial cells from co-culture spheroids. The current study employed real time imaging of spheres before and after stimulation with Ang-2 (Figure 2-2) and demonstrated that even 400-fold lower concentrations of Ang-2 (0.5 ng/ml) than previously reported (Scharpfenecker et al., 2005), led to a significant increase in the number of endothelial cells dissociating from the unaffected smooth muscle cell core (Figure 2-2). Because the in situ destabilization of endothelial/peri-endothelial cell interactions is modulated by the endogenous release of Ang-2 from endothelial cells, experiments also were undertaken to assess whether such Ang-2 release would affect the co-culture spheres. Ang-2 is stored in Weibel-Palade Bodies in endothelial cells and is rapidly secreted during both physiological and pathological angiogenic stimulus (Fiedler et al., 2004; Huang et al., 2002; Krikun et al., 2000; Lowenstein et al., 2005; Rondaij et al., 2006). Chemical stimulation of endothelial cells with PMA or thrombin leads to Weibel-Palade Bodies exocytosis and results in Ang-2 secretion (Fiedler et al., 2004); a finding confirmed in the present investigation by ELISA analysis of endogenous Ang-2 secretion from endothelial cells following PMA stimulation (Figure 2-3). When co-

culture spheroids were treated with PMA (50 ng/ml), the number of endothelial cells released was found to be increased ~ 6-fold without an effect on the smooth muscle cell core (Figure 2-4). Similar results were noted when spheroids were treated with the clotting factor thrombin (data not shown).

The current studies focused on real time imaging of Ang-2 initiated endothelial cell dissociation from smooth muscle cell contacts and the ability of the Ang-2 inhibitor to impair that dissociation. Endothelial/smooth muscle co-culture spheres were exposed to either exogenous Ang-2 (Figure 2-2) or agents to initiate endogenous Ang-2 expression (Figure 2-4) in the presence or absence of the inhibitor. Loss of endothelial cells from the co-culture spheres was monitored using cell trackers. The results showed that treatment with low dose Ang-2 inhibitor (0.5 nM) counteracted the effects of exogenous Ang-2 and inhibited the dissociation of endothelial from smooth muscle cells (Figure 2-2). Similarly, endothelial cell loss from the co-culture spheroids exposed to PMA could be significantly impaired (~ 3-fold) in the presence of the Ang-2 inhibitor (Figure 2-4). In a recent in vivo study Mazzieri and colleagues (Mazzieri et al., 2011) reported that treatment with the Ang-2 inhibitor 3.19.3 reduced the number of tumor blood vessels but the remaining tumor vasculature was heavily coated with pericytes. It should be noted, however, that the observation was made at the end of an extended treatment period; i.e. at the end of prolonged Ang-2 depletion which ultimately favors an Ang-1 based stabilization of the endothelial cell/pericyte interaction. The present investigation demonstrates that the Ang-2 inhibitor blocks the early phases of the angiogenesis process by impairing the initial destabilization of the endothelial/smooth muscle cell contacts. Therefore, this study demonstrates that Ang-2 targeting with the

inhibitor may have beneficial anti-angiogenic effects by blocking the initial phase of angiogenesis, the dissociation of endothelial and smooth muscle cells in a stable blood vasculature.

The presence of Ang-2 in the tumor microenvironment and circulation has been associated with disease progression (Anargyrou et al., 2008; Helfrich et al., 2009; Lind et al., 2005; Sie et al., 2009; Tait and Jones, 2004; Tsutsui et al., 2006) and enhanced metastatic potential (Carlson et al., 2001; Hu et al., 2006; Imanishi et al., 2007). In the angiogenic process, the angiopoietin/Tie2 axis is currently considered to be a non-redundant pathway involved in maintaining blood vessel integrity. Consequently, Ang-2 has been considered as a novel anticancer target. The current study supports the rationale for Ang-2 targeting as a therapeutic intervention in the angiogenic process. Experiments using fluorescent imaging of co-culture endothelial/smooth muscle cell spheres clearly demonstrate a significant impact of the Ang-2 inhibitor, a human monoclonal Ang-2 antibody, on Ang-2 dependent destabilization of the endothelial/muscle cell interaction in vitro. The present results support the concept that this antibody has inhibitory effects on new blood vessel formation via inhibition of Ang-2 induced endothelial/peri-endothelial cell contact loss. Since minimizing the dissociation of endothelial and peri-endothelial cells by targeting Ang-2 could hinder tumor-induced angiogenesis by blocking the endothelial cells from the influence of pro-angiogenic factors, the Ang-2 inhibitor warrants further investigation as a vascular directed anticancer therapy.

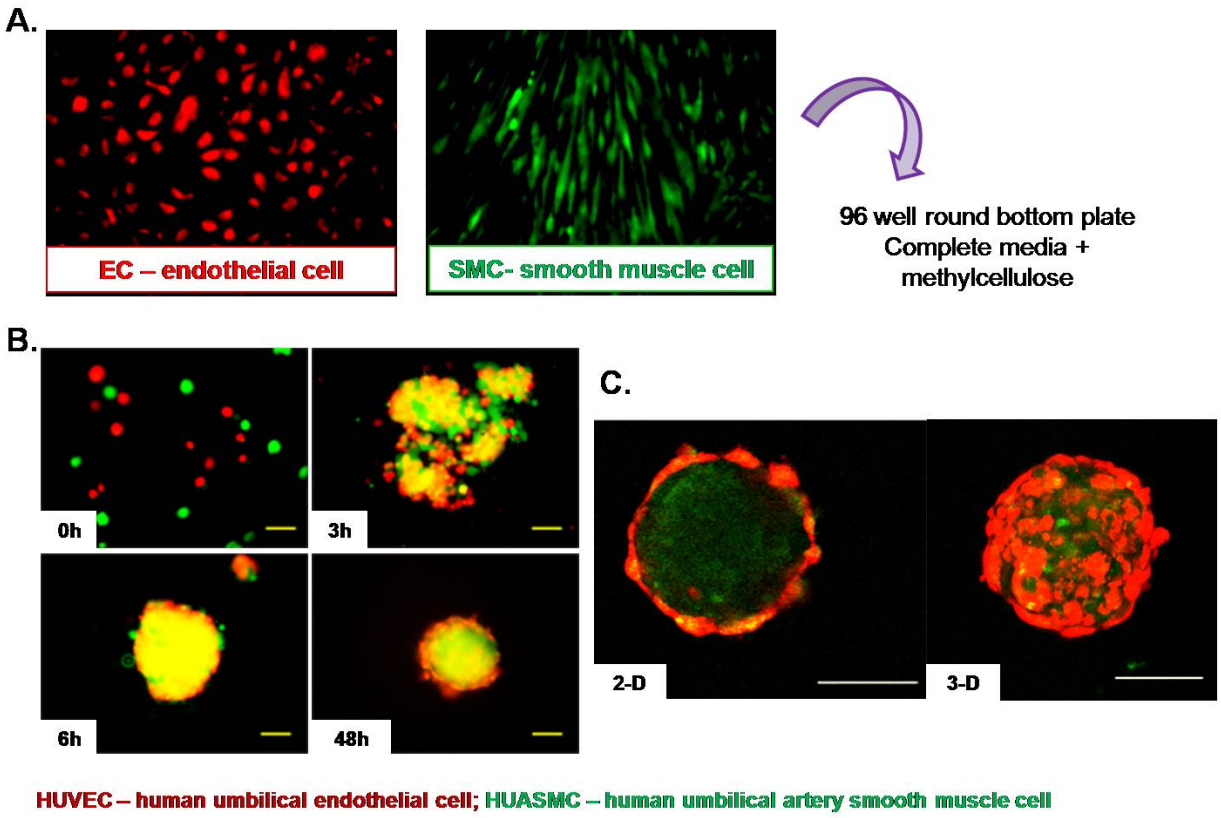

Figure 2-1. Endothelial and smooth muscle cells form co-culture spheres. (A) Monolayer of endothelial and smooth muscle cells are added to a 96 well-round bottom plate with complete media and methylcellulose. (B) Formation of an endothelial-smooth muscle cell sphere over a 48 hr time period. Smooth muscle cells (HUASMC) are shown in green (CellTrace Oregon Green) and endothelial cells (HUVEC) are shown in red (CellTracker Orange CMRA). Merged images are also shown. Images were taken with Nikon eclipse TS100. Scale bar represents 100 µm (C) Coronal and 3-dimensional view of co-culture sphere with smooth muscle cells located in the core and endothelial cells at the periphery. Live images were taken with a Leica SP5 confocal microscope. Scale bar represents 100 µm. Adapted with permission from ELSEVIER: *Microvascular Research* 2012; 83(3):290-7.

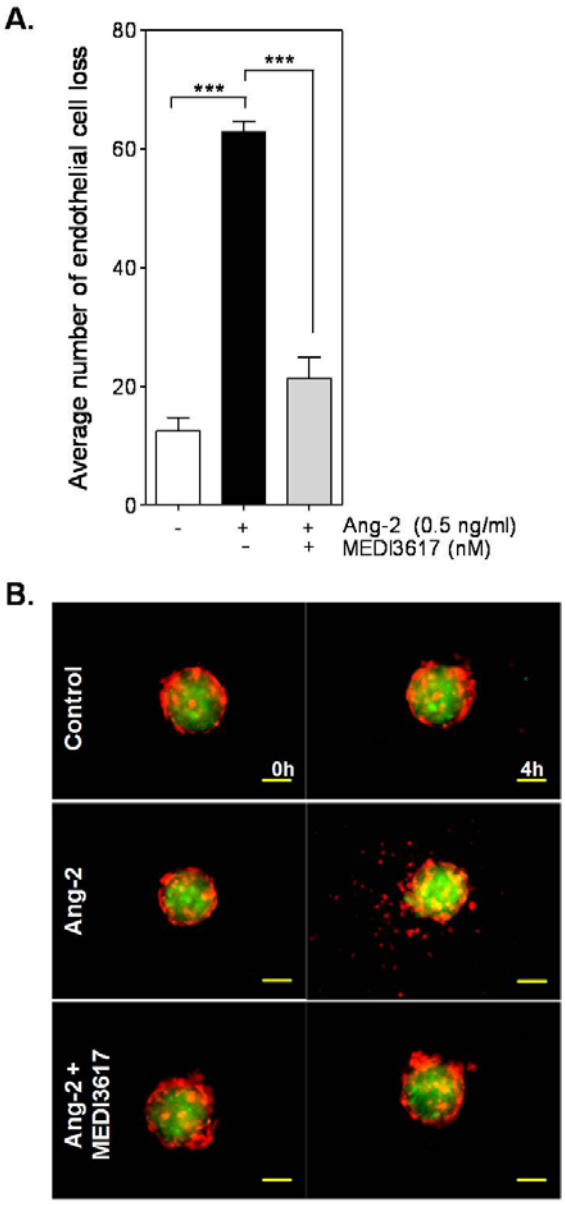

Figure 2-2. Exogenous Ang-2 causes sphere destabilization, Ang-2 antibody inhibits this response. (A) Stimulation of HUVEC with exogenous human recombinant Ang-2 (0.5 ng/ml) for 4 hr led to the loss of endothelial cells from the smooth muscle cell core. The presence of MEDI3617 (0.5 nM) impairs this loss. Column, mean; bars, SD (n=12 spheres per experimental group). ***, $p<0.0001$; Mann-Whitney U test. (B) Images demonstrating control, Ang-2 (0.5 ng/ml) induced destabilization of co-culture spheres and its inhibition in the presence of MEDI3617 (0.5 nM) at 4 hr. Images as in Figure 2-1. Adapted with permission from ELSEVIER: *Microvascular Research* 2012; 83(3):290-7.

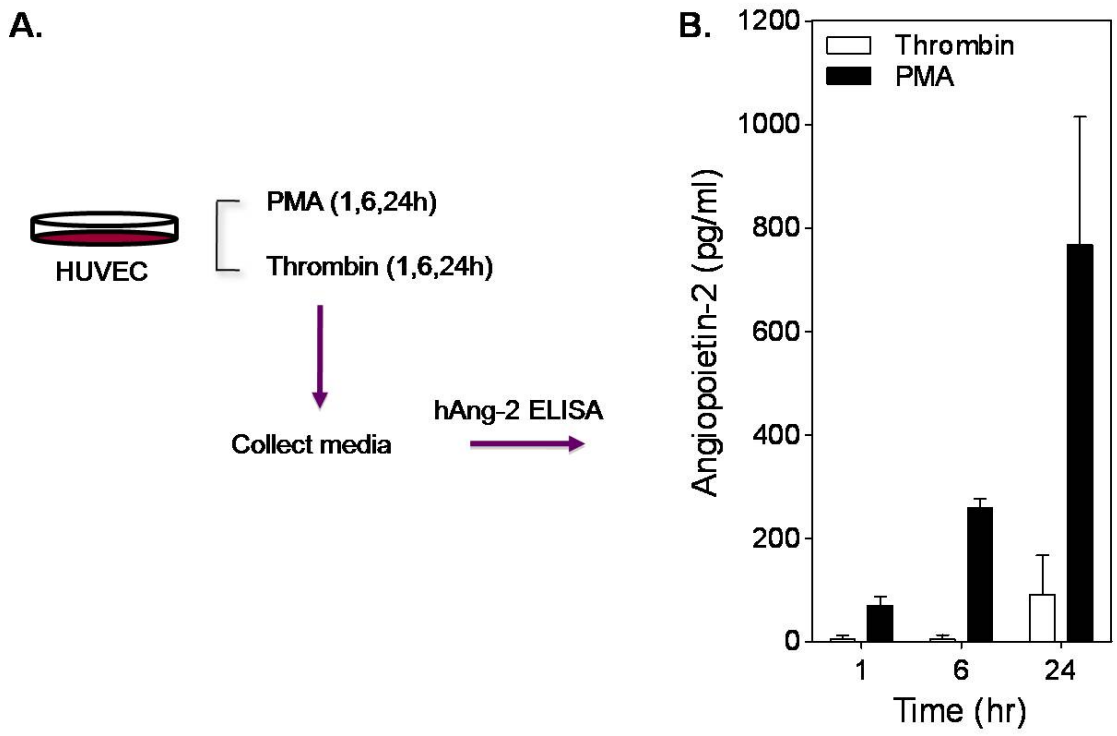

Figure 2-3. PMA and thrombin are stimulators of Ang-2 secretion from endothelial cells. (A) HUVEC were exposed to PMA (50 ng/ml) or thrombin (1 unit/ml) for 1,6, or 24 hours. Stimulation of HUVEC with PMA or thrombin led to Ang-2 secretion over time. (B) Ang-2 secretion was measured by ELISA and data were normalized to 10^5 cells, baseline readings from basal levels were set at 0. Column, mean; bars, SD of three independent experiments. Used with permission from ELSEVIER: *Microvascular Research* 2012; 83(3):290-7.

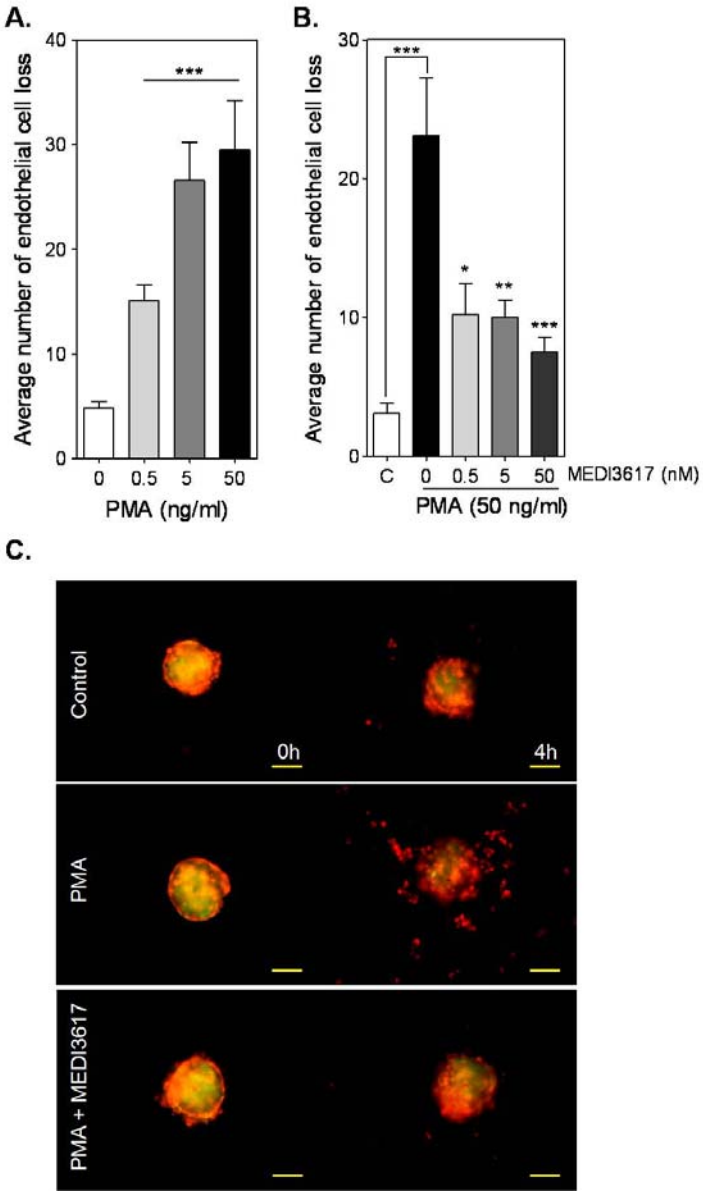

Figure 2-4. PMA induced endogenous Ang-2 causes sphere destabilization, Ang-2 inhibitor impairs this response. (A) Stimulation of HUVEC with PMA for 4 hr resulted in loss of endothelial cells from the smooth muscle cell core in a dose dependent manner. Column, mean; bars, SD (n=12 spheres per experimental group). ***, P<0.0001; Mann-Whitney U test. (B) MEDI3617 inhibited co-culture sphere destabilization when stimulated with PMA (50 ng/ml) for 4 hr. Column, mean; bars, SD (n=12 spheres per experimental group). *, p<0.05: **, p<0.01: ***, p<0.0001; Mann-Whitney U test. (C) Images demonstrating control, PMA (50 ng/ml) induced destabilization of co-culture spheres and its inhibition in the presence of MEDI3617 (0.5 nM) at 4 hr. Images as in Figure 2-1.Adapted with permission from ELSEVIER: *Microvascular Research* 2012; 83(3):290-7.

CHAPTER 3
IMPLICATIONS OF THE EFFECTS OF TUMOR MICROENVIRONEMNTAL FACTORS ON THE ANG-2 AXIS AND SPHERE DESTABILIZATION

Chapter 2 demonstrated the use of an endothelial-smooth muscle cell sphere model to evaluate Ang-2 dependent sphere destabilization and its inhibition in the presence of the Ang-2 inhibitor. Chapter 3 explored an area that remains controversial, the involvement of the tumor microenvironment in the Ang-2 axis. The following studies explored the involvement of tumor cells, hypoxia and VEGF in the Ang-2 axis. Results demonstrate that renal cell carcinoma tumor cells indirectly influence vessel destabilization by secretion of factors that stimulate endothelial cells to secrete Ang-2. Studies also evaluated factors commonly found in the tumor microenvironment such as hypoxia and VEGF. As demonstrated in Chapter 2, the presence of the Ang-2 inhibitor impairs Ang-2 dependent sphere destabilization.

Background

The tumor microenvironment highly influences tumor angiogenesis (Betof and Dewhirst, 2011; Weis and Cheresh, 2011). A growing tumor that exceeds the oxygen diffusion limit from normal vasculature experiences hypoxia and nutrient deprivation (Hanahan and Folkman, 1996). This triggers a pro-angiogenic phenotype, tumor cells respond to hypoxia by secreting pro-angiogenic factors such as VEGF that activates sprouting of new vessels toward the tumor (Betof and Dewhirst, 2011; Charlesworth and Harris, 2008; Dewhirst, 2006). The tumor microenvironment is also abundant in tumor associated macrophages (TAMs) and fibroblasts that have been shown to promote tumor growth and angiogenesis (Biswas et al., 2013; Cirri and Chiarugi, 2011; De Palma and Naldini, ; Xing et al., 2010).

The Angiopoietin/Tie2 axis is a key component of angiogenesis and vascular remodeling both in physiology and pathology (Augustin et al., 2009; Davis et al., 1996; Maisonpierre et al., 1997; Partanen et al., 1992; Schnurch and Risau, 1993). Angiopoietin-1 (Ang-1) interaction with Tie2 receptor promotes a quiescent vascular phenotype, maintaining endothelial and peri-endothelial cell interactions found in normal, non-proliferating vasculature (Augustin et al., 2009). On the other hand, Angiopoietin-2 (Ang-2) ligand is a Tie2 antagonist leading to vascular destabilization, the loss of peri-endothelial cell coverage off the endothelial layer of vasculature (Augustin et al., 2009; Scharpfenecker et al., 2005). Ang-2 is stored in endothelial cells in Weibel-Palade Bodies (WPB) co localized with von Willebrand Factor (vWF), but not other WPB proteins, and is rapidly secreted in response to microenvironmental changes (Fiedler et al., 2004). Ang-2 expression is elevated in various tumor settings, both within tumor tissue and in serum; increased levels have been correlated to poorer disease prognosis (Tait and Jones, 2004).

Due to the role of Ang-2 in tumor angiogenesis it has been increasingly pursued as an anti-angiogenic modality in cancer therapy. Currently there are several agents being evaluated in all phases of clinical development (Gerald et al., 2013). However, our understanding of this axis and its interplay in the tumor microenvironment remains limited. As a tumor develops and grows it surpasses the available oxygen and nutrients provided by nearby normal vasculature. Upon low oxygen conditions such as hypoxia tumor cells express the hypoxia inducible factor (HIF-1α) and upregulate several genes involved in a variety of processes such as angiogenesis and metabolism (Charlesworth and Harris, 2008; Dewhirst, 2006; Maxwell et al., 1997). Tumor cells promote

angiogenesis by directly secreting pro-angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF) especially under hypoxic conditions (Maxwell et al., 1997). Whether tumor cells directly affect the Ang-2/Tie2 axis in angiogenesis is still unclear. Several reports have shown that tumor cells do express Ang-2, however there is limited evidence for tumor cell secretion of Ang-2 into the microenvironment (Detjen et al., 2010; Engin et al., 2012; Li et al., 2013; Schulz et al., 2011). Furthermore, the understanding of the effects of hypoxia and VEGF on the Ang-2 axis is limited (Fiedler et al., 2004; Matsushita et al., 2005; Pichiule et al., 2004; Tsigkos et al., 2006).

Chapter 2 demonstrated the use of endothelial-smooth muscle cell sphere model to mimic normal vasculature and evaluate Ang-2 dependent cell-cell contact loss (destabilization) and its inhibition in the presence of the Ang-2 inhibitor (Molnar and Siemann, 2012). The current study evaluated the tumor microenvironmental effects such as the interplay between renal cell carcinoma, endothelial cell, hypoxia and VEGF on the Ang-2 dependent destabilization. The Ang-2 inhibitor was evaluated in the co-culture sphere models *in vitro*.

Materials And Methods

Reagents

Methylcellulose (viscosity 4000 cP) was obtained from Sigma; it was prepared as previously described in Chapter 2. Human Ang-2 Quantikine ELISA Kits and human recombinant Vascular Endothelial Growth Factor (VEGF) were purchased from R&D Systems®. CellTracker™ Orange CMRA and CellTrace™ Oregon Green® 488 Carboxylic Acid Diacetate Succinimidyl Ester (Molecular Probes – Invitrogen) were dissolved in DMSO and stored at -20 °C.

Cell Culture

Human umbilical vein endothelial cells (HUVEC), human microvascular endothelial cells from the lungs (HMVEC-L), and human umbilical artery smooth muscle cells (HUASMC) were purchased from Clonetics®. HUVEC were cultured in Nutrient mixture F-12 Ham, Kaighn's supplemented with 0.03 mg/ml Endothelial Cell Growth Supplement (ECGS) and 0.1 mg/ml heparin (Sigma) with 10% fetal bovine serum (FBS). HMVEC-L were grown in EGM-2 MV and HUASMC were cultured in SmGM-2 (Clonetics®). The human clear cell renal cell carcinoma Caki-1 and Caki-2 cell lines were received as a gift from Dr. Susan Knox (Stanford University); A498 and 786-0 human renal cell carcinoma cell lines were obtained from Dr. Kyung-Mi Bae (University of Florida). Caki-1 and Caki-2 were grown in Dulbecco's modified minimum essential medium (D-MEM, Invitrogen), A498 were grown in Eagles minimum essential medium (MEM, Cellgro), 786-0 were grown in RPMI-1640 medium (Sigma). All tumor cell media were supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 1% 200-mmol/L $_{L}$-glutamine (Invitrogen). Cells were kept at 37 °C, 5% CO_2. HUVEC, HMVEC-L and HUASMC were used between passages 2 and 4. Caki-1, Caki-2, A498 and 786-0 cells were used between passages 2 and 10.

Drug Preparation

Angiopoietin-2 inhibitor, MEDI3617, was kindly provided by MedImmune, LLC. 5 mg/ml stock solutions were diluted to 10 μM working solutions in sodium citrate buffer before subsequent dilutions in sterile saline. Stock solutions were kept at -80 °C and working concentrations at 4 °C.

Endothelial-Smooth Muscle Cell Co-Culture Sphere Formation

Co-culture spheres of HUVEC and HUASMC were generated as previously reported in Chapter 2. Confluent monolayer cultures of HUVEC were traced with 1 µg/ml CellTracker™ Orange CMRA in serum free media for 15 min. Confluent monolayer culture of HUASMC were traced with either 1 µg/ml CellTrace™ Oregon Green® 488 Carboxylic Acid Diacetate Succinimidyl Ester (imaging with Nikon eclipse TS100 microscope) or 1 µg/ml CellTracker™ Violet BMQC (imaging with Leica SP5 confocal microscope) in serum free media for 15 min. A 1:1.5 ratio of HUVEC to HUASMC (1000 cells per spheroid) were suspended in a 1:1 ratio of endothelial and smooth muscle cell complete medium in the presence of 4.2% carboxymethylcellulose into a non-adherent round-bottom 96 well plate (Sigma). After 48 hr, spheres were used for subsequent experiments.

Stimulation Of Endothelial-Smooth Muscle Cell Co-Culture Spheres

Endothelial and smooth muscle cells were initially allowed to form spheres for 48 hr at which point they were transferred to a non-adhesive round bottom 96 well plate (1 sphere per well) and were live imaged (Nikon eclipse TS100 microscope) at t=0 hr prior to any stimulation. Stimulants, tumor cell conditioned media, were then added into the wells (1:1 ratio of tumor cell conditioned media and endothelial cell media) and spheres were live imaged at t=4 hr. The number of fluorescent tagged HUVEC that detached from the smooth muscle cell cores was counted in the field of view. Statistical significance between stimulated groups and control (unstimulated) spheres was determined using the Mann-Whitney U test at $p<0.05$.

Imaging Co-Culture Sphere Formation And Destabilization

Co-culture sphere formation and destabilization, endothelial cell loss, was imaged with a Nikon eclipse TS100 inverted microscope. HUVEC were tagged with CellTracker™ Orange CMRA and HUASMC were tagged with CellTrace™ Oregon Green® 488 Carboxylic Acid Diacetate Succinimidyl Ester prior to sphere formation. HUVEC were fluorescently visualized using TRIC filter and HUASMC were viewed using FITC filter with a Nikon Intensilight C-HGFI lamp. Spheroids were imaged at t=0 and t=4 hr at 10x magnification and NIS Elements D 3.2 software was used to obtain individual and merged images.

Human Angiopoietin-2 ELISA

Renal cell carcinoma cells (Caki-1, Caki-2, 786-0, and A498) were grown in either 100 mm or 60 mm dishes and grown to 70-80% confluence. Following media removal and washing, either 10 ml (100 mm dish) or 2 ml (60 mm dish) media was added to the plates. Plates were either kept at normal oxygen levels (37 °C incubator) or placed under hypoxia for various time points. HUVEC and HMVEC-L were cultured in 60 mm dishes and grown to confluence (70-80%). Following media removal and washing, 2 ml of media containing either media alone (basal readings), media and tumor cell conditioned media (1:1), or human recombinant VEGF (5, 25, 50 ng/ml) was added to the plates for various times. The media was collected, centrifuged (1000 rpm, 4 °C, 10 min) and 1.5 ml of supernatant collected and stored at -20 °C until analysis. Cells were trypsinized, harvested and counted for each sample. A sandwich ELISA of human Angiopoietin-2 (Quantikine ELISA Kit R&D Systems DY623) was used to analyze Angiopoietin-2 secretion. The manufacturers' protocol was followed; briefly, 96-well plates were coated with the capture antibody and allowed to sit overnight at room

temperature. Standards and samples were added to the plate followed by the detection antibody, streptavidin HRP and substrate solution. The concentration of Ang-2 in samples was determined using the optical density at 450 nm. Duplicates of each sample were run and secreted Ang-2 (pg/ml) was normalized to 10^5 cell number. Statistical significance between groups was determined using the student t-test at $p<0.05$.

Hypoxia

Special aluminum chambers designed by Dr. Cameron Koch (University of Pennsylvania) were used as hypoxia chambers. Tumor (Caki-1, Caki-2, 786-0, A498) or endothelial cells (HUVEC, HMVEC-L) were plated on glass dishes in their respective full serum media. For hypoxia 1% oxygen, 5% CO_2 conditions were established. Upon flushing normal oxygen in exchange to hypoxic conditions the chambers were placed in 37 °C incubators for various time points.

Results

Tumor Cells Secrete Minimal Levels Of Ang-2 But Their Conditioned Media Stimulate Ang-2 Secretion From Endothelial Cells And Ang-2 Dependent Destabilization, Ang-2 Inhibitor Impairs This Loss

There is still controversy whether tumor cells can secrete Ang-2 thus directly affecting angiogenesis (as they do with VEGF) or whether Ang-2 is solely provided by the endothelial cells and found in the tumor stroma. Results show that human renal cell carcinoma cells, Caki-1, Caki-2, 786-0, and A498 secreted minimal levels of Ang-2; 13.4, 8, 8.6 and 24.8 pg/ml of Ang-2 respectively (Figure 3-2A-D). When conditioned media from tumor cells were added onto endothelial cells the endothelial cells secreted 2.2-2.4-fold higher levels of Ang-2 compared to basal conditions; 34.5-, 58-, 50-, and 15-fold higher levels compared to levels that Caki-1, Caki-2, 786-0, and A498 secreted

respectively ($p<0.01$ C,D; $p<0.0001$ A,B) (Figure 3-2A-D). When co-culture spheres were stimulated with Caki-1 conditioned media there was a significant loss of endothelial cell loss from the sphere core (2.7-fold); in the presence of the Ang-2 inhibitor Caki-1 conditioned media induced endothelial cell loss is significantly reduced by 2.2-fold ($p<0.01$) (Figure 3-2E). Figure 3-2F illustrates the endothelial cell loss due to Caki-1 conditioned media and its inhibition in the presence of the Ang-2 antibody.

Hypoxia Does Not Affect Tumor Cell Secretion of Ang-2

The tumor microenvironment is fairly hypoxic, which triggers tumor cells to secrete pro-angiogenic factors such as VEGF (Maxwell et al., 1997). The possibility of tumor cells secreting Ang-2 under hypoxic conditions was evaluated; both short term (acute) and long term (chronic) were assessed. The results showed that there was no significant difference in Ang-2 secretion from human renal cell carcinoma cells Caki-1 (Figure 3-3C), Caki-2 (Figure 3-3D), 786-0 (Figure 3-3E) or A498 (Figure 3-3F) under hypoxic conditions compared to control; tumor cells secreted minimal levels of Ang-2 regardless of the oxygen status in the environment.

Tumor Cell Media From Hypoxic Conditions Do Not Affect Endothelial Cell Secretion Of Ang-2 Compared to Tumor Cell Media From Normal Oxygen Levels

The addition of tumor cell conditioned media significantly increased Ang-2 secretion from endothelial cells under both normal (Figure 3-2A-D) and hypoxic conditions (Figure 3-4A-D). Endothelial cell secreted Ang-2 by an increase of 2.4-, 2.5-, 2.4-, and 2.6-fold in the presence of Caki-1, Caki-2, 786-0, and A498, respectively, conditioned media from hypoxic conditions compared to basal levels ($p<0.05$) (Figure 3-4A-D). However, the exposure of tumor cells to hypoxia did not lead to a difference in Ang-2 secretion from endothelial cells compared to normal oxygen conditions. Figure 3-

4E shows that co-culture spheres in the presence of conditioned media from Caki-1 cells, both from normal and hypoxic conditions, led to a significant loss of endothelial cells from the sphere 3.3- ($p<0.05$) and 4.1-fold ($p<0.0001$) compared to control respectively. Figure 3-2E,F shows that the Ang-2 inhibitor significantly reduced the number of endothelial cell loss from spheres in the presence of Caki-1 conditioned media from normal oxygen conditions; Figure 3-4E demonstrates that the same occurred in the presence of Caki-1 media from hypoxic conditions with a 1.9-fold ($p<0.05$) decrease in endothelial cell loss.

Endothelial Cell Secretion Of Ang-2 Was Not Altered Due to Hypoxia

Endothelial cells, HUVEC (Figure 3-5A) and HMVEC-L (Figure 3-5B), under acute or chronic hypoxic conditions did not secrete heightened levels of Ang-2 as compared to normal oxygen levels.

VEGF Does Not Significantly Affect Ang-2 Release From Endothelial Cells

The possibility of VEGF, found in abundance in the tumor microenvironment, being the factor tumor cells secrete and in turn stimulate Ang-2 release from endothelial cells was assessed. VEGF did not significantly affect HUVEC (Figure 3-8A) or HMVEC-L (Figure 3-8B) secretion of Ang-2 when evaluated at various time points (Figure 3-8).

Discussion

The tumor microenvironment plays an important role in tumor angiogenesis (Betof and Dewhirst, 2011; Weis and Cheresh, 2011). Several factors such as hypoxia, immune cells and stromal cells can have an impact on this process (Weis and Cheresh, 2011). The current study focused on three important aspects of the microenvironment; tumor cells, hypoxia, and pro-angiogenic growth factors. Increased expression of Ang-2 within the tumor microenvironment have been shown to correlate to advanced disease

(Detjen et al., 2010; Engin et al., 2012; Li et al., 2013; Schulz et al., 2011). However, it remains controversial how the microenvironment influences the Ang-2 axis and the origin of Ang-2 in the microenvironment.

During the growth of a tumor, hypoxia in the microenvironment leads to tumor cell secretion of pro-angiogenic factors (e.g. VEGF) and the direct influence on new vessel growth (Hanahan and Folkman, 1996). Whether tumor cells contribute to Ang-2 levels in the microenvironment and directly stimulate angiogenesis, as with VEGF (Figure 3-1) is controversial. Reports have demonstrated mRNA levels of Ang-2 within tumor cells, however, there is lack of evidence for tumor cell secretion of Ang-2 into the microenvironment (Currie et al., 2002; Tait and Jones, 2004; Yamakawa et al., 2004). Chapter 3 evaluated the involvement of renal cell carcinoma, a highly angiogenic disease, in the Ang-2/Tie2 axis during angiogenesis initiation focusing on the interaction of endothelial and peri-endothelial cells found in normal vasculature; the effect of the Ang-2 inhibitor on cell-cell contacts was determined.

Results indicate that renal cell carcinoma cells do express Ang-2 as been previously reported (Currie et al., 2002; Tait and Jones, 2004; Yamakawa et al., 2004), however, secrete minimal levels into the microenvironment in the range of 8-24 pg/ml (Figure 3-2). Nevertheless, the media collected off of tumor cells significantly stimulate endothelial cells to release their Ang-2 contents into the microenvironment (Figure 3-2). Basal levels of Ang-2 secretion ranged from 170-210 pg/ml but in the presence of tumor cell conditioned media this range was elevated to 370-470 pg/ml. The elevated Ang-2 secretion from endothelial cells in the presence of tumor cell conditioned media led to significant destabilization of endothelial and peri-endothelial cell contacts in the sphere

model (Figure 3-3). These studies were conducted during a short period of time, 4 hrs, therefore the levels of Ang-2 that were detected unlikely attribute to the synthesis of Ang-2 but rather the release of Ang-2 that were stored in Weibel-Palade Bodies (WPB).These results indicate that the tumor cells secrete certain factors that in turn stimulate WPB exocytosis/Ang-2 secretion from endothelial cells, therefore suggesting that tumor cells indirectly affect Ang-2 dependent vascular destabilization. The indirect effects of tumor cells on Ang-2 dependent vessel destabilization can be hindered in the presence of the Ang-2 inhibitor (Figure 3-3).

Hypoxia is a major factor in the tumor microenvironment that alters tumor cell behavior to promote angiogenesis (Bergers and Benjamin, 2003). A well-known example in tumor angiogenesis is the upregulation of VEGF by tumor cells as a response to hypoxia in the microenvironment and thus the promotion of angiogenesis. Hypoxia in the tumor microenvironment can occur due to diffusion or perfusion affects both of which tumor cells experience in the course of the tumors' growth (Dewhirst et al., 1996; Vaupel et al., 1998). Diffusion limited hypoxia is referred to as chronic or long term hypoxia and occurs as the tumor grows and expands in size; the oxygen diffusion limit from normal vasculature is 70-100 μm and the rapidly growing tumor quickly surpasses this range (Dewhirst et al., 1996; Vaupel et al., 1998). Acute or short term hypoxia occurs due to perfusion limitations; the tumor vasculature is irregular in shape, leaky and has highly proliferative endothelium. These vessels can suddenly shut down and cause hypoxia for minutes to hours to tumor cells that otherwise had access to oxygen and nutrients (Dewhirst et al., 1996). In the present study the response of tumor cells in regards to Ang-2 secretion was evaluated both in the acute and chronic hypoxic

environments. Results show that neither acute nor chronic hypoxia led to an increase in Ang-2 secretion from tumor cells; in all cases the range of Ang-2 secretion remained low in the range of 5-25 pg/ml (Figure 3-4).

Hypoxia alters the behavior of tumor cells towards a pro-angiogenic phenotype. The effect of tumor cell conditioned media, collected under hypoxia, on endothelial cell secretion of Ang-2 was evaluated. Results show that Ang-2 secretion from endothelial cells in the presence of conditioned media collected from hypoxic conditions was in the range of 410-450 pg/ml (Figure 3-5). These levels were not significantly different from the Ang-2 levels secreted by endothelial cells in the presence of tumor cell conditioned media collected under normal oxygen levels, 420-470 pg/ml (Figure 3-5). Furthermore, tumor cell conditioned media collected under hypoxic conditions did not significantly increase endothelial-smooth muscle cell contact loss in co-culture spheres compared to results obtained in the presence of tumor cell conditioned media from normal oxygen levels (Figure 3-6). Collectively, these results suggest that the tumor cell secreted factors that influence Ang-2 secretion from endothelial cells are not altered by hypoxia. Finally, these effects can be impaired in the presence of the Ang-2 inhibitor (Figure 3-6).

In the tumor microenvironment tumor cells are not the only cells that are exposed to hypoxic conditions, the endothelial cells in the microenvironment also experience the same conditions that tumor cells do. Therefore, next the effect of hypoxia on the endothelial cells and their behavior in regards to Ang-2 secretion was evaluated. Endothelial cell secretion of Ang-2 did not change from normoxic (180-290 pg/ml) to hypoxic conditions (140-230 pg/ml) (Figure 3-7) despite previous reports demonstrating hypoxia as a potent trigger for WPB exocytosis in endothelial cells (Lowenstein et al.,

2005; Rondaij et al., 2006). These observations could be attributed to the plasticity and dynamics of specific WPB protein release and even though hypoxia has been shown to enhance Ang-2 mRNA levels it may not be a potent stimulator of Ang-2 release from endothelial cells (Rondaij et al., 2006).

As mentioned previously, VEGF is highly secreted by renal cell carcinoma cells especially by VHL null or mutant cells and is, therefore, an abundant growth factor in the tumor microenvironment even in the absence of hypoxia (Bergers and Benjamin, 2003). The possibility of VEGF as the tumor cell secreted factor that stimulates Ang-2 release from endothelial cells, as observed in this study, was evaluated. The role of VEGF in stimulating Ang-2 release from endothelial cells is controversial. Matsushita and colleagues (Matsushita et al., 2005) showed that VEGF triggers exocytosis of WPB leading to Von-Willebrand Factor (vWF) release from endothelial cells. Tsigkos and colleagues (Tsigkos et al., 2006) demonstrated that VEGF leads to Ang-2 release from bovine lung microvascular endothelial cells; however Fiedler and colleagues (Fiedler et al., 2004) reported that VEGF does not cause Ang-2 release from human umbilical vein endothelial cells. In the present study human recombinant VEGF did not significantly affect Ang-2 release from endothelial cells (Figure 3-8). Even though an upward trend was observed the levels of Ang-2 secreted were much lower compared to those secreted in the presence of tumor cell conditioned media, therefore VEGF is most likely not the tumor cell secreted factor significantly stimulating the endothelial cells. Sphingosine-1 phosphate could be such factor; it is a potent stimulator of WPB exocytosis, is secreted from tumor cells and currently pursued as a target in various disease settings (Jang et al., 2009; Lowenstein et al., 2005; Zu Heringdorf et al., 2013).

In conclusion, this study indicates that the renal cell carcinoma tumor microenvironment has a potent effect on Ang-2 release into the microenvironment by endothelial cells. Renal cell carcinoma cells do not have a direct but rather an indirect effect on the Ang-2/Tie2 axis in tumor angiogenesis; the minimal levels of Ang-2 secretion from tumor cells most likely does not significantly contribute to Ang-2 dependent vessel destabilization. However, tumor cells secrete factors that potently stimulate Ang-2 release from endothelial cells. Further investigation into such factors is needed that may lead to better understanding of the tumor microenvironment and potentially new therapeutic targets. Nevertheless, Ang-2 inhibition shows promise as an anti-angiogenic modality as its inhibition led to a decrease in endothelial-peri-endothelial cell contact loss *in vitro*. The reported results support the pursuit of the Ang-2 inhibitor, MEDI3617, in the clinic for cancer therapy.

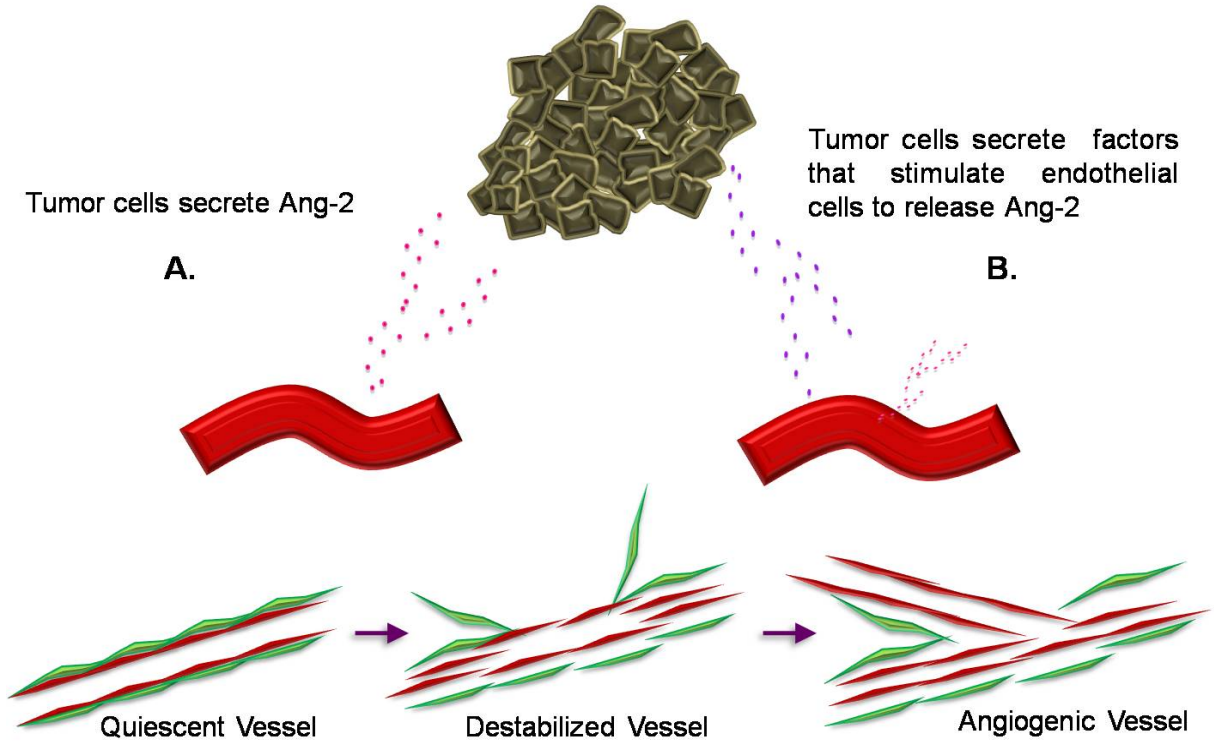

Figure 3-1. Tumor cell involvement in angiogenesis through the Ang-2-Tie2 axis. Tumor cells may directly influence vascular destabilization by secreting Ang-2 (A), or they may indirectly influence this process by secreting factors that in turn stimulate Ang-2 secretion from the endothelium of vessels (B).

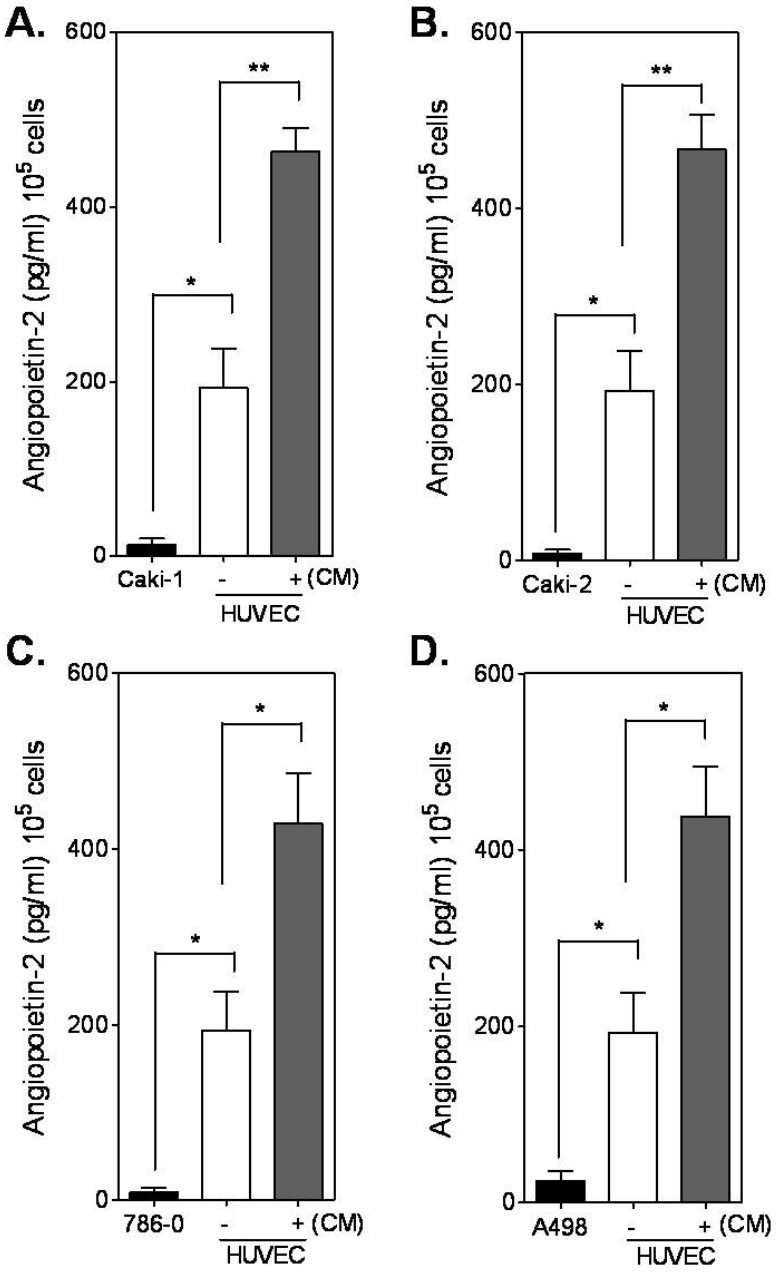

Figure 3-2. Tumor cell conditioned media stimulate Ang-2 secretion from endothelial cells and Ang-2 dependent destabilization, Ang-2 inhibitor hinders this loss. Renal cell carcinoma cell conditioned media (CM) was collected at 6 hr post media change. HUVEC were exposed to tumor cell CM for 4 hr. Ang-2 secretion was measured by ELISA, data were normalized to 10^5 cells. Tumor cells, Caki-1 (A), Caki-2 (B), 786-0 (C), and A498 (D) secreted low levels of Ang-2; tumor cell CM however elevated basal Ang-2 secretion from endothelial cells. Column, mean; bars, SEM of three independent experiments. *, $p<0.05$; **, $p<0.01$; Student t-test.

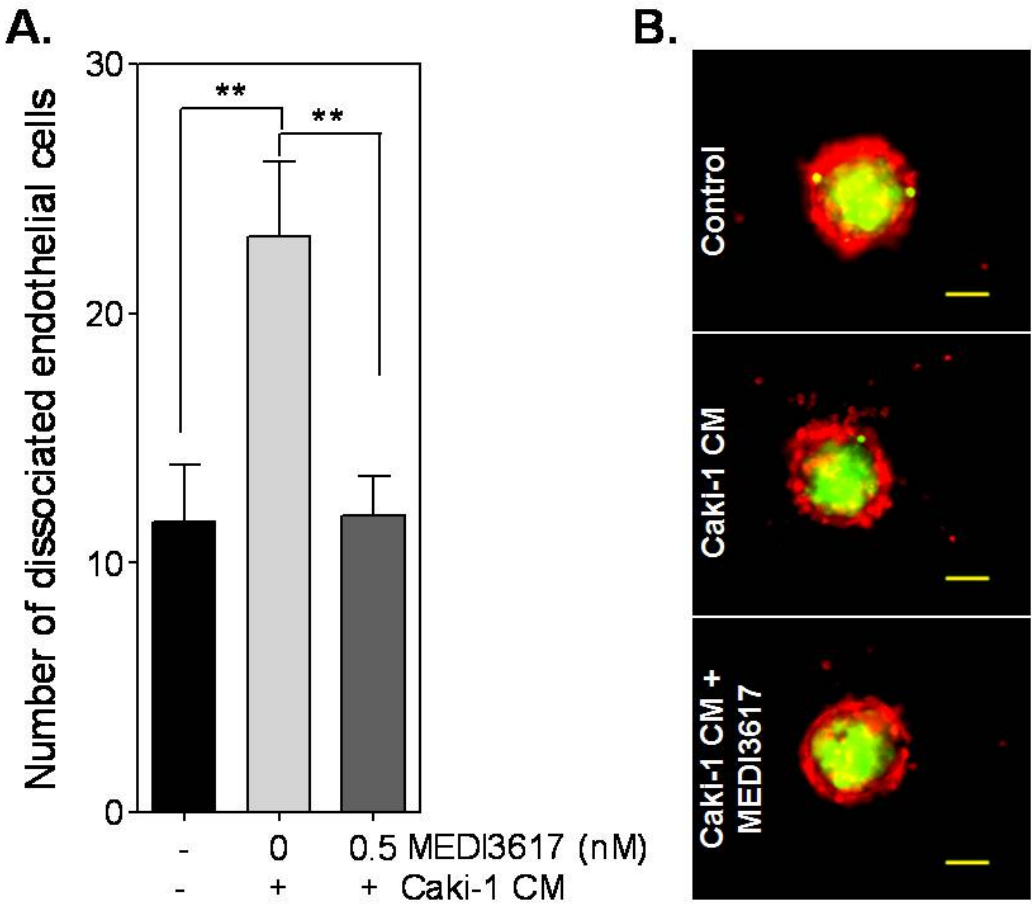

Figure 3-3. Tumor cell conditioned media leads to sphere destabilization. (A) Stimulation of spheres with Caki-1 CM for 4 hr led to significant endothelial cell loss from the sphere core compared to control. The presence of MEDI3617 (0.5 nM) impaired this loss. Column, mean; bars, SEM. Control and Caki-1 CM (n=12), Caki-1 CM plus MEDI3617 (n=7) spheres/group). **, $p<0.01$; Mann-Whitney U test. (B) Images demonstrating control, Caki-1 CM induced destabilization of co-culture spheres and its inhibition in the presence of MEDI3617 at 4 hr. Endothelial cells (HUVEC) shown in red (CellTracker Orange CMRA) and smooth muscle cells (HUASMC) shown in green (CellTrace Oregon Green). Images taken with Nikon eclipse TS100; scale bar, 100 μm.

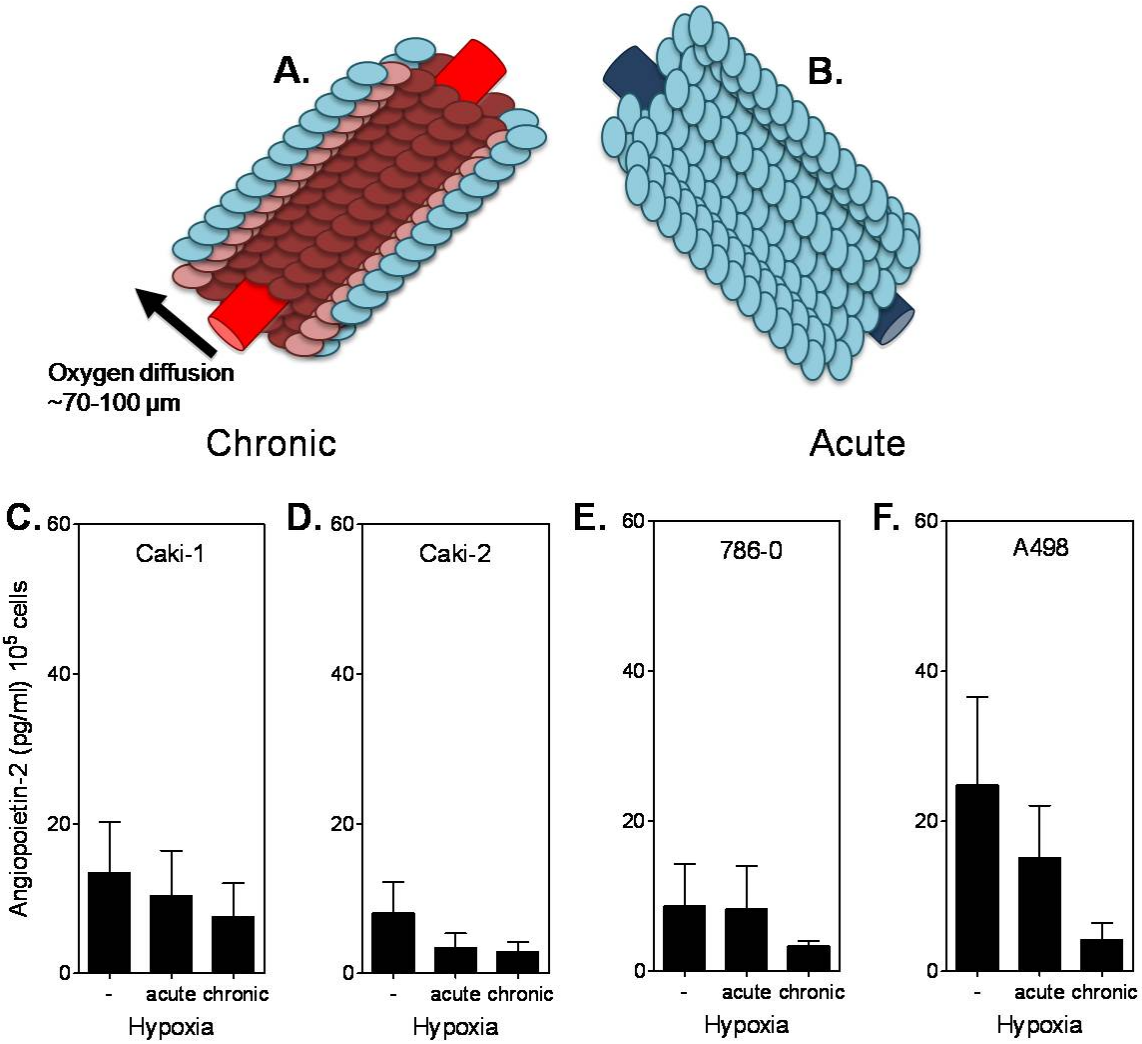

Figure 3-4. Tumor cells secrete minimal levels of Ang-2 under both normoxic and hypoxic conditions. (A) The oxygen diffusion limit is about 70-100 µm, tumor cells past this distance are deprived of oxygen and experience chronic or long term hypoxia. (B) Tumor vasculature is very unstable, vessels can shut down and reopen for minutes to hours. Tumor cells around such vessels experience acute or short term hypoxia. (C-D)Tumor cell media was collected at normal oxygen conditions 6 hr post media change, 6 (acute) and 48 (chronic) hr post hypoxia exposure. Ang-2 secretion was measured by ELISA, data were normalized to 10^5 cells. Normal oxygen levels, short term (acute) or long term (chronic) hypoxic conditions did not affect Caki-1 (C), Caki-2 (D), 786-0 (E), and A498 (F) secretion of Ang-2. Column, mean; bars, SEM of three independent experiments.

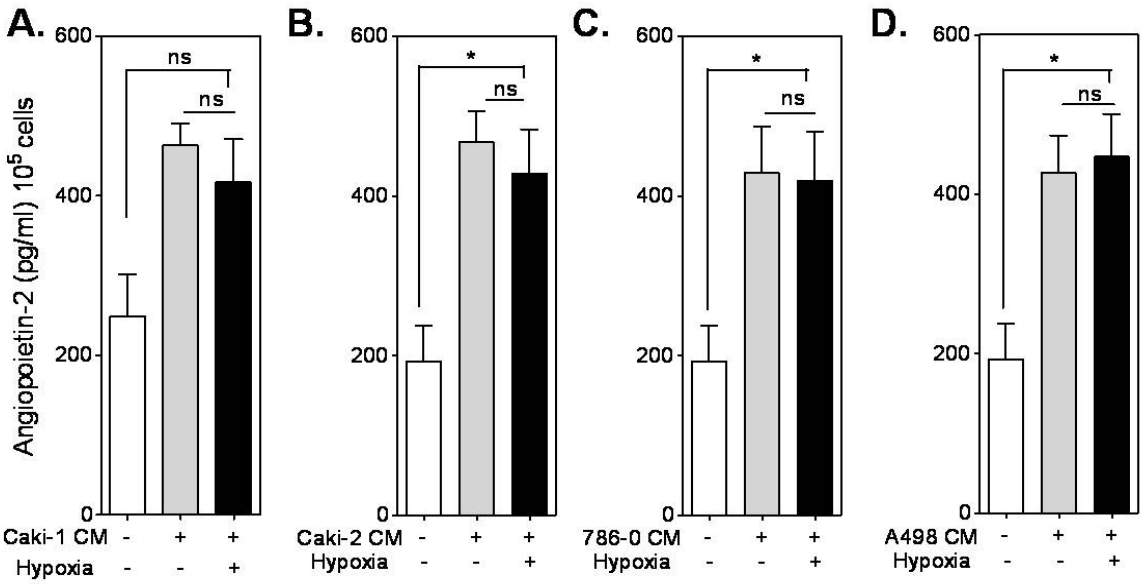

Figure 3-5. Tumor cell conditioned media from hypoxia do not enhance Ang-2 secretion from endothelial cells. HUVEC were exposed to tumor cell CM (normal or acute hypoxia) for 4 hr. Ang-2 secretion was measured by ELISA, data were normalized to 10^5 cells. Tumor cell CM from Caki-1 (A), Caki-2 (B), 786-0 (C), and A498 (D) significantly elevated Ang-2 secretion from endothelial cells in both conditions (normal or hypoxic conditions) compared to basal levels, however no difference in Ang-2 secretion from endothelial cells was shown between tumor cell CM collected under normal oxygen conditions or hypoxic conditions. Column, mean; bars, SEM of three independent experiments. *, $p<0.05$; Student t-test.

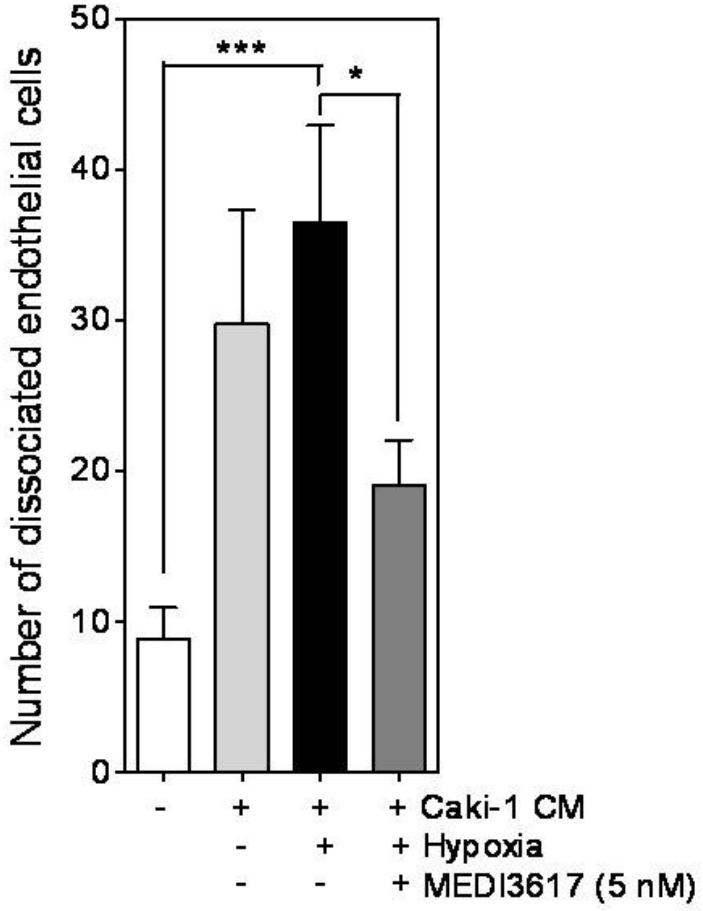

Figure 3-6. Hypoxia does not enhance tumor cell conditioned media mediated sphere destabilization. The presence of tumor cell CM from either normal or hypoxic conditions led to endothelial cell loss from the sphere core. No significant difference in endothelial cells loss was seen between tumor cell CM from normal or hypoxic conditions. The presence of MEDI3617 (0.5 nM) impaired endothelial cell loss due to tumor cell CM from both normal (Figure 3-3) or hypoxic conditions. Column, mean; bars, SEM. Control (n=13), Caki-1 CM normal (n=5); Caki-1 CM hypoxia (n=13); Caki-1 CM plus MEDI3617 (n=8) spheres/group). *, $p< 0.05$; ***, $p<0.0001$; Mann-Whitney U test.

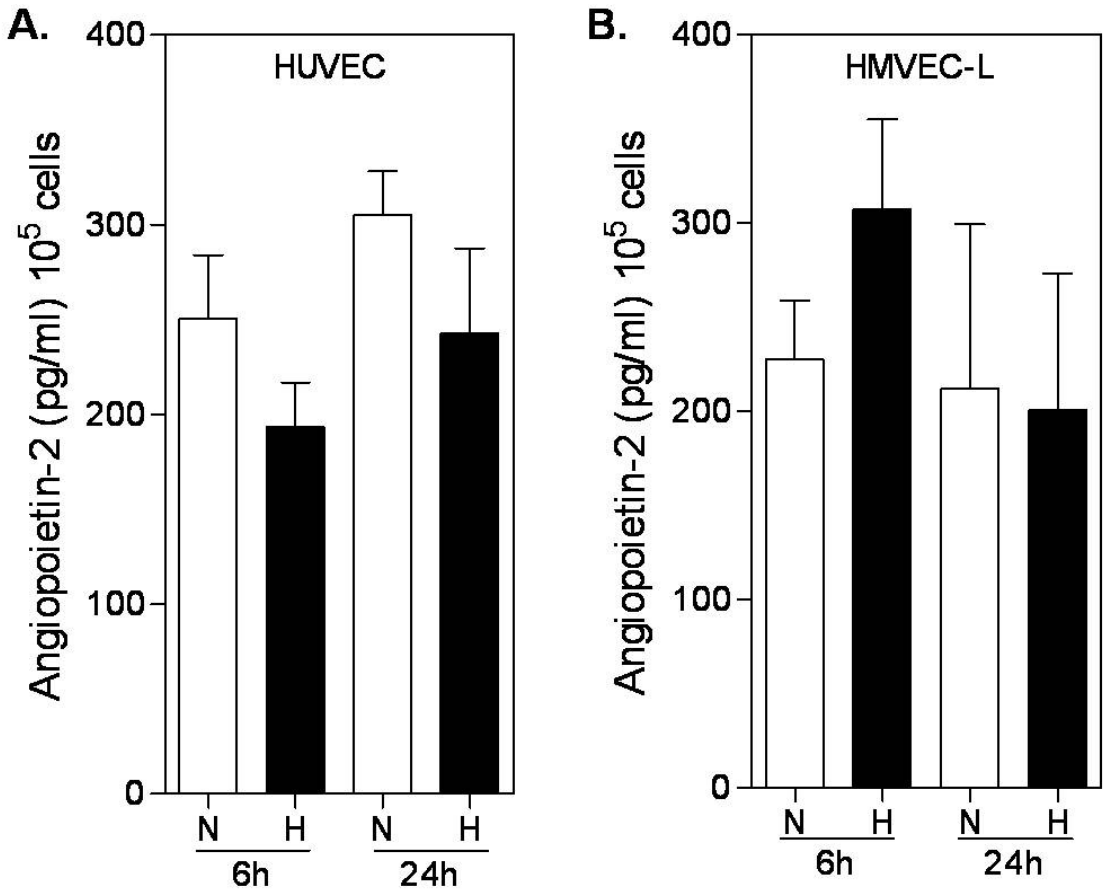

Figure 3-7. Hypoxia alone has no significant effect on Ang-2 secretion from endothelial cells. HUVEC (A) or HMVEC-L (B) was exposed to either acute (6 hr) or chronic (24 hr) hypoxia. Neither condition seemed to significantly affect Ang-2 secretion compared to normal oxygen levels. Column, mean; bars, SEM of three independent experiments.

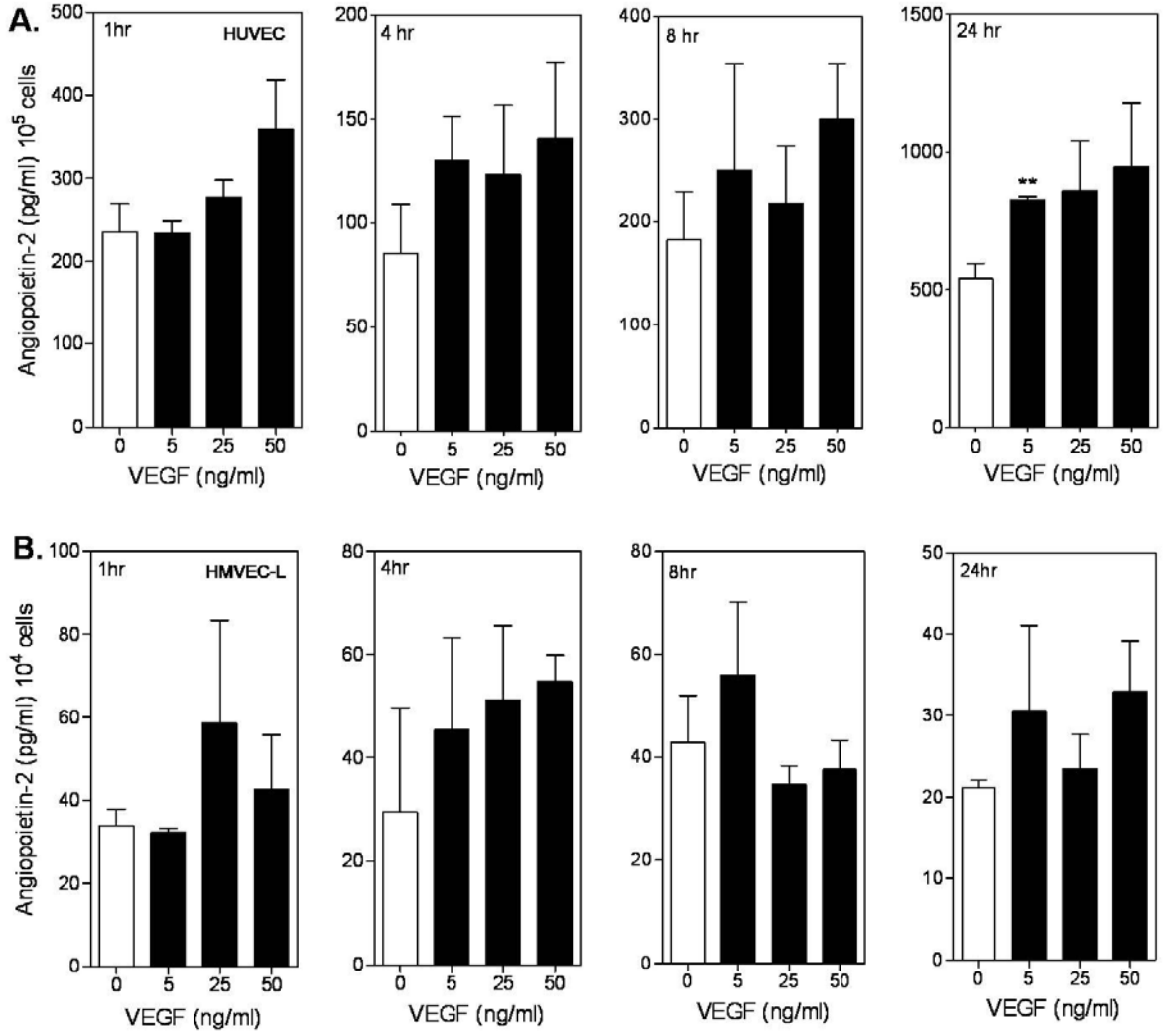

Figure 3-8. VEGF does not significantly stimulate endothelial cell secretion of Ang-2. HUVEC (A) and HMVEC-L (B) were exposed to VEGF (5, 25, or 50 ng/ml) for 1, 4, 8, or 24 hr; overall no significant difference in endothelial cell secretion of Ang-2 was observed. Ang-2 secretion was measured by ELISA, data were normalized to 10^5 cells (A) and 10^4 cells (B). Column, mean; bars, SEM of three independent experiments. **, $p<0.01$; Student t-test.

CHAPTER 4
ANGIOPOIETIN-2 INHIBITION ENHANCES THE NORMAL VASCULAR PHENOTYPE

Chapters 2 and 3 focused on the *in vitro* evaluation of Ang-2 dependent vessel destabilization and alluded to microenvironmental effects on this axis. In all cases, the presence of the Ang-2 inhibitor led to reduction of sphere destabilization suggesting that the inhibitor does neutralize the function of Ang-2. Chapter 4 evaluated the inhibitor as an anti-angiogenic agent; the inhibitors' effect on the tumor vasculature was assessed.

Background

Anti-angiogenic agents targeting the formation of new vasculature have become a standard of care in various cancer settings in the last decade (Ebos et al., 2009; Loges et al., 2010). The FDA approved agents currently used in the clinic mainly target the VEGF pathway, however some also inhibit the PDGF, cMET and mTOR pathways. In general, these drugs do not show sufficient anti-tumor effects alone, however, complement other conventional therapies such as chemotherapy, radiation therapy and surgery. Even though there are various success stories with these agents the full potential of anti-angiogenic therapies has not been met (Ebos et al., 2009; Loges et al., 2010). Patients are often resistant or become resistant over time with prolonged treatment with anti-angiogenics; such resistance stems from the redundancy of endothelial cell activation and therefore various other pathways may compensate when one is blocked, allowing the tumor to resume growth (Ebos et al., 2009; Loges et al., 2010). With the recent discovery of the Angiopoietin and Tie2 axis there is potential to overcome this resistance. This pathway is involved in a different process in angiogenesis than all the currently used anti-angiogenic agents; inhibiting vascular

destabilization rather than endothelial cell activation. There are several agents now being evaluated both preclinically and clinically that inhibit this axis (Gerald et al., 2013).

Normal vasculature is comprised of endothelial and peri-endothelial cells and the Ang-2/Tie2 axis destabilizes the contacts between the two cells types during angiogenesis. This exposes the normally protected endothelial cells to pro-angiogenic factors found in the microenvironment that in turn stimulate the endothelial cells to proliferate, migrate and form tubes (Armulik et al., 2005; Augustin et al., 2009; Carmeliet, 2005). Therefore, the current anti-angiogenic agents inhibit the second step in angiogenesis or the activation of the endothelium. The Ang-2/Tie2 axis is prevalent in the first step in angiogenesis or the destabilization of normal vasculature. In the present study the effect of the Ang-2 inhibitor was evaluated in renal cell carcinoma induced angiogenesis. The following studies focused on the early initiation of angiogenesis and development of tumor nodules. The structure of the tumor and surrounding vasculature was evaluated upon Ang-2 inhibition.

Materials And Methods

Reagents

MECA-32 was purchased from BioLegend (San Diego, CA), NG2 was obtained from Millipore (Temecula, CA). AlexaFluor 488 and 594 were purchased from Invitrogen (Grand Island, NY). VectaShield mounting medium with DAPI was purchased from Vector Labs Inc. (Burlingame, CA). Tissue-Tek OCT Compound was purchased from Sakura Finetek (Torrance, CA). 2-methylbutane was obtained from Thermo Fisher Scientific (Waltham, MA).

Cell Culture

Human clear cell renal cell carcinoma Caki-1 and Caki-2 cell lines were received as a gift from Dr. Susan Knox (Stanford University). They were grown in Dulbecco's modified minimum essential medium (D-MEM, Invitrogen) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 1% 200-mmol/L L-glutamine (Invitrogen). Cells were kept at 37 °C, 5% CO_2. Cells were used between passages 2 and 10.

Drug Preparation

Angiopoietin-2 inhibitor, MEDI3617 was kindly provided by MedImmune, LLC. The stock solution (5 mg/ml) was diluted to working concentrations in sodium citrate buffer solution. Stock solutions were kept at -80 °C and working concentrations at 4 °C.

Intradermal Angiogenesis Assay

All *in vivo* procedures were conducted in agreement with a protocol approved by the University of Florida Institutional Animal Care and Use Committee. Female athymic nu/nu mice were injected intradermally with 10^5 Caki-2 cells in 10 μl volume at four sites on the ventral surface. Beginning the day prior to tumor cell injection, mice were treated with IP injection of MEDI3617 (2, 10 mg/kg) every 3 days (3 doses total) up to six days post tumor cell inoculation. Mice were then euthanized, tumors measured via calipers and tumor volume (mm^3) calculated, assuming the tumor volume to be an ellipsoid, using the following equation: tumor volume = $\pi/6 \times d_1 \times d_2 \times$ height. Skin flaps were then removed and vessels growing into tumor nodules were counted using a Leica MZ16F dissecting microscope with Leica KL 1500 LCD fiber optic illuminator (Leica Microsystems Inc., Buffalo Grove, IL) at 2.5x original magnification (1,2,3). Images were captured with a Retiga EXi Fast1394 digital CCD camera (QImaging, British Columbia,

Canada) and OpenLab5 software (PerkinElmer Inc., Waltham, MA). Statistical significance between control and treated groups was determined using the Mann-Whitney U-Test at $p<0.05$.

Immunohistochemistry

Intradermal tumors were fresh frozen in OCT and methylbutane and cryosectioned at 5 μm thickness using Leica CM 3050S cryostat (Leica Microsystems Inc., Buffalo Grove, IL); sections were placed on superfrost plus gold slides (Thermo Fisher Scientific Inc., Waltham, MA) and kept at -80 °C until immunohistochemical staining. Tissue sections were acetone fixed for 10 minutes, blocked in 2% normal horse serum in 1x TBS, and incubated overnight at 4 °C with MECA-32 and NG2 primary antibodies. Secondary antibodies AlexaFluor 488 and 594 were added onto slides for 1 hr. Tissue sections were imaged with a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Inc., Thornwood, NY) with EXFO X-Cite 120 light source (Lumen Dynamics Group Inc., Ontario, Canada). Images were taken with a Retiga EXi Fast digital CCD camera (Qimaging, British Columbia, Canada) and processed in OpenLab5 software (PerkinElmer Inc., Waltham, MA); Rhodamine for MECA-32/AlexaFluor594, FITC for NG-2/AlexaFluor488 and DAPI filters were used. Vessel counts were obtained by taking up to ten random fields/tumor with 20x objective, counting the number of vessels in each random field. The number of peri-endothelial cell covered vessels was counted in the tumor periphery for each tumor. Statistical significance between control and treated groups was determined using the Mann-Whitney U-Test at $p<0.05$.

Results

Ang-2 Inhibitor Impairs Angiogenesis In Both VHL Normal And Mutated Renal Cell Carcinoma Models

The effect of Ang-2 inhibition on tumor cell induced angiogenesis was evaluated in both a VHL normal and mutated renal cell carcinoma model. Athymic nude mice were injected with $1x10^5$ Caki-1 (VHL normal) or Caki-2 (VHL mutated) human renal cell carcinoma cells (Figure 4-1). The mice received a single dose of the Ang-2 inhibitor and tumor nodules were evaluated 3 days post tumor cell injection (Figure 4-2A). Results show that Ang-2 inhibition led to a 1.2-, 2.2- ($p<0.01$), and 3-fold ($p<0.001$) reduction in the number of Caki-1 tumor nodule associated vessels in a dose dependent manner at 2, 10, and 20 mg/kg respectively (Figure 4-2B). Similar results were obtained with treatment of Caki-2 nodules, the Ang-2 antibody led to a 1.4- ($p<0.01$), 1.8- ($p<0.001$), and 2-fold ($p<0.001$) reduction in tumor nodule associated vasculature in a dose dependent manner (Figure 4-2C,D).

Ang-2 Inhibitor Hinders Both Tumor Growth And Angiogenesis In Renal Cell Carcinoma Xenograft Model

To evaluate the effects of Ang-2 targeting on initiation of angiogenesis and tumor growth, nude mice were inoculated intradermally with Caki-2 (mutated VHL) human renal cell carcinoma cells ($1x10^5$) and treated with the Ang-2 inhibitor (Figure 4-3A). Results showed that the Ang-2 inhibitor reduced the tumor volume by 2.1 and 3.9-fold ($p<0.01$) (Figure 4-3B), and vessel number by 1.4 and 2.1-fold ($p<0.01$, $p<0.0001$) (Figure 4-3C) at 2 and 10 mg/kg respectively compared to control. Immunohistochemical analysis revealed that the Ang-2 inhibitor significantly reduced (1.6 and 1.7-fold for 2 and 10 mg/kg respectively) the number of vessels in the tumor core compared to control ($p<0.01$) (Figure 4-4).

Ang-2 Inhibition Increases Peri-Endothelial Cell Covered Vessels In Both The Tumor Periphery And Core

Normal vasculature is composed of an endothelial cell layer surrounded by a peri-endothelial cell layer. Vascular structure of tumors from the intradermal assay (Figure 4-3, 4-4) was determined using immunohistochemistry. Figure 4-5 demonstrates normal vasculature; nuclei for both the endothelial (red, MECA-32) and peri-endothelial cell layer (green, NG2) are visible with the peri-endothelial cell layer surrounding the endothelium. Treatment of tumors with the Ang-2 inhibitor led to a 1.5- and 3.4-fold increase in the 2 and 10 mg/kg groups, respectively, compared to control in the tumor periphery ($p<0.05$, $p<0.0001$ respectively) (Figure 4-6). A significant difference of 2.2-fold was noted in the 10 mg/kg group compared to the 2 mg/kg group ($p<0.05$) (Figure 4-6). Similar trends were seen within the tumor, treatment with the inhibitor led to 2- and 4.2-fold increase in the number of peri-endothelial cell covered vessels in the 2 and 10 mg/kg groups, respectively, compared to control ($p<0.05$, $P<0.01$ respectively) (Figure 4-7). A significant difference was seen between the 2 and 10 mg/kg groups with a 2.1-fold increase in the number of peri-endothelial cell covered vessels in the 10 mg/kg group ($p<0.05$) (Figure 4-7).

Discussion

Renal cell carcinoma is a highly vascularized disease and anti-angiogenic agents are commonly used as both first and second line treatments in patients. Although in general these agents are beneficial especially in the combination of conventional cancer therapies there are patients who fail to respond or become resistant to such treatments (Ebos et al., 2009). The intradermal angiogenesis assay (Figure 4-1) allows for the rapid evaluation of anti-angiogenic agents by the assessment of vasculature penetrating the

tumor nodule. The current studies evaluated a new class of anti-angiogenic agents that inhibit the Angiopoietin/Tie-2 axis, the Ang-2 inhibitor, MEDI3617, and its effect on tumor cell induced angiogenesis was determined.

Results show that the Ang-2 inhibitor with a single dose effectively impaired the early development of human renal cell carcinoma induced angiogenesis (Figure 4-2). Since renal cell carcinomas commonly have VHL mutations rendering the disease highly aggressive and vascularized, both a VHL normal (Caki-1) and mutated (Caki-2) cell lines were evaluated. In either case the Ang-2 inhibitor significantly impaired the number of tumor nodule associated vessels in a dose dependent manner by reducing the number of vessels 1.2-3 fold in Caki-1 model (Figure 4-2B) and 1.4-2 fold in Caki-2 model (Figure 4-2C,D). The difference in angiogenic potential of the two cell lines were clearly visible, the VHL mutated cell line, Caki-2, had a 2.3-fold higher number of tumor nodule associated vessels than the VHL normal Caki-1 cells did. However the Ang-2 inhibitor was able to impair angiogenesis significantly although it is evident that the range of inhibition was lower in the VHL mutated tumor (Figure 4-2). This observation most likely account for the fact that the VHL mutated Caki-2 cells constitutively expresses VEGF and may escape the Ang-2/Tie2 axis early on.

Even though there was a slightly lower inhibitory response to the Ang-2 inhibitor in VHL mutated Caki-2 cells, when the tumor cells were allowed to grow and develop for twice as long (Figure 4-3A) receiving three doses of the Ang-2 antibody, the results showed a reduction of both the tumor volume (Figure 4-3B) and vessel number (Figure 4-3C). Impairment of tumor vasculature was further confirmed by immunohistochemical analysis of tumor nodules (Figure 4-4). These results are consistent with studies

involving the Ang-2 inhibitor in other tumor settings as well as various other Ang-2 targeting agents (Brown et al., 2010; Daly et al., 2013; Falcon et al., 2009; Leow et al., 2012).

The Ang-2/Tie2 axis is involved in the destabilization of normal vasculature by disrupting the endothelial and peri-endothelial cell contacts therefore the structure of the tumor and surrounding vasculature were determined after treatment with the Ang-2 inhibitor. Results show that Ang-2 inhibition led to a dose dependent increase in the number of vessels that had peri-endothelial cell coverage both in the tumor periphery (Figure 4-6) and within the tumor core (Figure 4-7). The increase in peri-endothelial cell coverage upon treatment with the Ang-2 inhibitor was also observed with the Ang-1/2 peptibody Trebananib (AMG386). Therefore this phenomenon is not exclusive to MEDI3617 but supports the role of Ang-2 in vascular destabilization (Falcon et al., 2009). Since the sections obtained for these tumors only show a snapshot in time it can not be determined whether the peri-endothelial cell coverage of vessels was maintained as the tumor nodule developed or whether upon Ang-2 inhibition the tumor associated vessels were able to recruit peri-endothelial cells to the tumor vessels via the Ang-1/Tie2 interactions. In either case treatment with the Ang-2 inhibitor led to an increase in normal vascular phenotype and reduced the number of tumor vessels. These data support the advancement of this agent as an anti-angiogenic drug for the treatment of cancer.

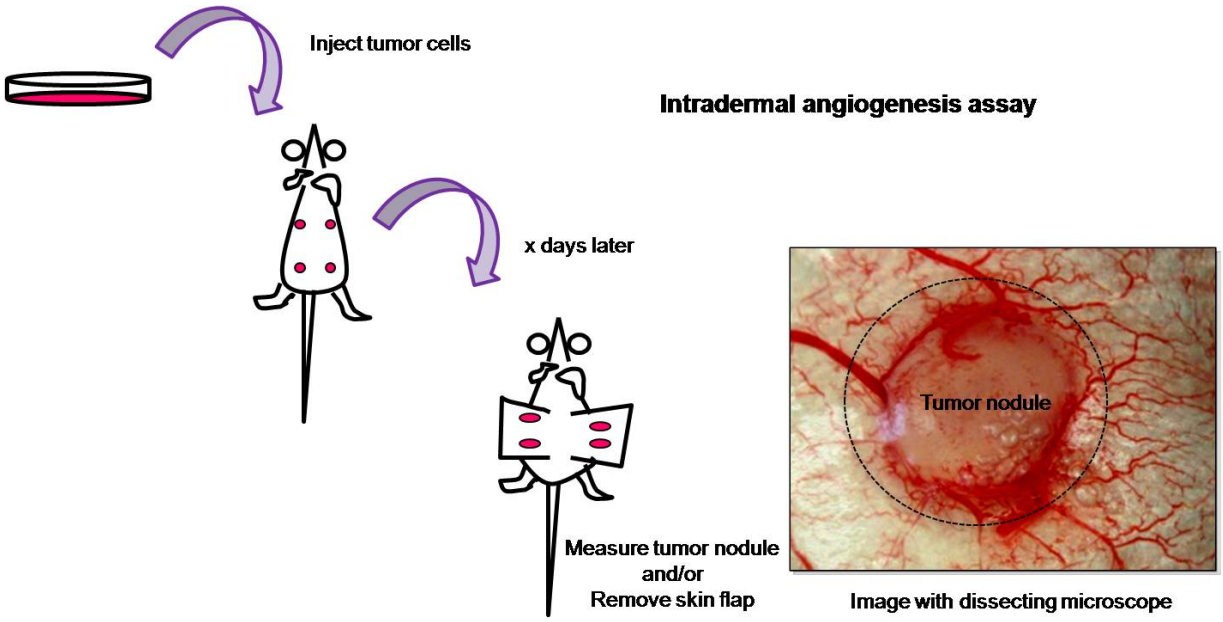

Figure 4-1. Intradermal angiogenesis assay. Tumor cells were injected at 10^5 cells underneath the skin on the ventral side at four sites. Tumor cells were allowed to grow for either 3 or 6 days post injection at which point tumor nodules were measured (6 day) and skin flaps removed and the number of blood vessels growing into the tumor counted (3,6 day)

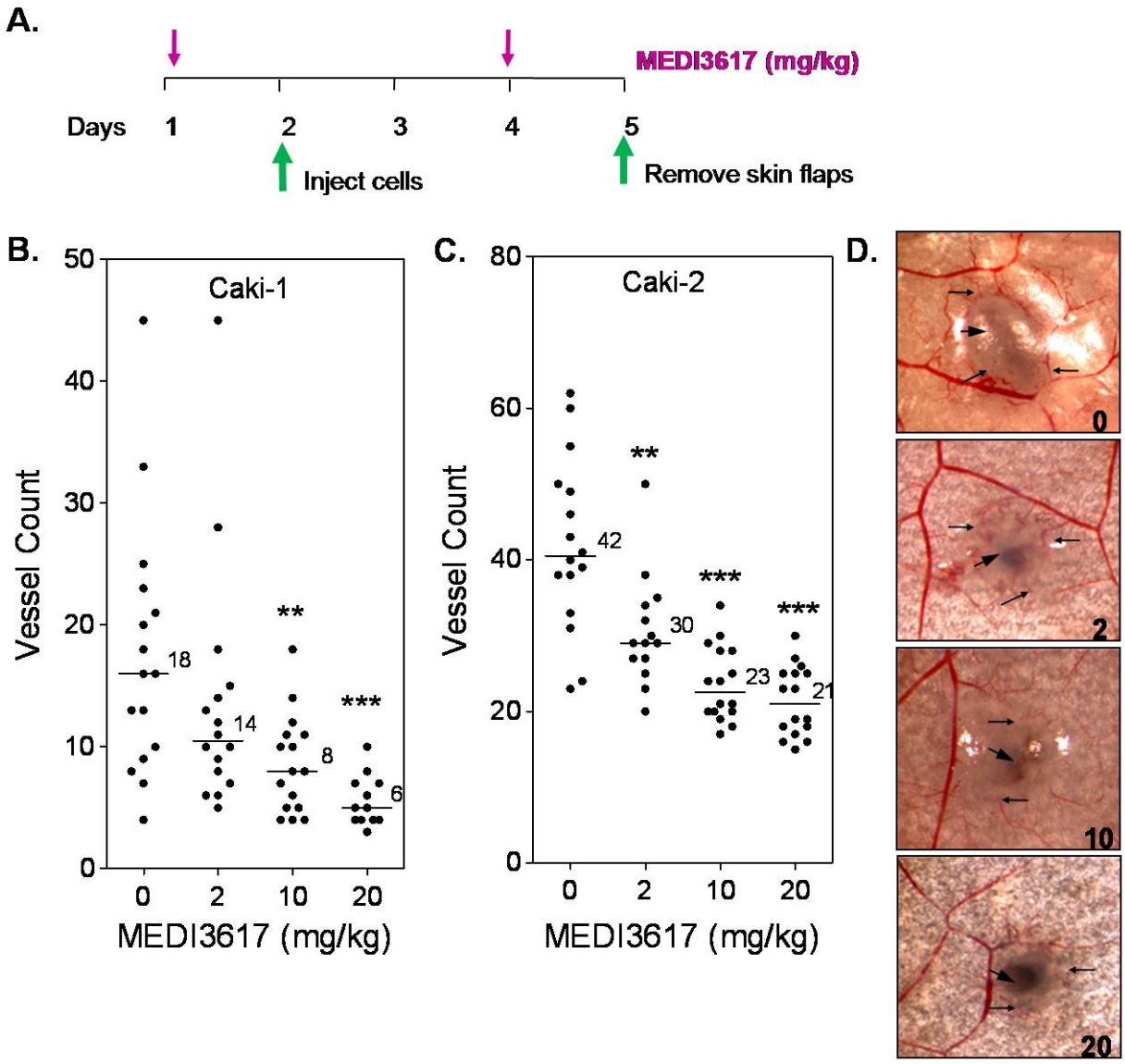

Figure 4-2. Ang-2 inhibition reduces tumor cell induced blood vessel formation. Mice were injected intradermally with tumor cells (Figure 4-1) and treated with MEDI3617 (2 ,10, and 20 mg/kg) beginning the day prior to tumor cell inoculation (A). The number of blood vessels initiated by (B) human renal cell carcinoma Caki-1 and (C) Caki-2 tumor cells were determined at the end of a 3 day period. Lines, mean; bar, SD (n=16). **, $p<0.01$: ***, $p<0.0001$; Mann-Whitney U test. (D) Representative images of blood vessels induced by Caki-2 tumor cells using a dissecting microscope at 2x magnification. Large arrow head, tumor nodule; small arrows, tumor induced blood vessels. Adapted with permission from ELSEVIER: *Microvascular Research* 2012; 83(3):290-7.

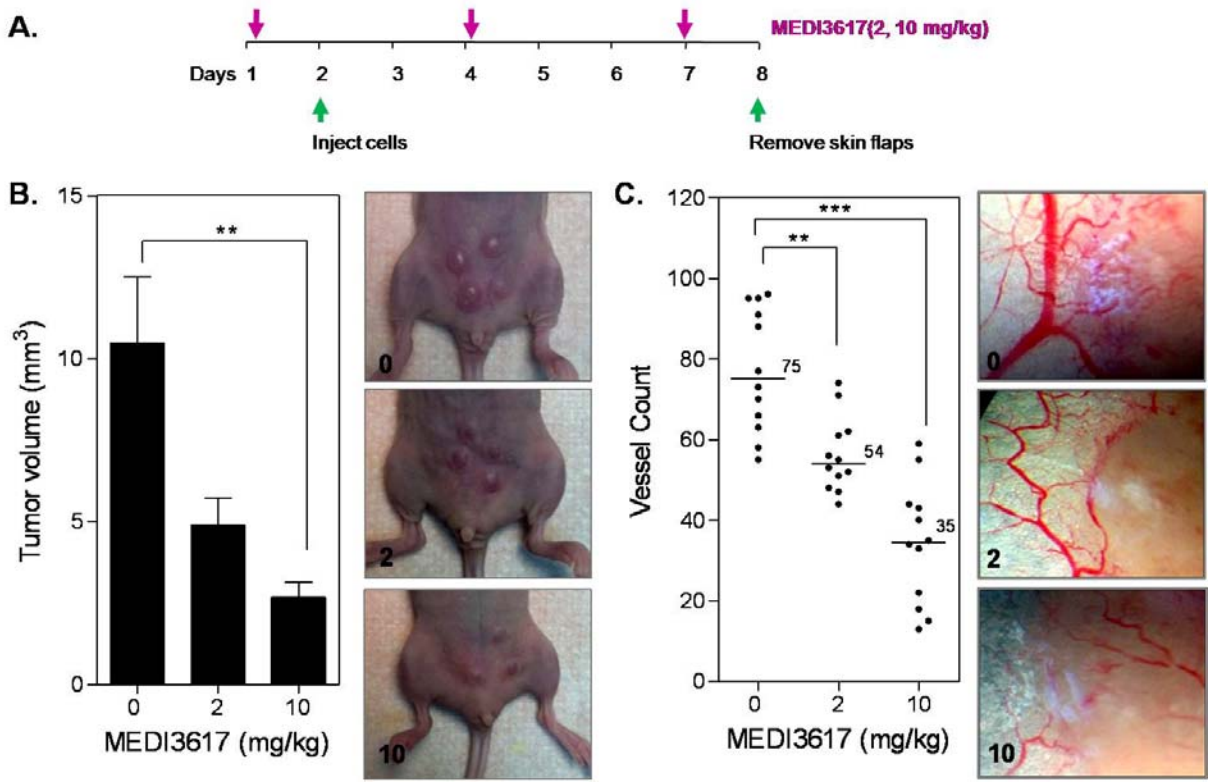

Figure 4-3. Ang-2 inhibition impedes both tumor growth and angiogenesis. Mice were injected intradermally with Caki-2 renal cell carcinoma cells (Figure 4-1) and treated with MEDI3617 (2 or 10 mg/kg) beginning the day prior to tumor cell inoculation (A). Tumor volume (B) and the number of tumor cell induced blood vessels (C) were determined at the end of a 7 day period. Bar, mean with SEM (n=16); line, median (n=12). **, $p<0.01$; ***, $p<0.0001$; Mann-Whitney U-Test.

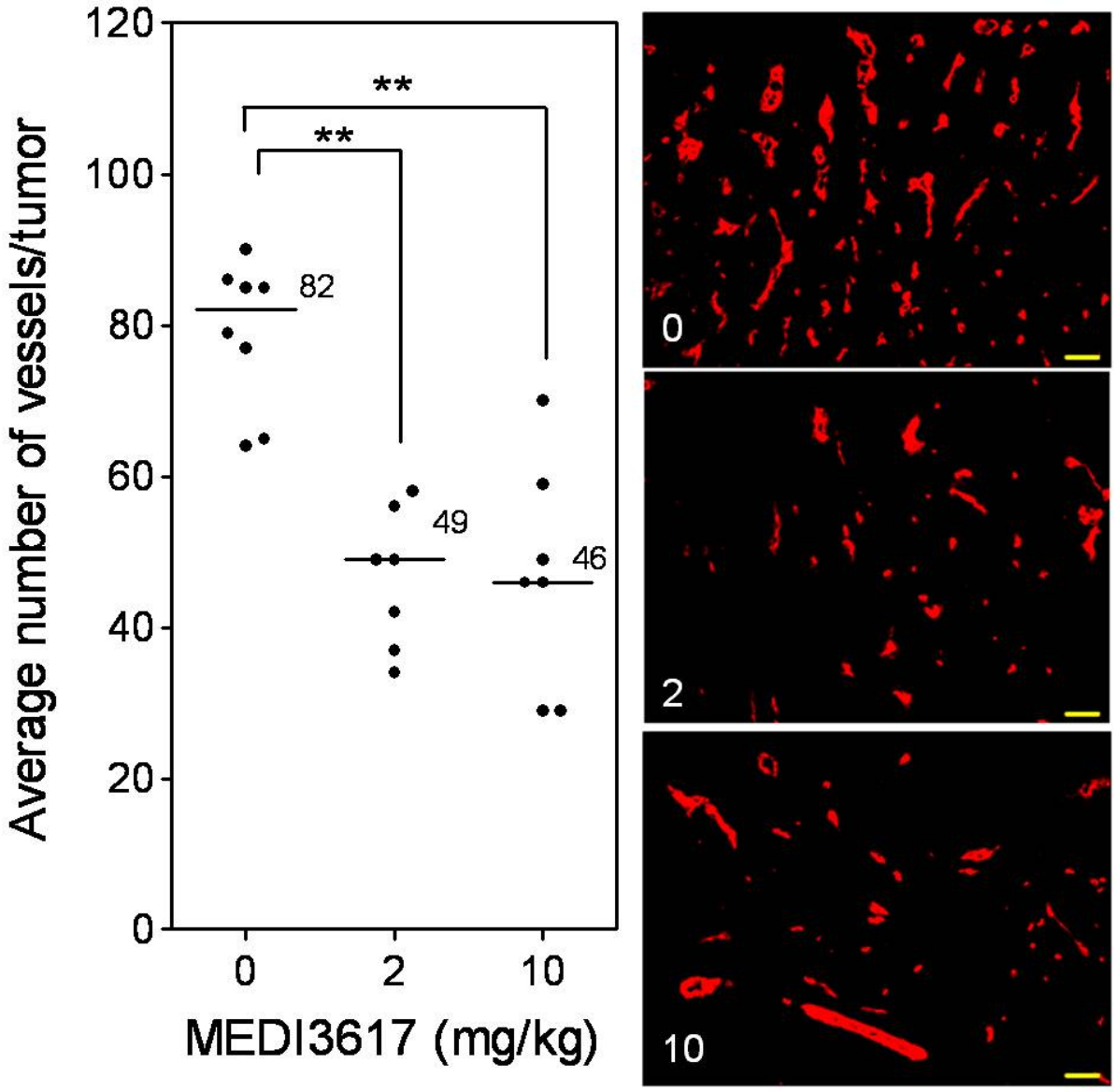

Figure 4-4. Ang-2 inhibition reduces the number of tumor vessels. Mice were treated with MEDI3617 (Figure 4-1). The number of blood vessels within the tumor were evaluated using immunohistochemistry of fresh frozen tissue. Vasculature was stained with MECA-32. Line, median. 0, 2 mg/kg (n=8), 10 mg/kg (n=7) **, $p<0.01$; Mann-Whitney U-Test. (B) Representative images of the median of each group. Images taken with Zeiss Axioplan Imaging2 microscope with 20x objective; scale bar = 140 μm.

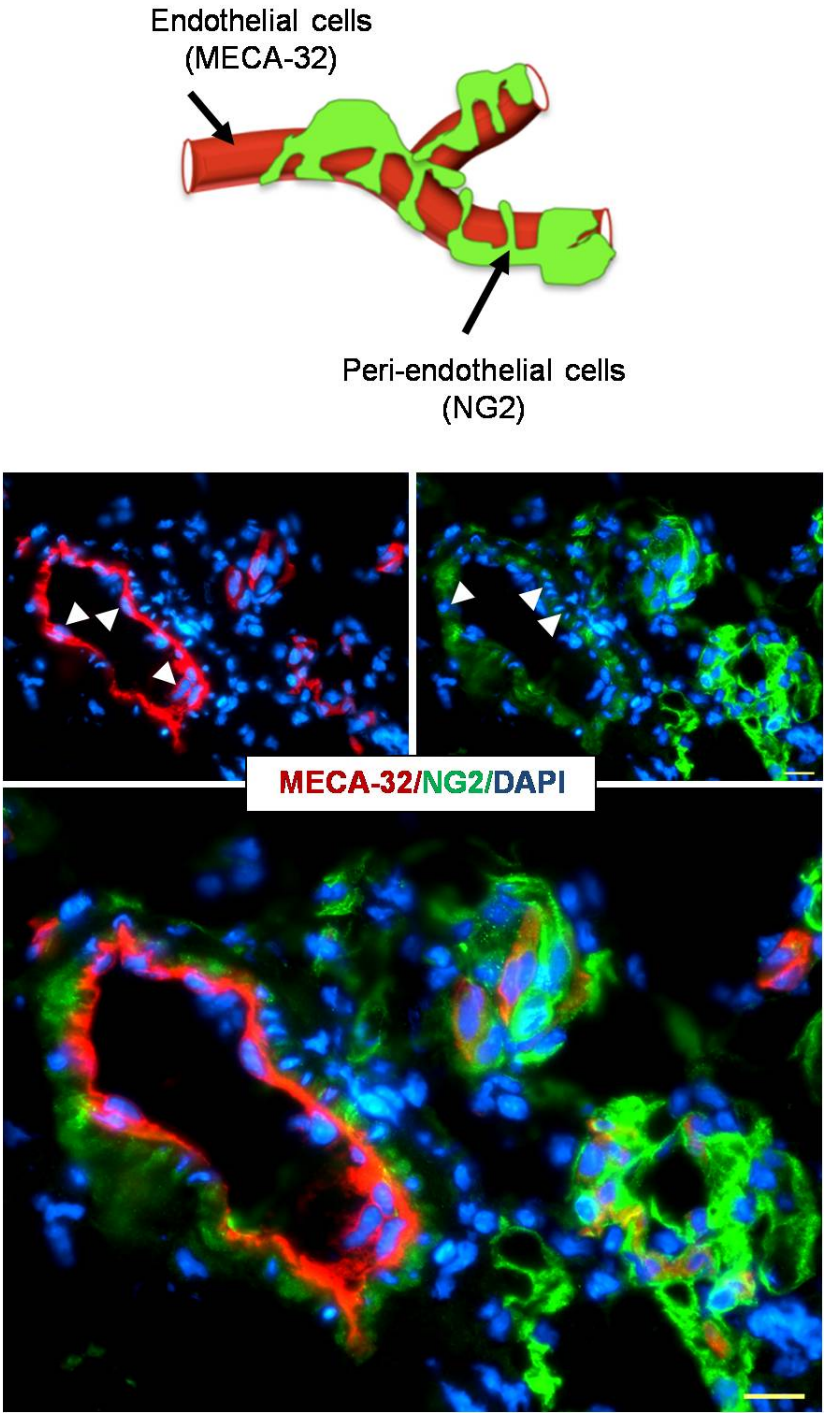

Figure 4-5. Normal vasculature is composed of endothelial and peri-endothelial cells. Arrows, nuclei associated with endothelial and peri-endothelial cells. Red, MECA-32 (endothelium); green, NG2 (peri-endothelial cells), blue, DAPI. Images taken with Zeiss Axioplan Imaging2 microscope with 63x objective; scale bar = 46 μm.

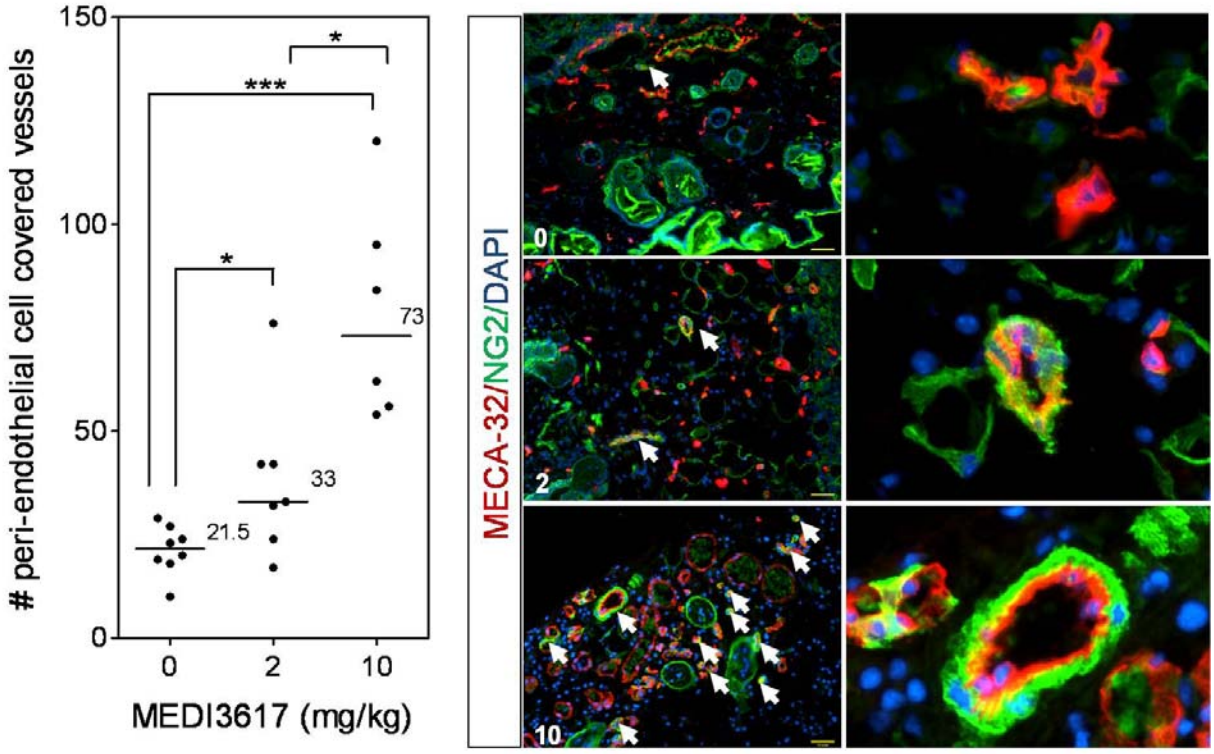

Figure 4-6. Ang-2 inhibition leads to increased normal vascular phenotype in the tumor periphery. The number of peri-endothelial cell covered vessels increased in a dose dependent manner in the tumor periphery. Line, median. 0 (n=8), 2 mg/kg (n=7), 10 mg/kg (n=6) *, $p<0.05$; ***$p<0.0001$; Mann-Whitney U-Test. White arrows show peri-endothelial cell covered vessels. Red, MECA-32 (endothelium); green, NG2 (peri-endothelial cells); blue, DAPI. Images taken with Zeiss Axioplan Imaging2 microscope with 20x objective; scale bar = 140 µm

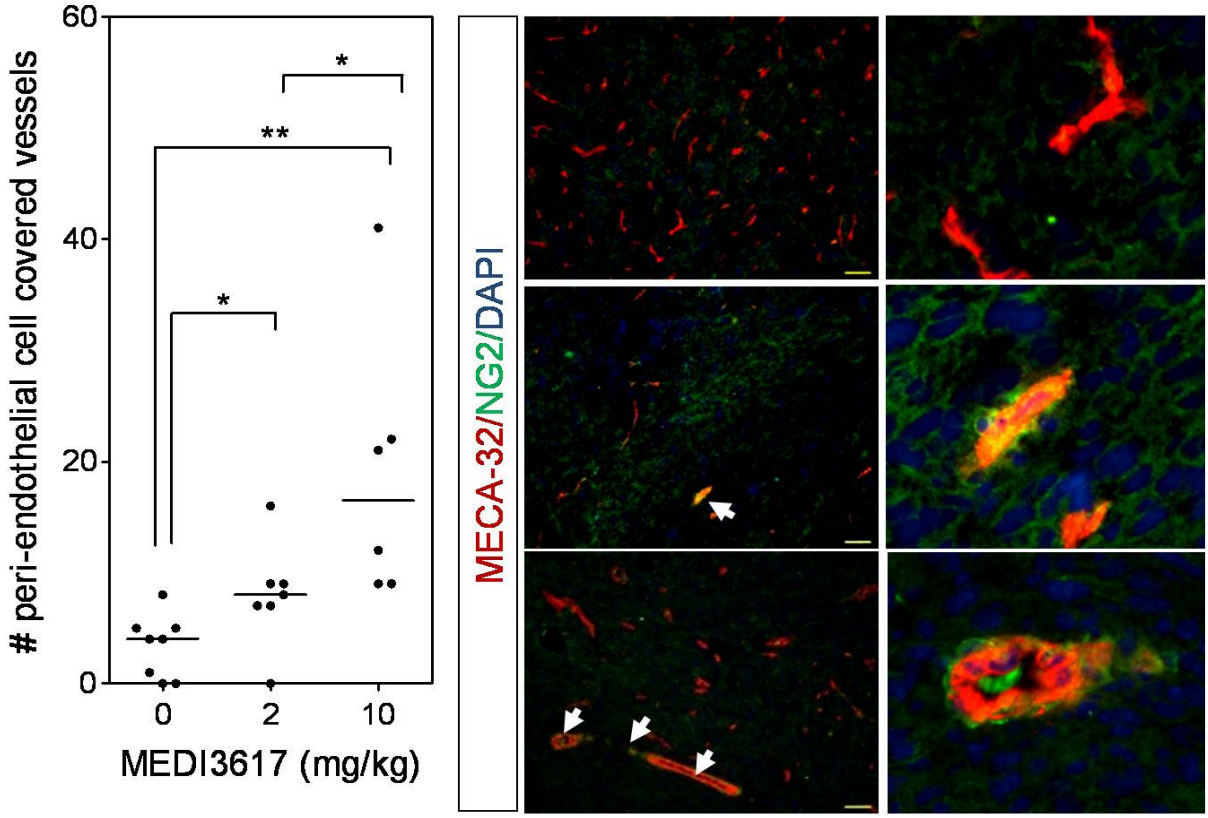

Figure 4-7. Ang-2 inhibition leads to increase normal vascular phenotype within the tumor. The number of peri-endothelial cell covered vessels increased in a dose dependent manner within the tumor core. Line, median. 0 (n=8), 2 mg/kg (n=7), 10 mg/kg (n=6) *, $p<0.05$; **$p<0.001$; Mann-Whitney U-Test. White arrows show peri-endothelial cell covered vessels. Red, MECA-32 (endothelium); green, NG2 (peri-endothelial cells); blue, DAPI. Images taken with Zeiss Axioplan Imaging2 microscope with 20x objective; scale bar = 140 µm.

CHAPTER 5
DORSAL SKINFOLD WINDOW CHAMBER MODEL TO EVALUATE EARLY TUMOR CELL INDUCED ANGIOGENESIS AND ANTI-ANGIOGENIC THERAPY

Chapter 4 evaluated the anti-angiogenic effect of Ang-2 inhibition using an intradermal angiogenesis assay. The assay is an endpoint evaluation of impacts on the tumor vasculature. Chapter 5 used an *in vivo* murine dorsal skinfold window chamber model to evaluate anti-angiogenic therapy real time. This model allows for the assessment of tumor and vascular growth on a daily basis by imaging mice surgically implanted with titanium chambers bearing tumor. The following work was conducted in collaboration with Jennifer Lee and Dr. Brian Sorg in the Biomedical Engineering Department at the University of Florida.

Background

The dorsal skinfold window chamber is commonly used to evaluate the microvasculature in various settings *in vivo*. The model involves surgical implantation of a titanium window onto exposed microvasculature on the dorsal skin of mice (Moy et al., 2011; Palmer et al., 2011). This method has been used to evaluate for example angiogenesis in endometriosis and tumor development; it was also used to evaluate the early phases of preclinical development of Bevacizumab in the 1990s (Borgstrom et al., 1998; Borgstrom et al., 1999; Borgstrom et al., 1996; Laschke and Menger, 2007; Li et al., 2000; Yuan et al., 1996). Hyperspectral imaging of hemoglobin saturation has allowed for the evaluation of oxygenation and hypoxia, oxygen transport dynamics and characterization of the abnormal vascular physiology and acute oxygen fluctuations within the tumor microvasculature (Dedeugd et al., 2009; Hardee et al., 2009; Sorg et al., 2005; Wankhede et al., 2010a). Tumor microvascular response to various therapies

such as radiotherapy, vascular disrupting agents and sickled erythrocytes has also been evaluated using this imaging system (Dewhirst et al., 2007; Terman et al., 2013; Wankhede et al., 2010b). Settings other than the tumor microvasculature have also been explored for example the characterization of arteriovenous malformation in hereditary hemorrhagic talengiectasia, the formation of spontaneous and induced microvascular thrombosis and occlusions and immune cell localization and tissue damage in particle based vaccines (Choe et al., 2010; Park et al., 2009; Wankhede et al., 2010a).

Anti-angiogenic agents are commonly evaluated *in vivo* using either matrigel plug or intradermal assays as well as histological assessments of the number and function of tumor associated vasculature (Auerbach, 2008). To date, the murine dorsal skinfold window chamber model has not been widely utilized to evaluate tumor response to anti-angiogenic agents using hyperspectral imaging to assess not only the vascular density of the tumor but also its oxygenation status. This model allows for an *in vivo*, real time assessment of tumor vasculature at microvessel resolution and has tremendous potential to answer several important questions regarding aspects of vascular response to anti-angiogenics such as oxygenation status of the vasculature. The current study evaluated both a VEGF and Ang-2 targeted approach on the induction of early human renal cell carcinoma cell induced angiogenesis. Limitations of this model in this particular setting quickly became apparent and are discussed here.

Materials And Methods

Reagents

Mouse Ang-2 ELISA kit was purchased from MyBioSource (San Diego, CA). MECA-32 was purchased from BioLegend (San Diego, CA), NG2 was obtained from

Millipore (Temecula, CA). AlexaFluor 488 and 594 were purchased from Invitrogen (Grand Island, NY). VectaShield mounting medium with DAPI was purchased from Vector Labs Inc. (Burlingame, CA). Tissue-Tek OCT Compound was purchased from Sakura Finetek (Torrance, CA). 2-methylbutane was obtained from Thermo Fisher Scientific (Waltham, MA).

Cell Culture

The human clear cell renal cell carcinoma, Caki-2, cell line was received as a gift from Dr. Susan Knox (Stanford University). Caki-2 was grown in Dulbecco's modified minimum essential medium (D-MEM, Invitrogen, Grand Island, NY) supplemented with 10% FBS (Invitrogen, Grand Island, NY), 1% penicillin-streptomycin (Invitrogen, Grand Island, NY), and 1% 200-mmol/L $_{L}$-glutamine (Invitrogen, Grand Island, NY). Cells were kept at 37 °C, 5% CO_2.

Drug Preparation

Ketamine and xylazine were purchased from Webster Veterinary (Devens, MA) and prepared in sterile saline. Angiopoietin-2 inhibitor, MEDI3617, was kindly provided by MedImmune, LLC. The stock solution (5 mg/ml) was diluted to the working concentration (10 mg/kg) in sodium citrate buffer solution. Stock solutions were kept at -80 °C and working concentrations at 4 °C. Sunitinib was obtained from LC Laboratories (Woburn, MA) and stored at -20 °C. Working concentration of Sunitinib was prepared fresh daily by making stock and diluent buffers of citric acid monohydrate and sodium citrate dihydrate at pH 6.8 and 3.2 respectively. A 1:7 stock to diluent solution was made (~pH 3.3) and acidified to pH 1.0, Sunitinib was dissolved, and the solution was adjusted to pH 3.5. Working concentration of Sunitinib was kept at room temperature.

Window Chamber Surgery And Tumor Initiation

All *in vivo* procedures were conducted in agreement with a protocol approved by the University of Florida Institutional Animal Care and Use Committee. Dorsal skinflap window chamber surgeries were carried out as previously described by Moy and colleagues (Moy et al., 2011). Briefly, female athymic nu/nu mice (Harlan Laboratories, Indianapolis, IN) were surgically implanted with a titanium window chamber on the dorsal skinflap. During the surgical procedure mice were anesthetized via intraperitoneal (IP) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Human renal cell carcinoma, Caki-2, tumor was initiated in the window chamber during surgery by injecting cells ($2x10^4$ in 10 μl volume) subqutaneously in the dorsal skinflap prior to placing a 12 mm diameter number 2 round glass cover slip (Erie Scientific, Portsmouth, NH) over the exposed skin. Post surgical procedure, animals were housed in an environmental chamber maintained at 33 °C and 50% humidity with standard 12 hr light/dark cycles for the remainder of the study.

Treatment Of Window Chamber Tumors

Mice were treated with Sunitinib (100 mg/kg) daily via oral gavage or with MEDI3617 (10 mg/kg) every 3 days via IP injection, starting the day of window chamber surgery/tumor initiation up to day 11 post-surgery when mice were euthanized. During the study, tumors were measured daily using calipers and tumor volume (mm^3) was calculated, assuming that the tumor volume was half of an ellipsoid, using the following equation: tumor volume = ½ [$\pi/6 \times d_1 \times d_2 \times$ height]. Statistical significance was determined using Mann-Whitney U-Test.

Hyperspectral Imaging

The spectral imaging system, image acquisition, and image processing methods have previously been described (Moy et al., 2011; Sorg et al., 2005). Briefly, window chamber tumors were imaged daily using a Zeiss AxioImager microscope (Carl Zeiss, Inc., Thornwood, NY) with 100-W tungsten halogen lamp, CCD camera thermoelectrically cooled to -20 °C (DVC Co., Austin, TX; Model no.1412AM-T2-FW) and C-mounted liquid crystal tunable filter (LCTF) (CRI Inc., Woburn, MA). Tuning of the LCTF and image acquisition with the CCD camera was automatically controlled with LabVIEW8 software (National Instruments Corp., Austin, TX). Vascular hemoglobin saturation measurements and images were created from the spectral image data, image processing was performed using Matlab software (The Mathworks Inc., Natick, MA). During imaging mice were placed on a heated platform and anesthetized with isofluorane (Webster Veterinary, Devens, MA).

Immunohistochemistry

Eleven days post surgery/tumor cell inoculation the mice were euthanized with euthasol (0.01 ml/g) (Webster Veterinary, Devens, MA), titanium window chambers were removed and tumors were fresh frozen in OCT and methylbutane. Tumors were sectioned at 5 μm thickness using Leica CM 3050S cryostat (Leica Microsystems Inc., Buffalo Grove, IL); sections were placed on superfrost plus gold slides (Thermo Fisher Scientific Inc., Waltham, MA) and kept at -80 °C until immunohistochemical staining. Tissue sections were acetone fixed for 10 minutes, blocked in 2% normal horse serum, and incubated overnight at 4 °C with MECA-32 and NG2 primary antibodies, at room temperature with secondary antibodies AlexaFluor 488 and 594 for 1 hr. Tissue sections

were imaged with a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Inc., Thornwood, NY) with EXFO X-Cite 120 light source (Lumen Dynamics Group Inc., Ontario, Canada). Images were taken with a Retiga EXi Fast digital CCD camera (QImaging, British Columbia, Canada) and processed in OpenLab5 software (PerkinElmer Inc., Waltham, MA); Rhodamine for MECA-32/AlexaFluor594, FITC for NG-2/AlexaFluor488 and DAPI filters were used. Vessel counts were obtained by taking up to ten random fields/tumor at 20x objective, counting the number of vessels in each field. The number of peri-endothelial cell covered vessels was counted in the tumor periphery for each tumor. Statistical significance between control and treated groups was determined using the Mann-Whitney U-Test at $p<0.05$.

Serum Angiopoietin-2 Levels

Blood from the tail vein was drawn from mice (2 mice/group) that (1) did not receive surgery (baseline), (2) that received surgery and ones (3) that received surgery with tumor cells implanted at days 3 and 5 post surgery. About 100 μl of blood per mouse was collected and placed on ice for 2 hrs to let the blood clot. Blood was then centrifuged for 15 minutes, 4 °C at 1000 g. Serum was collected and stored at -80 °C until analysis. Serum Ang-2 levels were calculated using a mouse Ang-2 ELISA kit. Based on company recommendation, manufacturers' protocol was altered to load 30 μl of either standards or serum and 50 μl of 3.3 fold diluted conjugate in 0.9% NaCl per well and incubate for 2 hrs at 37 °C; manufacturers' protocol was otherwise followed.

Intradermal Angiogenesis Assay

All *in vivo* procedures were conducted in agreement with a protocol approved by the University of Florida Institutional Animal Care and Use Committee. Female athymic

nu/nu mice were injected intradermally with 10^5 Caki-2 cells in 10 μl volume at four sites on the ventral surface. Beginning the day prior to tumor cell injection, mice were treated with either daily oral gavage of Sunitinib (100 mg/kg) or IP injection of MEDI3617 (10 mg/kg) every 3 days up to six days post tumor cell inoculation. Mice were then euthanized, tumors measured via calipers and tumor volume (mm^3) calculated, assuming the tumor volume to be an ellipsoid, using the following equation: tumor volume = $\pi/6 \times d_1 \times d_2 \times$ height. Skin flaps were then removed and vessels growing into tumor nodules were counted using a Leica MZ16F dissecting microscope with Leica KL 1500 LCD fiber optic illuminator (Leica Microsystems Inc., Buffalo Grove, IL) at 2.5x original magnification. Images were captured with a Retiga EXi Fast1394 digital CCD camera (QImaging, British Columbia, Canada) and OpenLab5 software (PerkinElmer Inc., Waltham, MA). Statistical significance between control and treated groups was determined using the Mann-Whitney U-Test at $p<0.05$.

Results

Human Renal Cell Carcinoma, Caki-2, Growth In The Window Chamber

Renal cell carcinoma is a highly vascularized disease and anti-angiogenic agents are currently used as both first and second line treatments in patients. Caki-2 cells, VHL mutant and highly vascular and aggressively growing cell line, were implanted into the window chamber (Figure 5-1A). Induction of tumor cell induced angiogenesis was seen at day 5-6 post tumor cell implantation at which point the tumors quickly expanded and became heavily vascularized with hemoglobin saturation 50-80% in the microvasculature at day 8 (Figure 5-1B).

VEGF Inhibition Impedes Tumor And Vessel Growth In Caki-2 Window Chamber Tumors

Caki-2 tumors grew rapidly and expanded their microvasculature greatly over time with fairly good oxygenation. Treatment of mice with the VEGF inhibitor led to a significant 5.2-fold reduction in tumor volume ($p<0.01$) (Figure 5-2A) as well as a dramatic impairment of tumor vasculature and oxygenation, a reduction from 60-80% hemoglobin saturation to 0-40% in the treated groups (Figure 5-2B). Immunohistochemical analysis of tumor vasculature at study endpoint revealed a 2.4-fold reduction in the treated groups compared to control ($p<0.0001$) (Figure 5-3).

Ang-2 Inhibition Does Not Affect Caki-2 Tumor Growth In The Window Chamber

Treatment of mice with the Ang-2 inhibitor did not show impairment of tumor growth or vascular development (Figure 5-4). However, immunohistochemical analysis of tumor vasculature at study endpoint revealed a 1.1-fold reduction in the treated groups compared to control ($p<0.05$) (Figure 5-5). Furthermore, analysis of the vascular structure revealed a 4-fold increase in the number of vessels that maintained peri-endothelial cell coverage in the treated groups compared to control ($p<0.05$) (Figure 5-6).

VEGF And Ang-2 Inhibition In The Intradermal Angiogenesis Model

To evaluate the inhibition of both the VEGF/VEGFR and Ang-2/Tie2 pathways in the absence of the surgery involved with the dorsal skinflold window chamber model an intradermal assay was used. VEGF/VEGFR pathway was inhibited once again with Sunitinib (100 mg/kg) and led to the reduction of both tumor and vessel growth by 43 ($p<0.001$) (Figure 5-7A) and 2.5-fold ($p<0.0001$) (Figure 5-7B) respectively compared to control. Ang-2/Tie2 pathway was inhibited by MEDI3617 (10 mg/kg) and also led to a

reduction of both tumor and vessel growth by 3.1 ($p<0.001$) (Figure 5-7C) and 1.6-fold ($p<0.0001$) (Figure 5-7D) respectively compared to control.

Surgery Associated With The Window Chamber Model Leads to An Increase In Serum Ang-2 Levels

The circulating Ang-2 levels were determined in mice that received surgery in order to explain the discrepancy between results in the window chamber model versus the intradermal assay regarding Ang-2 targeting in angiogenesis. The release of Ang-2 from endothelial cells as a wound healing response has been previously noted (Kampfer et al., 2001; Kong et al., 2010). Results show that mice that underwent surgery have an increased level of Ang-2 in their circulation compared to control mice that did not receive surgery (Figure 5-8). Furthermore, the presence of tumor cells in mice that received surgery further increased the Ang-2 levels in the circulation compared to mice that only received surgery.

Discussion

Vascular-targeted agents that inhibit the formation of new blood vessels from pre-existing ones have become a standard of care in several cancer settings over the last decade (Ebos et al., 2009; Loges et al., 2010). Our understanding of the complexity of the angiogenic process and the tumor microenvironment led to the realization that anti-angiogenic therapies have yet to reach their full potential (Ebos et al., 2009; Ferrara, 2010; Loges et al., 2010). With the current FDA approved agents there remains a cohort of patients who do not respond or stop responding to treatment; with this realization and discovery of new important pathways in tumor angiogenesis, there are many new agents currently being evaluated in preclinical and clinical trials (Gerald et al., 2013; Loges et al., 2010).

The murine dorsal skinfold window chamber model has been used in the last two decades to study and understand the microvasculature of not only tumors but other diseases as well (Borgstrom et al., 1998; Borgstrom et al., 1999; Borgstrom et al., 1996; Choe et al., 2010; Dedeugd et al., 2009; Dewhirst et al., 2007; Hardee et al., 2009; Laschke and Menger, 2007; Li et al., 2000; Moy et al., 2011; Palmer et al., 2011; Park et al., 2009; Sorg et al., 2005; Terman et al., 2013; Yuan et al., 1996). Recently, the ability to utilize hyperspectral imaging and evaluate vascular oxygenation status through hemoglobin saturation the window chamber model opened opportunities to study, among others, vascular-targeted agents and their effect on the microvasculature (Wankhede et al., 2010b). The dorsal skinfold window chamber model with high resolution hyperspectral imaging, to our knowledge, has not been widely used to evaluate anti-angiogenc therapy on the early initiation of angiogenesis and development of tumors. Ferrara and colleagues have used the window chamber model with basic imaging techniques to demonstrate the anti-angiogenic effects of Bevacizumab in preclinical development (Borgstrom et al., 1998; Borgstrom et al., 1999; Borgstrom et al., 1996; Yuan et al., 1996). To date, there are no data in the literature using this model to evaluate Ang-2 targeted agents.

In the present study, two different classes of anti-angiogenic agents were evaluated. Sunitinib is a small molecule tyrosine kinase inhibitor that targets the VEGFR1-3 and PDGFR and has been FDA approved in 2006 as first line treatment in metastatic kidney cancer (Goodman et al., 2007). The Ang-2 inhibitor, MEDI3617, was used as the Ang-2 targeted agent. Treatment of the tumor bearing mice began the day of tumor cell injection so that the effects of the anti-angiogenic agents could be

evaluated on the initiation of tumor cell induced angiogenesis. The current study used the VHL mutated Caki-2 human renal cell carcinoma cells at a small concentration in order to allow a few days before the initiation of angiogenesis in the window chamber.

Results show that Caki-2 cells injected at $2x10^4$ cells initiate angiogenesis 5-6 days post tumor cell injection (Figure 5-1). When Caki-2 tumor bearing mice are treated with 100 mg/kg Sunitinib during an 11 day period the growth of the tumor is significantly inhibited, 5.2-fold compared to untreated tumors (Figure 5-2A). The development of tumor microvasculature of treated mice is significantly impaired with sparse and poorly oxygenated vessels (Figure 5-2B). Immunohistochemical analysis of tumors at endpoint further demonstrates the significantly impaired vasculature of treated mice with a 2.4-fold reduction in the number of vessels compared to control tumors (Figure 5-3). The results clearly show that the window chamber model and hyperspectral imaging can be a useful tool to evaluate agents targeting the VEGF pathway and the response of the tumor and microvasculature to such treatment. Results nicely demonstrate not only the inhibition of vascular development but also the poor oxygenation of the microvasculature that the tumor does possess.

On the other hand, the results show a different tumor response to the Ang-2 inhibitor. Mice treated with the inhibitor did not show any impairment of tumor growth (Figure 5-4A) compared to untreated tumors nor did it seem to have an effect on the vascular density or oxygenation of the vessels (Figure 5-4B). These results were rather puzzling at first but immunohistochemical analysis revealed a slight but significant reduction in the vessel number (Figure 5-5) as well as a highly significant 4-fold increase in peri-endothelial cell covered vessels when tumors were treated with the

Ang-2 inhibitor (Figure 5-6). Furthermore, previous results presented in Chapter 4 clearly showed a significant anti-angiogenic effect of the Ang-2 inhibitor, which is the opposite response that is seen in the window chamber model. Therefore, the intradermal assay was repeated with both Sunitinib and MEDI3617 to closely resemble the experiment conducted in the window chamber model.

Results from the intradermal assay show that Sunitinib led to a significant 43- (Figure 5-7A) and 2.5-fold (Figure 5-7B) reduction in tumor volume and vessel number respectively compared to control correlating to the results seen in the window chamber model. On the contrary to results seen with the Ang-2 inhibitor in the window chamber, treatment with the Ang-2 inhibitor led to a significant 3.1- (Figure 5-7C) and 1.6-fold (Figure 5-7D) reduction in tumor volume and vessel number compared to control. Similar results with the VEGF targeted agent but opposing results with the Ang-2 targeted agent led to questions about the difference between the two models. In general, the sole difference between the intradermal assay and window chamber model is the initial surgery that is involved with the window chamber model. Based on the basic biology of the Ang-2 and VEGF axis in physiological response to injury led to the hypothesis that perhaps the surgery involved with the window chamber model leads to the rapid release of Ang-2 from damaged endothelial cells when the skinflap is cut to expose the vasculature on the skin that is spared. The angiopoietin axis is not only involved with the rapid response to vascular injury as a wound healing response but is also a pro-inflammatory factor (Fiedler and Augustin, 2006; Fiedler et al., 2006; Kampfer et al., 2001; McDonald, 2008 ; Roviezzo et al., 2005; Staton et al.). One can imagine

that the surgery to implant the window chamber would not only elicit a wound healing but also a pro-inflammatory response.

To support this hypothesis serum samples from mice that have not received surgery, mice that received surgery and ones with surgery and tumor cell injection were evaluated at various time points after surgery. Results demonstrate mice that received surgery had 1.4- and 1.5-fold higher Ang-2 levels in the serum compared to basal levels in mice that did not receive surgery at days 3 and 5 post surgery respectively (Figure 5-8). Furthermore, mice that received surgery and were injected with tumor cells had a slightly higher increase of 1.6-fold compared to baseline at days 3 and 5 post surgery (Figure 5-8). Due to small animal numbers and restrictions on the amount of blood that could be obtained from each mouse the statistical significance between groups could not be calculated. Nevertheless it is clear that the window chamber surgery did elevate the circulating Ang-2 levels in mice.

Evaluating the data between the window chamber, intradermal angiogenesis assay and the Ang-2 levels in the serum it appears that the surgery led to a release of Ang-2 into the microenvironment of the window chamber and even though Ang-2 inhibition with the antibody was initiated the day of surgery, perhaps the amount of the inhibitor to Ang-2 ligand was low yielding minimal anti-angiogenic response only noticeable when the tumors were evaluated using immunohistochemical analysis. It is evident that treatment with the inhibitor led to changes in the vasculature both in number (Figure 5-5) and structure (Figure 5-6) suggesting that the inhibitor was attempting to block vascular destabilization, however, the presence of the inhibitor at that particular dose did not elicit a broader anti-tumor effect. Perhaps an increased

concentration of the inhibitor could lead to significant anti-tumor and anti-angiogenic effects.

In conclusion, the murine dorsal skinfold window chamber model is a valuable model to evaluate tumor microvasculature and vascular oxygenation using hyperspectral imaging. Caution with this model should be taken when assessing the vascular response to therapeutic strategies. In this study two different classes of anti-angiogenic agents were evaluated. The Ang-2/Tie2 axis is important in vascular destabilization while the VEGF/VEGFR axis plays a role in endothelial cell activation to proliferate, migrate and form new vessels. While both axes are essential in physiological angiogenesis such as wound healing, they nevertheless have very different roles. Endothelial cells store Ang-2 in Weibel-Palade Bodies to be able to quickly respond to environmental changes such as vascular injury (Fiedler et al., 2004; Lowenstein et al., 2005; Rondaij et al., 2006) while VEGF is essential in the formation of new vasculature, a later response in wound healing (Carmeliet, 2005). It is clear that the surgery involved with the window chamber model upsets the normal balance of Ang-2 in the microenvironment leading to skewed results to Ang-2 inhibition. The window chamber model, however, is a great tool to evaluate the inhibition of endothelial cell activation and could be utilized to rapidly and effectively evaluate anti-angiogenic agents in preclinical studies.

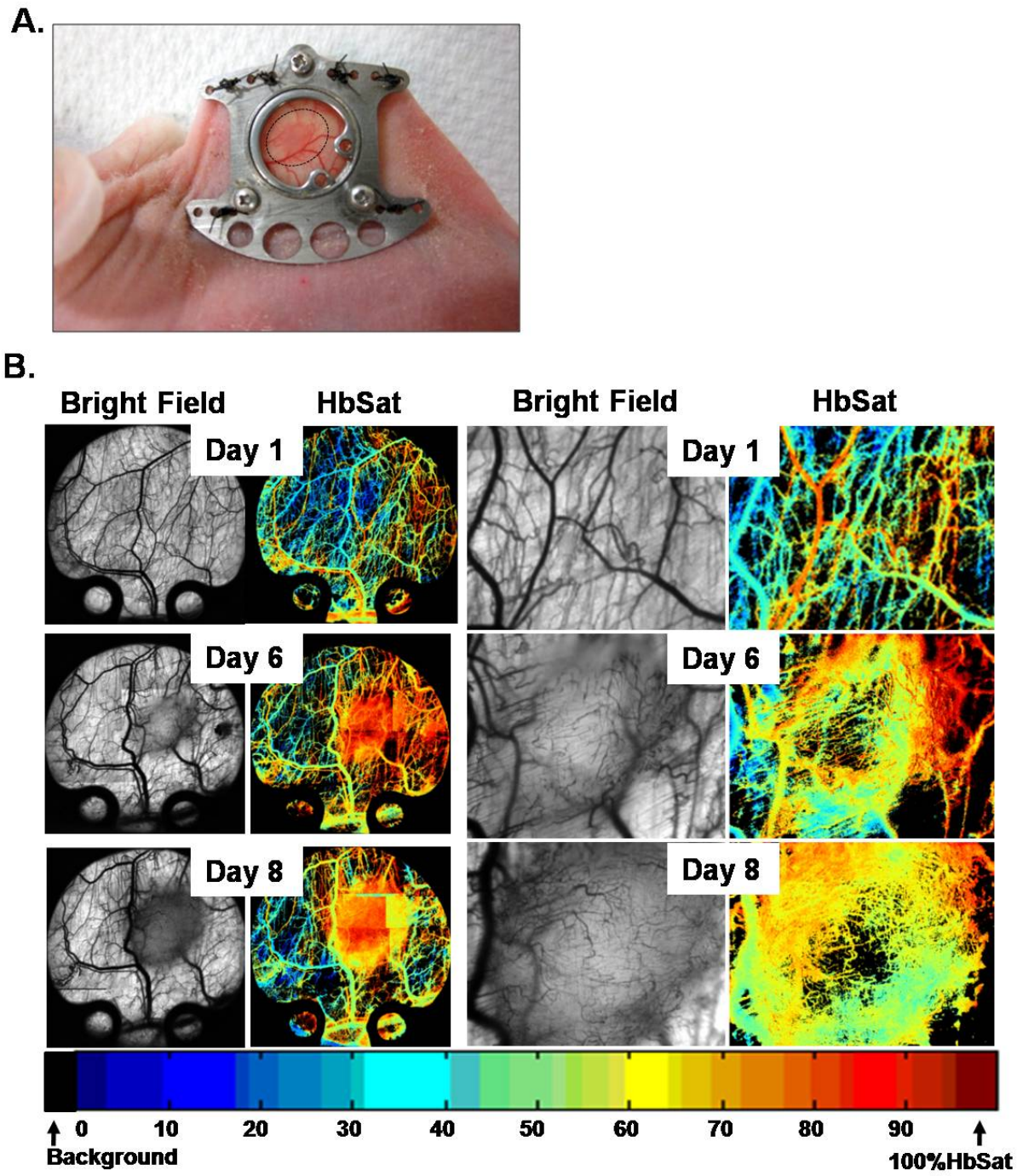

Figure 5-1. Human renal cell carcinoma, Caki-2, tumor growth in the window chamber model. Titanium chambers were surgically implanted in nude mice (A) and tumor cells injected ($2x10^4$) subcutaneously into the window. (B) Signs of vascular change were visible at about 5-6 days post tumor cell injection at which point tumors rapidly became more densely vascularized and oxygenated with tumor volume increasing daily.

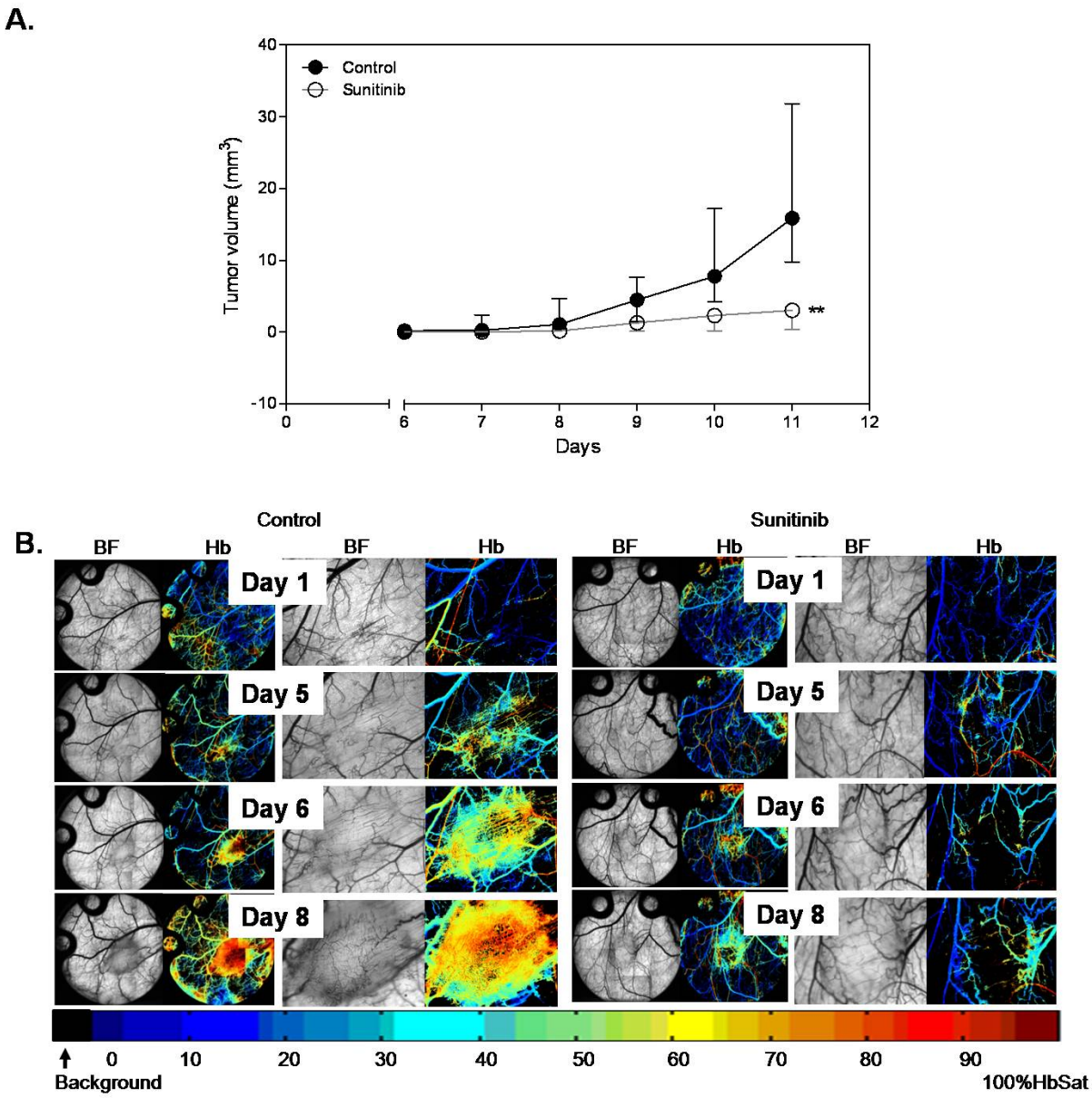

Figure 5-2. VEGF inhibition impedes tumor and vessel growth in Caki-2 tumors. Mice bearing window chambers with Caki-2 tumors were treated with Sunitinib. Treatment led to significant reduction in tumor volume (A) and visible inhibition of and poorly oxygenated tumor vasculature (B). Median + 90/10 percentile; control (n=8), Sunitinib (n=7) (combination of two independent experiments). **, $p<0.01$, Mann-Whitney U-Test.

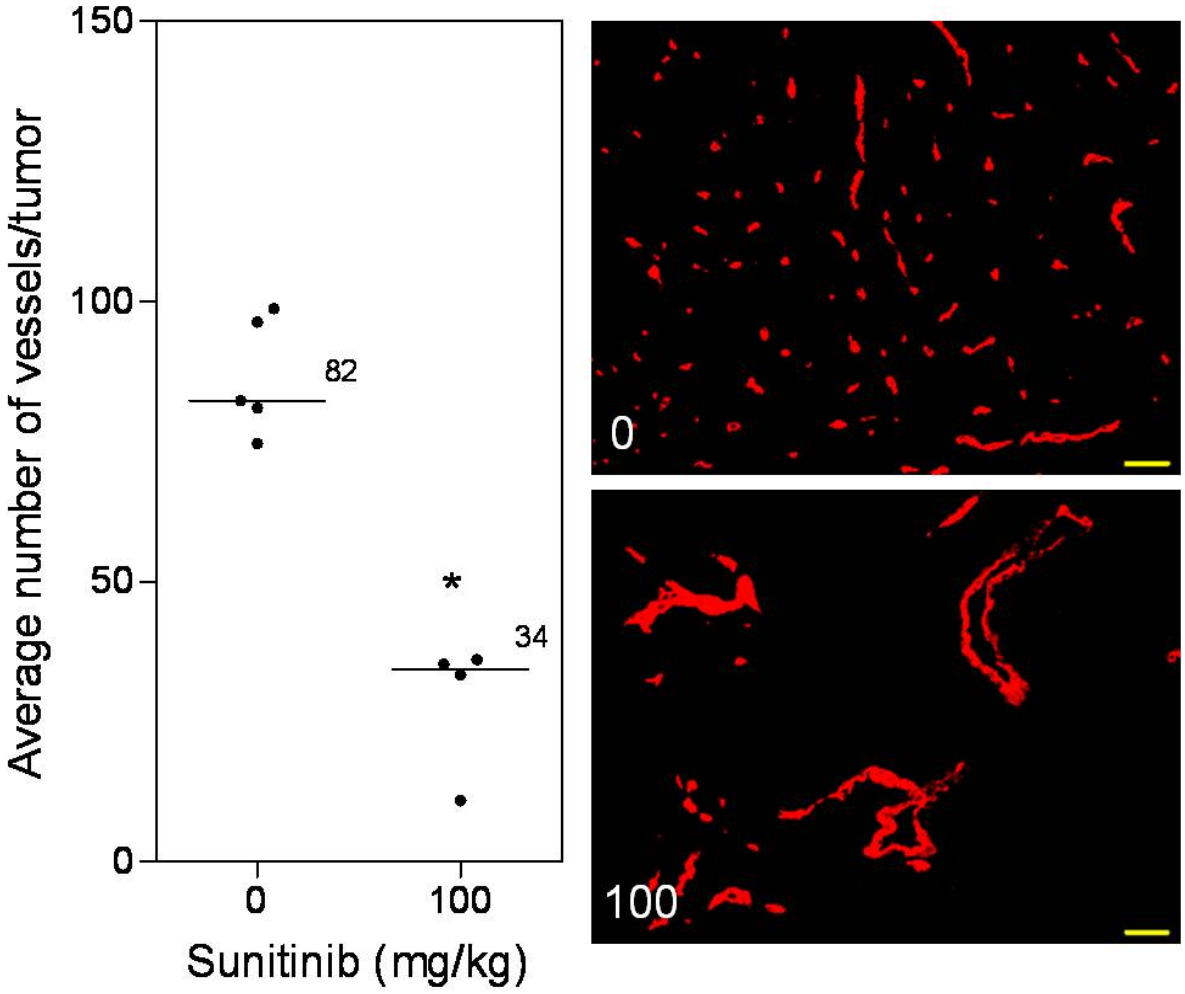

Figure 5-3. VEGF inhibition impedes vessel growth in Caki-2 tumors. Tumors (Figure 5-2) were frozen and evaluated using immunohistochemistry. Sunitinib treatment led to significant reduction of vessel number within the tumor. Line, median; *, $p<0.05$ Mann-Whitney U-Test. Representative images of the median of each group. Red, MECA-32. Images taken with Zeiss Axioplan Imaging2 microscope with 20x objective; scale bar = 140 μm.

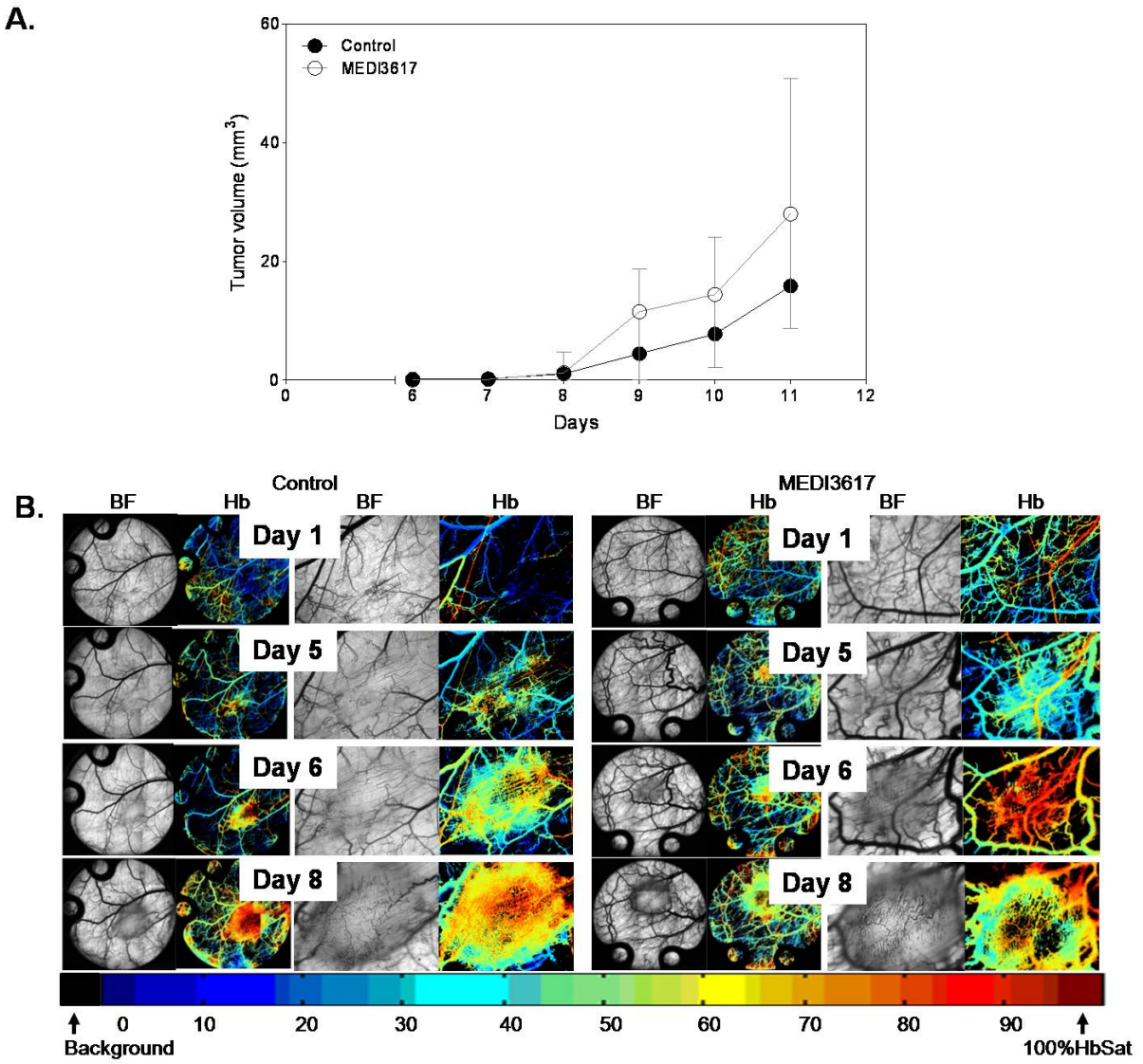

Figure 5-4. Ang-2 inhibition does not affect Caki-2 tumors. Mice bearing window chambers with Caki-2 tumors were treated with MEDI3617. Treatment did not affect tumor volume (A) or vasculature (B). Median + 90/10 percentile; control (n=8), MEDI3617 (n=7) (combination of two independent experiments).

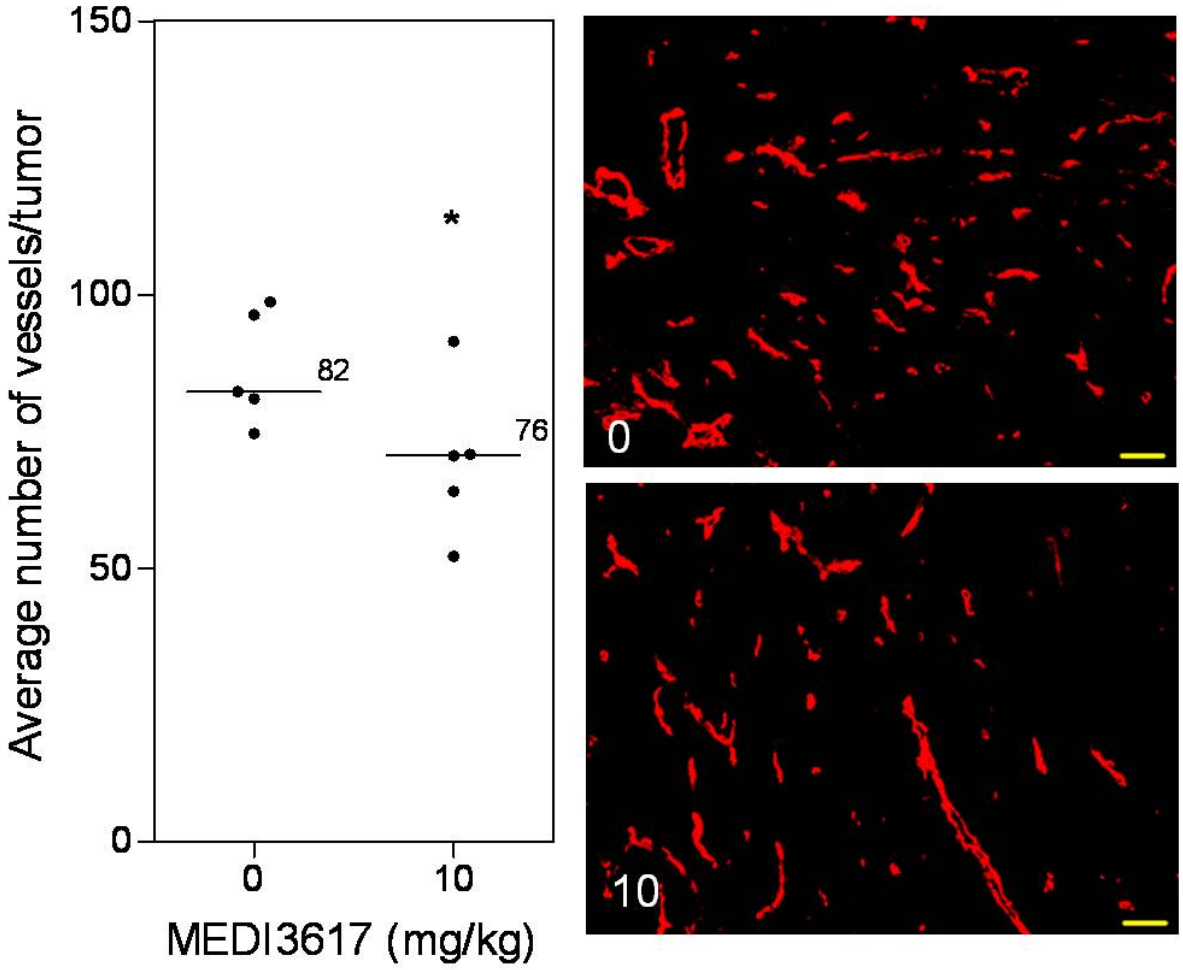

Figure 5-5. Ang-2 inhibition led to reduced tumor vasculature. Tumors (Figure 5-4) were frozen and evaluated using immunohistochemistry. MEDI3617 treatment led to significant reduction of vessel number within the tumor. Line, median; *, $p<0.05$ Mann-Whitney U-Test. Representative images of the median of each group. Red, MECA-32. Images taken with Zeiss Axioplan Imaging2 microscope with 20x objective; scale bar = 140 μm.

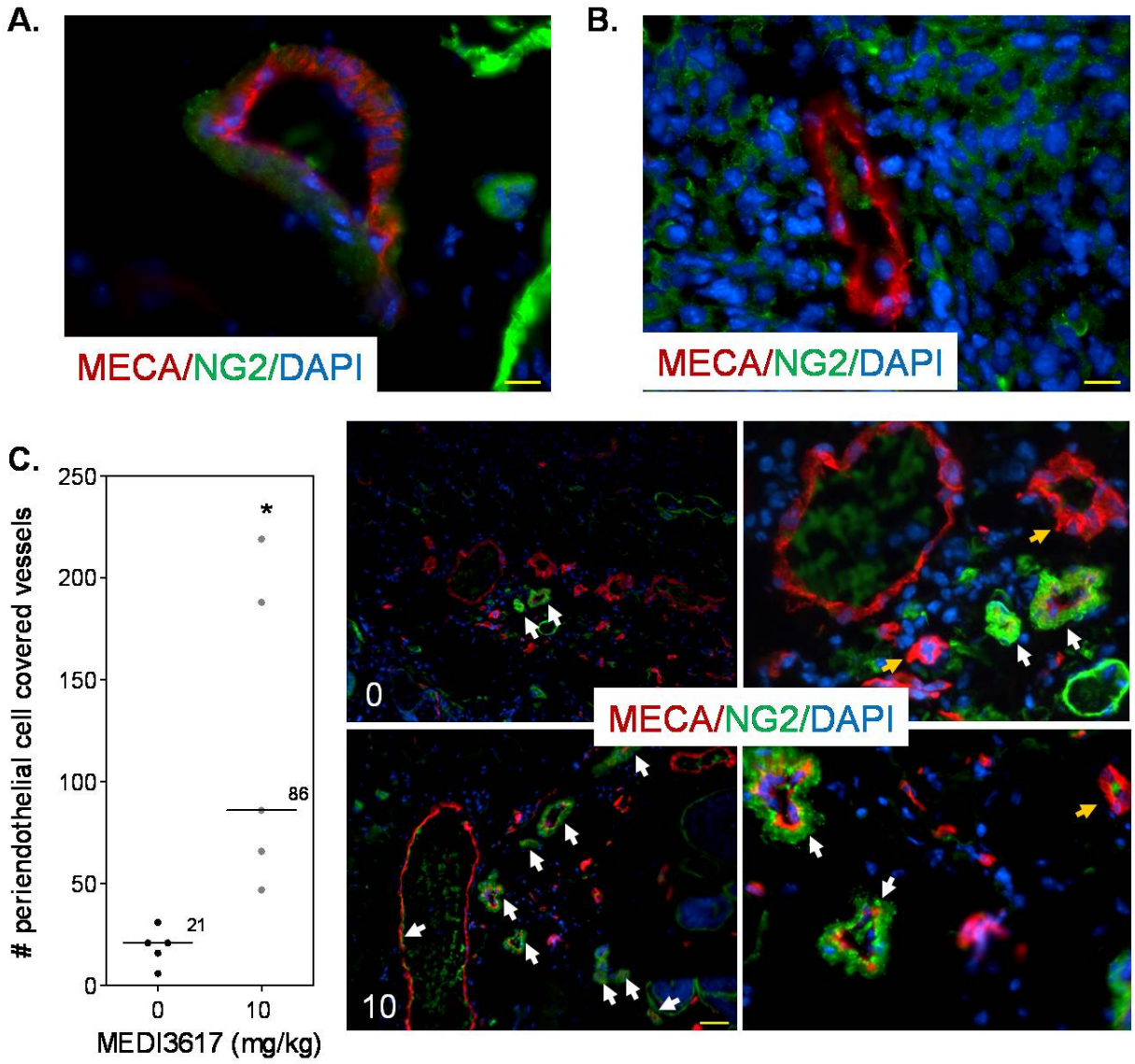

Figure 5-6. Ang-2 inhibition leads to increased peri-endothelial cell coverage in the tumor periphery. Window chamber tumors treated with MEDI3617 were evaluated immunohistochemically for vascular structure. (A) Normal vasculature with peri-endothelial cell coverage. (B) Shows tumor vasculature without peri-endothelial cell coverage. Scale, 46 μm. (C) Ang-2 inhibition led to increased number of vessels that had peri-endothelial cell coverage. Line, median; *, $p<0.05$; Mann- Whitney U-Test. Representative images of each group. White arrows show peri-endothelial cell covered vessels; yellow arrow shows vessels without peri-endothelial cell coverage. Red, MECA-32 (endothelium); green, NG2 (peri-endothelial cells); blue, DAPI. Images taken with Zeiss Axioplan Imaging2 microscope with 20x objective; scale bar = 140 μm.

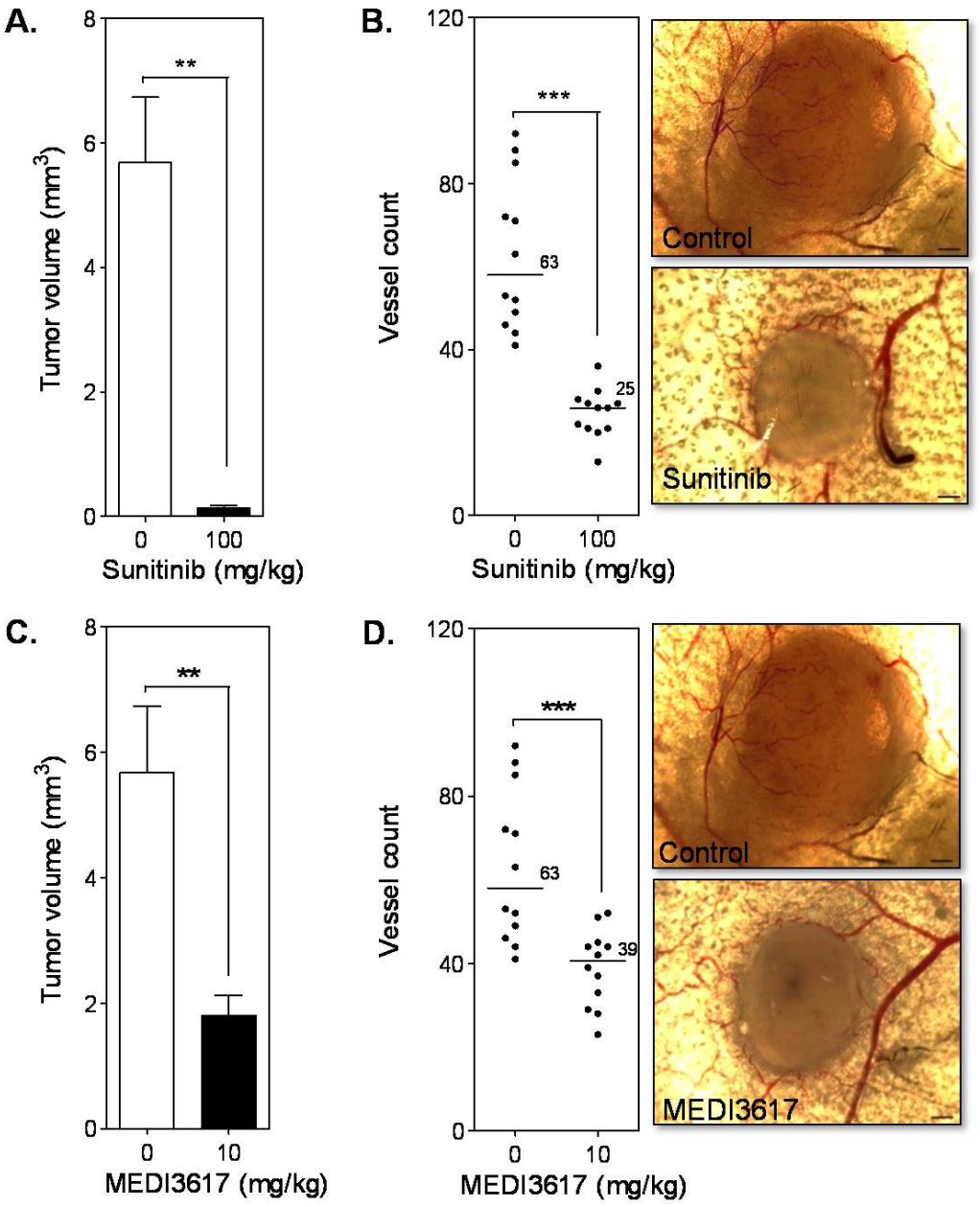

Figure 5-7. Ang-2 and VEGF inhibition in the intradermal assay. Mice were injected intradermally with Caki-2 renal cell carcinoma cells and treated with either MEDI3617 (10 mg/kg) or Sunitinib (100 mg/kg) beginning the day prior to tumor cell inoculation. Tumor volume (A,C) and the number of tumor cell induced blood vessels (B,D) were determined at the end of a 7 day period. Bar, mean with SEM (n=12); line, median (n=12); **, $p<0.01$; ***, $p<0.0001$; Mann-Whitney U-Test.

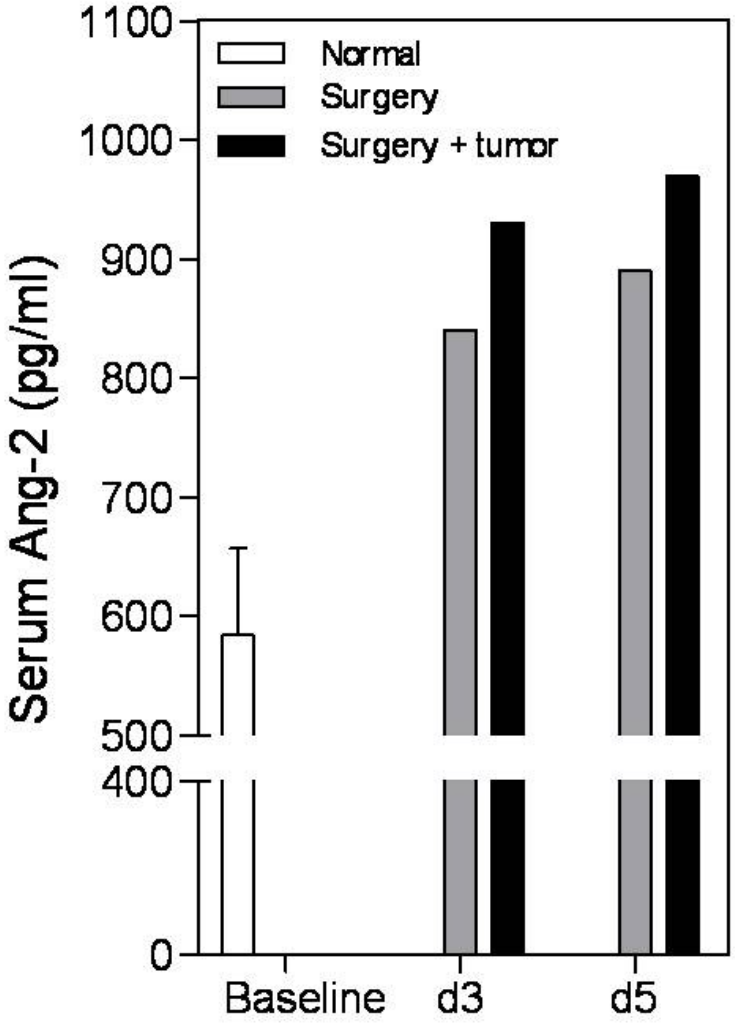

Figure 5-8. Window chamber surgery leads to increase serum Ang-2 levels. The surgery involved with the window chamber morel led to increase Ang-2 levels in the circulation compared to mice that did not undergo surgery. The presence of Caki-2 tumor cells further led to an increase in Ang-2 levels compared to surgery alone.

CHAPTER 6
ANTI-TUMOR EFFECT OF ANGIOPOIETIN-2 INHBITION AS A MONO- AND COMBINATION THERAPY WITH VEGF TARGETED AGENTS

Part A: Anti-Tumor Effect Of Ang-2 Mono-Therapy

Chapters 4 and 5 focused on the *in vivo* evaluation of the anti-angiogenic effects of Ang-2 inhibition. Chapter 6 explored the anti-tumor effect of Ang-2 inhibition both in the primary tumor and metastatic tumor setting (Part A) as well as combination therapy with VEGF targeted agents (Part B).

Background

Tumors cannot grow beyond a certain size without the initiation of angiogenesis (Folkman, 1971; Folkman, 2007). Over the last four decades there has been tremendous interest and progress in angiogenesis targeted therapies (Folkman, 2007). Anti-angiogenic agents have been shown to slow tumor growth and significant tumor growth delay in preclinical studies has led to the approval of several agents for treatment of various solid tumors (Borgstrom et al., 1998; Borgstrom et al., 1999; Borgstrom et al., 1996; Folkman, 2007; Goodman et al., 2007). A new class of anti-angiogenic agents the Ang-2/Tie2 target agents has been significantly pursued in the treatment of cancer. Several reports show significant anti-tumor effects with these agents (Brown et al., 2010; Coxon et al., 2010; Daly et al., 2013; Leow et al., 2012).The current study evaluated the anti-tumor effect of the Ang-2 inhibitor in both VHL normal and mutated human renal cell carcinoma models as well as a metastatic setting.

Materials And Methods

Reagents

Protease (P-5147), DNase (DN-25) and collagenase (CO-130) and Bouin's solution were obtained from Sigma.

Cell culture

The human clear cell renal cell carcinoma Caki-1 and Caki-2 cell lines were received as a gift from Dr. Susan Knox (Stanford University); the cells were grown in Dulbecco's modified minimum essential medium (D-MEM, Invitrogen) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 1% 200-mmol/L $_{L}$-glutamine (Invitrogen). Cells were kept at 37 °C, 5% CO_2. The cells were used between passages 2 and 10.

Drug preparation

Angiopoietin-2 inhibitor, MEDI3617, was kindly provided by MedImmune, LLC. Stock solution (5 mg/ml) was diluted to working concentrations in sodium citrate buffer solution. Stock solutions were kept at -80 °C and working concentrations at 4 °C.

Xenograft dissociation

All *in vivo* procedures were conducted in agreement with a protocol approved by the University of Florida Institutional Animal Care and Use Committee. Female athymic nu/nu mice were initially injected intramuscularly (IM) with Caki-1 or Caki-2 cells at about $2x10^6$ in 20 µl volume. Tumors were allowed to grow to about 1000 mm^3 at which point tumors were harvested, and tumor cells dissociated from the stroma. Briefly, tumors were harvested from mice and minced with a scissor then placed in 35 ml/tumor enzyme cocktail made of 0.25 mg/ml collagenase, 0.5 mg/ml protease, and 0.4 mg/ml DNase in PBS. Tumor chunks were then incubated for 1 hr at 37 °C on a rotator.

Dissociated cells were filtered, transferred to a new conical tube and centrifuged at 1000 rpm, 10 min at 4 °C. Cells were counted and prepared for growth delay injections (as described below).

Growth delay tumor initiation and treatment

All *in vivo* procedures were conducted in agreement with a protocol approved by the University of Florida Institutional Animal Care and Use Committee. Female athymic nu/nu mice were injected IM at 10^6 cells in 20 µl volume with Caki-1 or Caki-2 cells dissociated from previous tumors (as described above). Anti-tumor effects of the Ang-2 inhibitor were evaluated by (1) allowing tumors to reach a volume of 200 mm^3 before treatment began (Caki-1, Caki-2) or (2) beginning treatment the day of tumor cell injection (Caki-2). Mice were treated with the Ang-2 inhibitor at various doses every three days (twice/week). Tumors were measured with calipers daily and once they reached a volume of 1000 mm^3 the mice were euthanized.

Lung metastasis tumor initiation and treatment

All *in vivo* procedures were conducted in agreement with a protocol approved by the University of Florida Institutional Animal Care and Use Committee. Female athymic nu/nu mice were injected with human renal cell carcinoma, Caki-2, cells at 10^4 in 200 µl volume via tail vein. Mice were treated with the Ang-2 inhibitor (2,10 mg/kg) every 3 days (twice/week, 8 doses total) starting the day of tumor cell injection. Mice were euthanized 24 days post tumor cell injection, the lungs removed and fixed overnight in Bouin's solution. The number of metastases as well as size of each metastasis was counted. Statistical significance between control and treated groups was determined using the Mann-Whitney U-Test at $p<0.05$.

Results

Ang-2 inhibition does not significantly impact renal cell carcinoma xenograft growth

To evaluate the anti-tumor effect of the Ang-2 inhibitor mice were injected intramuscularly with either Caki-1 or Caki-2 cells. Tumors were grown to a volume of 200 mm^3 before treatment was initiated. Tumor growth delay to 1000 mm^3 was evaluated. Results show that in Caki-1 tumors treatment with the Ang-2 inhibitor did not have a significant effect on tumor growth delay yielding only a 1-3 day delay compared to control (Figure 6-1A,B). Even smaller effects of 0-1 day delay were seen in Caki-2 tumors treated with the Ang-2 inhibitor even though the dose was increased compared to doses the Caki-1 bearing mice received (Figure 6-1 C,D). Since significant results were seen with the Ang-2 inhibitor in the early tumor growth and development in the intradermal assay in Chapters 4 and 5, a growth delay experiment was conducted where treatment of mice was initiated the day of tumor cell injection. However, results from this study were also negative with no growth delay effects in treated groups compared to control (Figure 6-2).

Ang-2 inhibition does not impact renal cell carcinoma lung metastases

Angiogenesis is an important process not only for the growth of the primary tumor but also for the development and growth of metastatic lesions. The effect of Ang-2 inhibition on lung colonization after tail vein injection of Caki-2 tumor cells was evaluated. Treatment of mice began the day of tumor cell injection and resumed for a 3-week period (Figure 6-3A). Results showed no significant effect on the number of lung metastases between control and treated mice, the median number of lung metastases were 33, 28 and 29 for control, 2 and 10 mg/kg respectively (Figure 6-3B). Overall there

was no difference between the size of metastatic lesions between control and treated groups (Figure 6-3C) nor the distribution of the percentage of lesions in a particular size range (Figure 6-3D).

Discussion

Anti-angiogenic agents have been pursued in the laboratory and the clinic since Dr. Judah Folkman first proposed the importance of angiogenesis for tumor growth and the potential for a therapeutic intervention to cut off the blood supply to the tumor and impair its growth (Folkman, 1971). The past decade VEGF and other targeted agents have been approved for various solid tumors and are now used as part of the standard of care. The complexity of tumor angiogenesis and the microenvironment that influences the growth of the tumor has been sought after and the more knowledge we gain added complication to this process are presented. New targets to maximize the inhibition of tumor angiogenesis are pursued (Loges et al., 2010). Various groups in a variety of tumor settings are currently investigating the Angiopoietin/Tie2 axis targeting agents (Gerald et al., 2013). Chapters 4 and 5 focused on the anti-angiogenic effect of the Ang-2 inhibitor on early angiogenesis and tumor growth. The present study evaluated the anti-tumor effect of the inhibitor on both the VHL normal and mutated renal cell carcinoma cell lines. Treatment of tumors began once they have established a substantial size and vasculature to mimic the status of tumors upon a patients' diagnosis with cancer.

The results show that the Ang-2 inhibitor has a minor effect on the growth of the Caki-1 tumors, a 1-3 day growth delay compared to control (Figure 6-1A,B) however no effect was seen in the growth of the Caki-2 tumors compared to control (Figure 6-1C,D). These results were puzzling especially because Leow and colleagues (2012) have

conducted several experiments using this antibody and have seen successful growth delay effects. However when closely examining the xenograft models they have used and their growth characteristics it is evident that the tumor models used in this study grew much faster allowing for less dosing of the inhibitor before the study was terminated. For example, colon cancer LoVo xenografts grew 2.9-fold slower and were dosed 2-3 times more than the Caki-1 tumors used in this study. Renal cell carcinoma 786-0 xenografts grew 2.7-fold slower, colon Colo205 xenografts 1.8-fold slower each allowing for 2-3 more dosings of the inhibitor compared to Caki-1 xenografts. The most aggressive model, HeyA8 ovarian cell line grew 1.3-fold slower allowing for 1-2 more doses of the inhibitor (Leow et al., 2012).

The comparison between Caki-2 xenografts used in this model compared to the ones used by Leow and colleagues (2012) were even more drastic. Caki-2 xenografts grew 5.3-, 5-, 2.5-, and 4-fold faster than LoVo, 786-0, HeyA8 and Colo205 xenografts respectively reducing the number of doses by 2-4 throughout treatment (Leow et al., 2012). Furthermore, the Caki-2 xenografts were dosed with 2-5 times higher doses of the inhibitor than the ones reported by Leow and colleagues (2012). These results suggest that both the Caki-1 and especially the Caki-2 tumors grow more aggressively than either xenograft treated with this inhibitor before. Reports using other Ang-2 targeted agents also used comparable xenograft models that Leow and colleagues (2012) have used and there are currently no reports with Ang-2 targeted agents in either the Caki-1 or Caki-2 xenograft models (Brown et al., 2010; Daly et al., 2013; Leow et al., 2012; Neal and Wakelee, 2010). Due to the aggressive and highly angiogenic profile of both these tumor models we believe that once the tumor vasculature is established in

these tumors there is limited need for activation of the Ang-2 axis. Once a tumor establishes its vasculature it is highly irregular with minimal peri-endothelial cell coverage and high proliferation rate of the endothelial cells (Carmeliet and Jain, 2000; Siemann, 2011). The tumor most likely relies on pro-angiogenic factors such as VEGF and other pathways to stimulate endothelial cell proliferation and tube formation (Carmeliet, 2005; Carmeliet and Jain, 2000). Most likely there is activity at the border of the tumor where normal pre-existing vasculature is destabilized and stimulated, however it is unlikely to be the primary source of new blood vessels for the tumor.

To evaluate whether Ang-2 inhibition is more important in the early phase of tumor development another growth delay experiment was conducted. Treatment of mice began the day of tumor cell injection and thus the tumor was not given the opportunity to establish its vasculature. Results however were disappointing and there was no significant difference between the control and treated groups (Figure 6-2). There are several possible explanations such as the use of lower doses of the inhibitor 2 and 10 mg/kg rather than the 20 and 50 mg/kg used in Figure 6-1C,D. Also the tumor reached the endpoint size of 1000 mm^3 within 11-12 days allowing only 4 dosings. Perhaps treatment with higher doses or injection of 10-fold less tumor cells allowing longer time before study endpoint would allow more dosings throughout the treatment period. It is possible that the Ang-2 inhibitor has early phase effects on the tumor growth however once the tumor escapes the Ang-2 axis it aggressively takes advantage of the VEGF axis leading to rapid vascularization and thus growth of the tumor. Perhaps a higher dose of the inhibitor should also be evaluated, however once the tumor escapes the Ang-2 axis there is probably minimal effects it may have on the developing vasculature

especially in the inner core of the tumor since peri-endothelial cell coverage is sparse and loose.

Finally, Ang-2 inhibition was evaluated in a metastatic setting. Metastasizing tumor cells need to develop the same way that primary tumors do and therefore rely on angiogenesis to expand in size (Weidner et al., 1991). Since our results from the intradermal assay in Chapter 4 showed significant impairment of Caki-2 tumor growth and vascular development, here the impairment of lung colony formation was evaluated. Mice were injected with Caki-2 cells via tail vein and treated with the Ang-2 inhibitor during a 3 week period (Figure 6-3A). Results did not show a significant difference in the number of metastatic lesions (Figure 6-3B), size (Figure 6-3C) nor the range of size (Figure 6-3D) between treated and untreated mice. Although this was surprising at first there is a possibility that these tumors colonize the lung and begin growth at an early time point since by the 21 days most lesions are fairly large. There is a possibility that the Ang-2 inhibitor may have an effect very early on but once the tumor escapes the Ang-2 axis it aggressively takes over the VEGF pathway especially in the Caki-2 model since VHL is mutated and the cells produce high levels of VEGF even in the absence of hypoxia. An earlier time point may be evaluated to test this hypothesis. Another possibility may be that once Caki-2 cells infiltrate the lungs they do not rely on angiogenesis for obtaining vasculature but rather co-opt vessels in the heavily vascularized lungs (Aird, 2007). In this case neither Ang-2 nor VEGF pathway inhibiting agents would have an effect on lung colonization of Caki-2 tumor cells.

In conclusion, these studies showed minimal to no anti-tumor effect when treated with the Ang-2 inhibitor. A possible explanation for this may be the aggressive nature of

the tumor models chosen to evaluate since others have seen significant tumor growth delay in other less aggressive xenograft models. Another explanation may be that the Ang-2 axis and its inhibition may only be relevant very early on during tumor development when the tumor cells stimulate the nearby normal vasculature. Peri-endothelial cells cover normal vessels and therefore the Ang-2 axis is necessary to first destabilize the vasculature before the tumor cells can activate the endothelium to form new vessels (Augustin et al., 2009). Therefore once the tumor cells have established vasculature from normal pre-existing vessels they can stimulate the formation of new vessels from the highly proliferative and abnormal tumor vasculature leaving the Ang-2 axis less necessary.

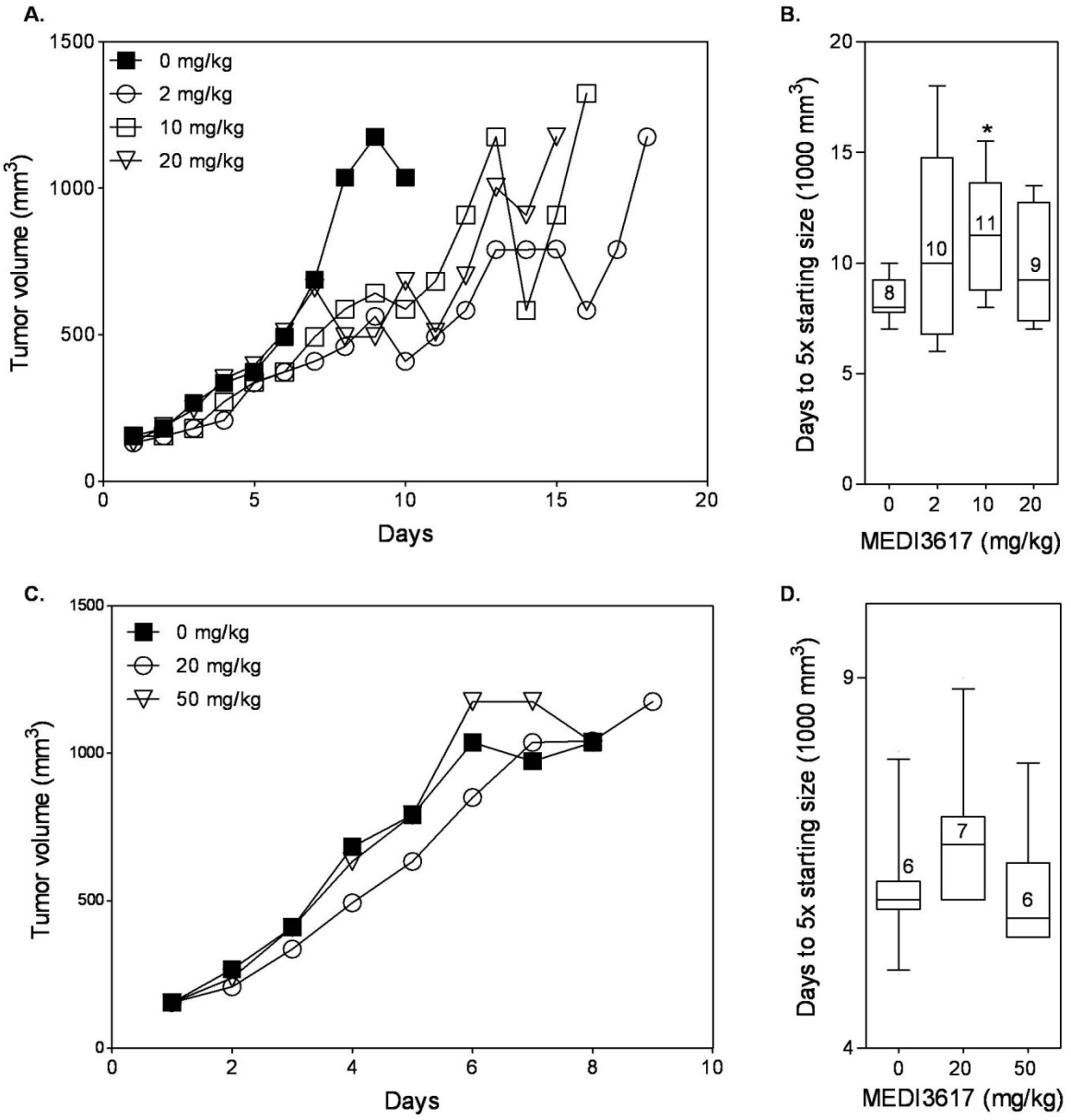

Figure 6-1. Ang-2 inhibition does not affect renal cell carcinoma tumor growth. Mice were injected with 10^6 human renal cell carcinoma Caki-1 (A,B) or Caki-2 (C,D) cells intramuscularly. Tumors were treated with MEDI3617 beginning when tumor volume reached 200 mm^3 and mice were euthanized once tumor reached 1000 mm^3. Ang-2 inhibition had no effect on tumor growth delay. (A,C) Median responders. (C,D) Median. (A,B) 0, 10, 20 mg/kg (n=6), 2 mg/kg (n=6). (C,D) 0, 20, 50 mg/kg (n=10). *, $p<0.05$; Mann-Whitney U-Test.

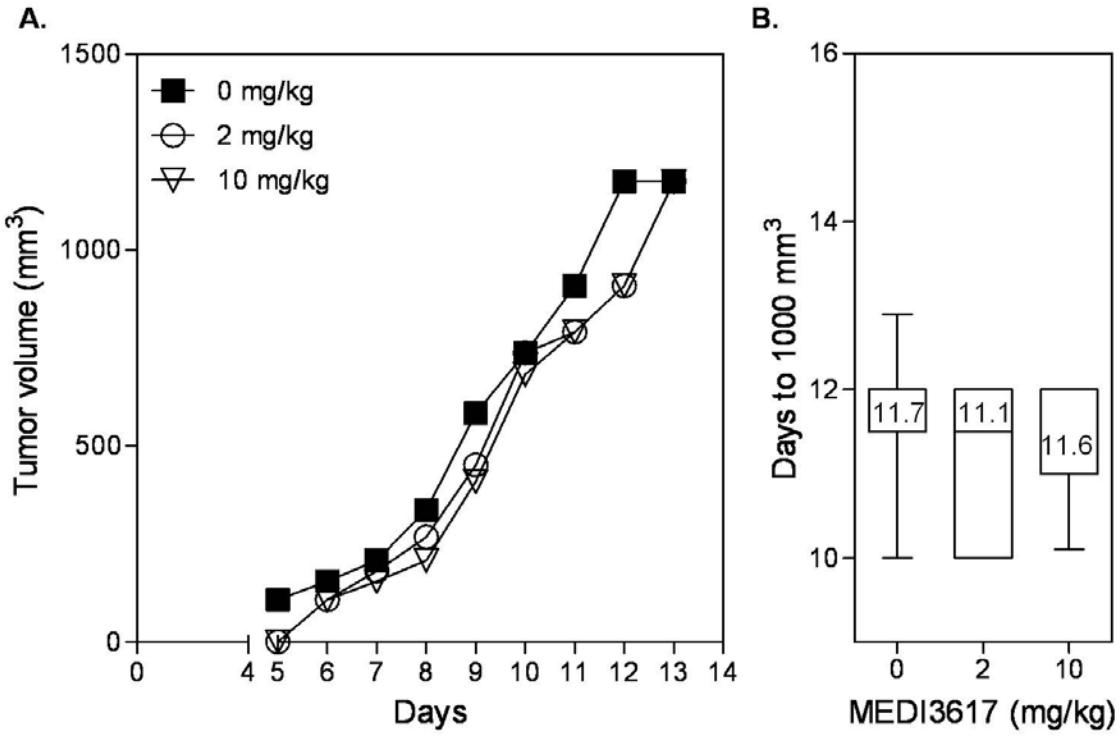

Figure 6-2. Early treatment with Ang-2 inhibitor does not affect renal cell carcinoma tumor growth. Mice were injected with 10^6 human renal cell carcinoma Caki-2 cells intramuscularly. Treatment of tumors began the day of tumor cell injection and continued until tumor volume reached 1000 mm^3. Ang-2 inhibition had no effect on tumor growth delay. (A) Median responders. (B) Median (n=10).

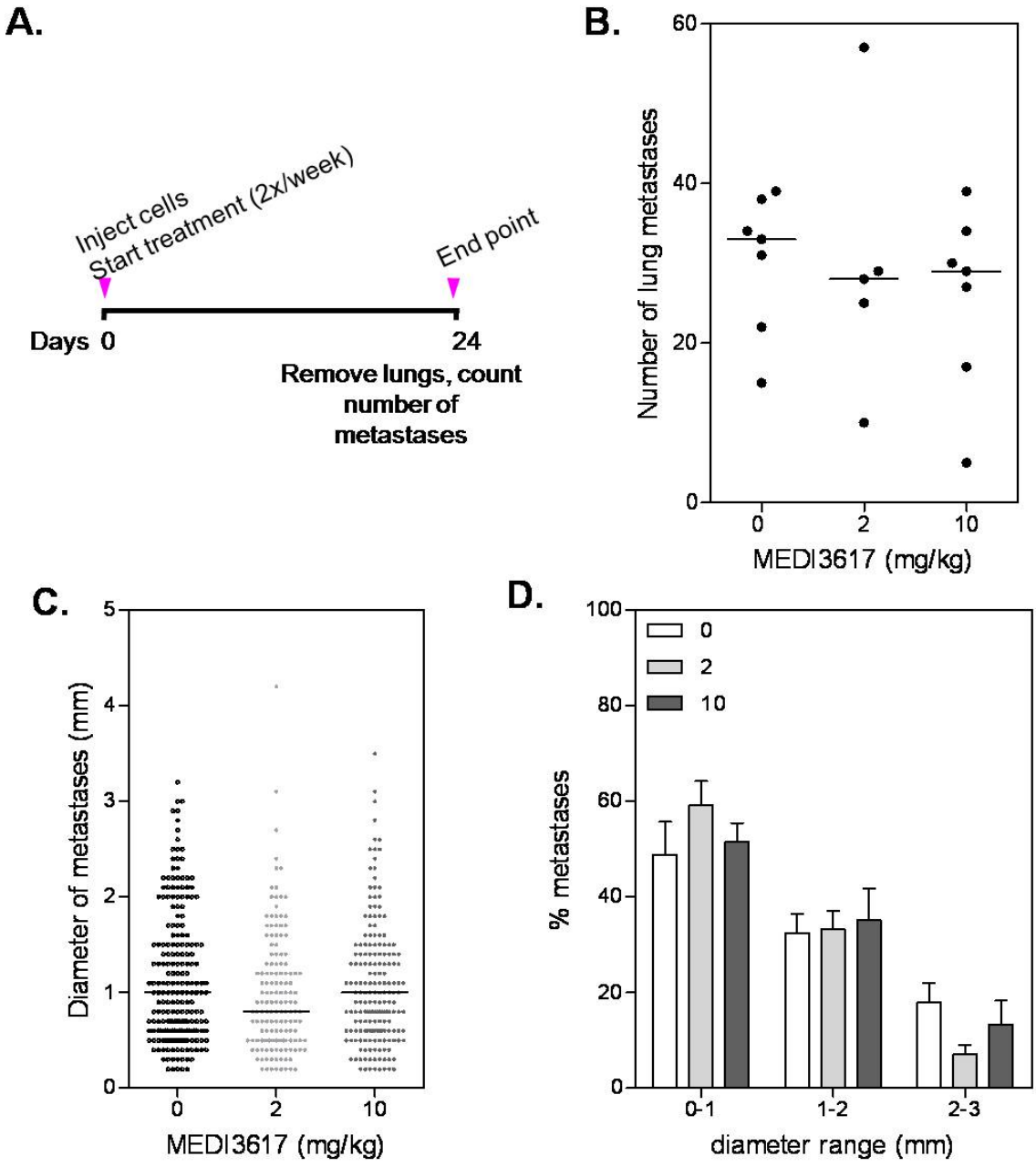

Figure 6-3. Ang-2 inhibition does not affect renal cell carcinoma lung metastasis growth. (A) Mice were injected with human renal cell carcinoma, Caki-2, cells at 10^4 via tail vein. Treatment with Ang-2 antibody began the day of tumor cell injection. (B) Ang-2 inhibition did not affect the number of lung metastases (C) the size of individual metastases (D) or the frequency of metastases size. (B,C) Line, median (D) mean with SEM 0, 10 mg/kg (n=7), 2 mg/kg (n=5).

Part B: Combination Therapy With VEGF Targeted Agents

Background

Angiogenesis is a two-step process (a) the normal vasculature is destabilized loosening the endothelial and peri-endothelial cell contacts in the vasculature at which point (b) pro-angiogenic factors such as VEGF activate the endothelium to proliferate and form new vessels. Currently the majority of FDA approved anti-angiogenic agents target the VEGF pathway and some have other targets as well such as PDGF, cMET and mTOR. In general anti-angiogenic agents have been complementary to conventional cancer therapies and are used in a variety of tumor settings, however, there are some patients who do not respond or stop responding after prolonged treatment (Bergers and Hanahan, 2008; Ebos et al., 2009; Loges et al., 2010). To circumvent this issue a new class of anti-angiogenic agents the Ang-2 targeted agents have been pursued both in the preclinical and clinical setting. There is some evidence that the combination of Ang-2 and VEGF targeted agents yield superior anti-angiogenic response than either agent alone (Brown et al., 2010; Dandu et al., 2008; Hashizume et al., 2010; Koh et al., 2010). Currently, the combination of the VEGF antibody Bevacizumab and the Ang-1/2 peptibody Trebananib and small molecule VEGFR inhibitor Axitinib with the Ang-2 specific CVX-060 are being evaluated in the clinic (Gerald et al., 2013). There is also an antibody in preclinical development that targets both the Ang-2 and VEGF ligands (Gerald et al., 2013).

The current study evaluated the combination of the Ang-2 inhibitor, MEDI3617, with two small molecule tyrosine kinase inhibitors Sunitinib and Cediranib. Sunitinib is a multikinase inhibitor that is FDA approved as first line treatment for kidney cancer

(Goodman et al., 2007). Cediranib is a VEGFR specific small molecule inhibitor that is currently in clinical development (Zhu et al., 2013). The effects on early tumor development and angiogenesis initiation were evaluated.

Materials And Methods

Reagents

MECA-32 was purchased from BioLegend (San Diego, CA), AlexaFluor 594 was purchased from Invitrogen (Grand Island, NY). VectaShield mounting medium with DAPI was purchased from Vector Labs Inc. (Burlingame, CA). Tissue-Tek OCT Compound was purchased from Sakura Finetek (Torrance, CA). 2-methylbutane was obtained from Thermo Fisher Scientific (Waltham, MA).

Cell culture

The human clear cell renal cell carcinoma Caki-2 cell line was received as a gift from Dr. Susan Knox (Stanford University); the cells were grown in Dulbecco's modified minimum essential medium (D-MEM, Invitrogen) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 1% 200-mmol/L $_{L}$-glutamine (Invitrogen). Cells were kept at 37 °C, 5% CO_2. The cells were used between passages 2 and 10.

Drug preparation

Angiopoietin-2 inhibitor, MEDI3617, was kindly provided by MedImmune, LLC. Stock solution (5 mg/ml) was diluted to working concentrations in sodium citrate buffer solution. Stock solutions were kept at -80 °C and working concentrations at 4 °C. Cediranib was kindly provided by AstraZeneca (Wilmington, DE) and stored at 4 °C. Working concentration of Cediranib was prepared fresh daily in 10% volume Tween 80 and 1M HEPES and stored at 4 °C. Sunitinib was obtained from LC Laboratories

(Woburn, MA) and stored at -20 °C. Working concentration of Sunitinib was prepared fresh daily in stock and diluent buffers of citric acid monohydrate and sodium citrate dihydrate at pH 6.8 and 3.2 respectively at 1:7 stock to diluent solution (~pH 3.3) and acidified to pH 1.0, Sunitinib was dissolved, and the solution adjusted to pH 3.5. Working concentration of Sunitinib was kept at room temperature.

Intradermal assay

All *in vivo* procedures were conducted in agreement with a protocol approved by the University of Florida Institutional Animal Care and Use Committee. Female athymic nu/nu mice were injected intradermally with 10^5 Caki-2 cells in 10 μl volume at four sites on the ventral surface. Beginning the day prior to tumor cell injection, mice were treated (1) with either daily oral gavage of Sunitinib (10 mg/kg) (7 doses total), IP injection of MEDI3617 (2 mg/kg) every 3 days (3 doses total) or both; or (2) with either daily oral gavage of Cediranib (2 mg/kg) (7 doses total), IP injection of MEDI3617 (2 mg/kg) every 3 days (3 doses total) or both up to six days post tumor cell inoculation. Mice were then euthanized, tumors measured via calipers and tumor volume (mm^3) calculated, assuming the tumor volume to be an ellipsoid, using the following equation: tumor volume = $\pi/6 \times d_1 \times d_2 \times$ height. Skin flaps were then removed and vessels growing into tumor nodules were counted using a Leica MZ16F dissecting microscope with Leica KL 1500 LCD fiber optic illuminator (Leica Microsystems Inc., Buffalo Grove, IL) at 2.5x original magnification (1,2,3). Images were captured with a Retiga EXi Fast1394 digital CCD camera (QImaging, British Columbia, Canada) and OpenLab5 software (PerkinElmer Inc., Waltham, MA). Statistical significance between control and treated groups was determined using the Mann-Whitney U-Test at $p<0.05$.

Immunohistochemistry

Intradermal tumors were fresh frozen in OCT and methylbutane and cryosectioned at 5 μm thickness using Leica CM 3050S cryostat (Leica Microsystems Inc., Buffalo Grove, IL); sections were placed on superfrost plus gold slides (Thermo Fisher Scientific Inc., Waltham, MA) and kept at -80 °C until immunohistochemical staining. Tissue sections were acetone fixed for 10 minutes, blocked in 2% normal horse serum in 1x TBS, and incubated overnight at 4 °C with MECA-32. Secondary antibody AlexaFluor 594 was added onto slides for 1 hr. Tissue sections were imaged with a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Inc., Thornwood, NY) with EXFO X-Cite 120 light source (Lumen Dynamics Group Inc., Ontario, Canada). Images were taken with a Retiga EXi Fast digital CCD camera (Qimaging, British Columbia, Canada) and processed in OpenLab5 software (PerkinElmer Inc., Waltham, MA); Rhodamine for MECA-32/AlexaFluor594 and DAPI filters were used. Vessel counts were obtained by taking up to ten random fields/tumor with 20x objective, counting the number of vessels in each field. Statistical significance between control and treated groups was determined using the Mann-Whitney U-Test at $p<0.05$.

Results

MEDI3617 and Sunitinib combination leads to greater antiangiogenic effect

Sunitinib is a multikinase small molecule inhibitor that is currently used as a first line treatment in renal cell carcinoma. Caki-2 renal cell carcinoma cells were injected intradermally and mice were dosed with the Ang-2 inhibitor alone, Sunitinib alone or the combination of the two agents (Figure 6-5A). Results show that both agents alone led to reduction of tumor volume a 1.5- fold ($p=0.06$) reduction with MEDI3617 and 4.6-fold

($p<0.0001$) reduction with Sunitinib treatment compared to control, however, the combination of the two agents led to a much more significant reduction of 15.8 fold ($p<0.0001$) compared to control. The combination of the two agents was also significantly lower than either agent alone with a 10.7- ($p<0.0001$) and 3.4-fold ($p<0.0001$) decrease compared to MEDI3617 and Sunitinib treatment alone respectively (Figure 6-5B).

Evaluating the effects on the tumor cell induced vasculature results show a 1.7- ($p<0.0001$) and 1.8-fold ($p<0.0001$) decrease in the number of vessel in MEDI3617 and Sunitinib treated groups compared to control. The combination of the two agents led to a much more significant reduction in vessel number by 3.5-fold ($p<0.0001$) compared to control. The combination treatment was also significantly better at reducing tumor cell induced blood vessels by 2- ($p<0.0001$) and 1.9-fold ($p<0.0001$) compared to MEDI3617 and Sunitinib alone respectively (Figure 6-5C). Figure 6.6 demonstrates these results using immunohistochemistry, evaluating the tumor core rather than the vessels visible around the tumor periphery as shown in Figure 6-5C. MEDI3617 treatment led to a 2.1- ($p<0.0001$) and Sunitinib a 1.7-fold ($p<0.0001$) decrease in tumor vasculature compared to control. The combination treatment led to a 4.1-fold ($p<0.0001$) reduction in vessel number compared to control and a 1.9- ($p<0.01$) and 2.3-fold ($p<0.0001$) reduction compared to MEDI3617 and Sunitinib treatments alone respectively.

MEDI3617 and Cediranib combination lead to greater anti-angiogenic effect

Cediranib is a small molecule inhibitor that is specific to VEGFR 1-3 unlike the multikinase small molecule inhibitor Sunitinib. Cediranib is currently in Phase II clinical trials in various disease settings. Caki-2 renal cell carcinoma cells were injected

intradermally and mice were dosed with the Ang-2 inhibitor alone, Cediranib alone or the combination of the two agents (Figure 6-7A). Results show that both agents alone led to significant reduction of tumor volume 3.5- fold ($p<0.05$) reduction with MEDI3617 treatment and a 3.8-fold ($p<0.05$) reduction with Cediranib compared to control, however, the combination of the two agents led to a much more significant reduction of 54.6-fold ($p<0.0001$) compared to control. The combination of the two agents was also significantly lower than either agent alone with a 15.6- ($p<0.0001$) and 14-fold ($p<0.01$) decrease compared to MEDI3617 and Cediranib treatments alone respectively (Figure 6-7B).

Evaluating the effects on the tumor cell induced vasculature results show a 1.6- ($p<0.01$) and 1.5-fold ($p<0.01$) decrease in the number of vessel in MEDI3617 and Cediranib treated groups compared to control. The combination of the two agents led to a much more significant reduction in vessel number by 2.7-fold ($p<0.0001$) compared to control. The combination treatment was also significantly better at reducing tumor cell induced blood vessels by 1.6- ($p<0.01$) and 1.8-fold ($p<0.0001$) compared to MEDI3617 and Cediranib alone respectively (Figure 6-7C). Figure 6.8 demonstrates these results using immunohistochemistry, evaluating the tumor core rather than the vessels visible around the tumor periphery as shown in Figure 6-7C. MEDI3617 treatment led to a 2.5- ($p<0.01$) and Cediranib a 1.4-fold ($p<0.01$) decrease in tumor vasculature compared to control. The combination treatment led to a 3.5-fold ($p<0.01$) reduction in vessel number compared to control and a 1.3- and 2.4-fold ($p<0.05$) reduction compared to MEDI3617 and Cediranib treatments alone respectively.

Discussion

Angiogenesis can be separated into two main events (a) the destabilization of normal vasculature, or the loosening of endothelial and peri-endothelial cell contacts, and (b) the activation of the endothelium to proliferate and form new vessels. The Angiopoietin/Tie2 axis is responsible for the first step in angiogenesis or the vessel destabilization while pro-angiogenic factors such as VEGF activate the endothelial cells. Currently FDA approved anti-angiogenic agents target the VEGF pathway and have been shown to complement conventional therapies such as chemotherapy (2013; Kerbel, 2006), however the issues of lack of patient response and tumor rebound due to acquired patient resistance has been a problem in the clinic (Bergers and Hanahan, 2008; Ebos et al., 2009; Loges et al., 2010).

In this study the combination of VEGF targeted agents with the Ang-2 inhibitor, MEDI3617, were evaluated. Targeting two key pathways in angiogenesis should show complementary anti-angiogenic effects. Two VEGF targeted agents were evaluated, the small molecule multi tyrosine kinase inhibitor Sunitinib that is FDA approved for treatment of metastatic kidney cancer and the VEGFR specific small molecule inhibitor Cediranib that is in clinical development for a variety of solid tumors (Figure 6-4). Results show that both in the Sunitinib (Figure 6-5) and Cediranib combination (Figure 6-6) treatments the combination of VEGF and Ang-2 targeting was significantly superior to either modality alone. Treatment of tumors with the Ang-2 inhibitor and Sunitinib led to a 1.5- and 4.6-fold reduction in the tumor volume (Figure 6-5B) and 1.7- and 1.8-fold (Figure 6-5C) reduction in tumor vasculature respectively. However, the combination of the two agents led to a 15.8-fold reduction in tumor volume (Figure 6-5B) and 3.5-fold reduction in tumor vasculature (Figure 6-5C). Similar results were seen with

combination studies with Cediranib. Both the Ang-2 inhibitor and Cediranib led to a 3.5- and 3.8-fold reduction in tumor volume (Figure 6-6B) and 1.6- and 1.5-fold reduction in tumor vasculature (Figure 6-6C) respectively, however, the combination of the two agents led to a much greater anti-tumor and anti-angiogenic effect with a 54.6- and 2.7-fold reduction respectively (Figure 6-6B,C).

In conclusion, the combined targeting of the Ang-2 and VEGF pathways showed much better anti-angiogenic effects than either therapeutic modality alone. Two different VEGF targeted agents were evaluated and showed comparable effects in combination with the Ang-2 inhibitor supporting the pursuit of agents that target both pathways in angiogenesis. These results support the combination of the Ang-2 inhibitor, MEDI3617, especially with Sunitinib that is already used in the clinic.

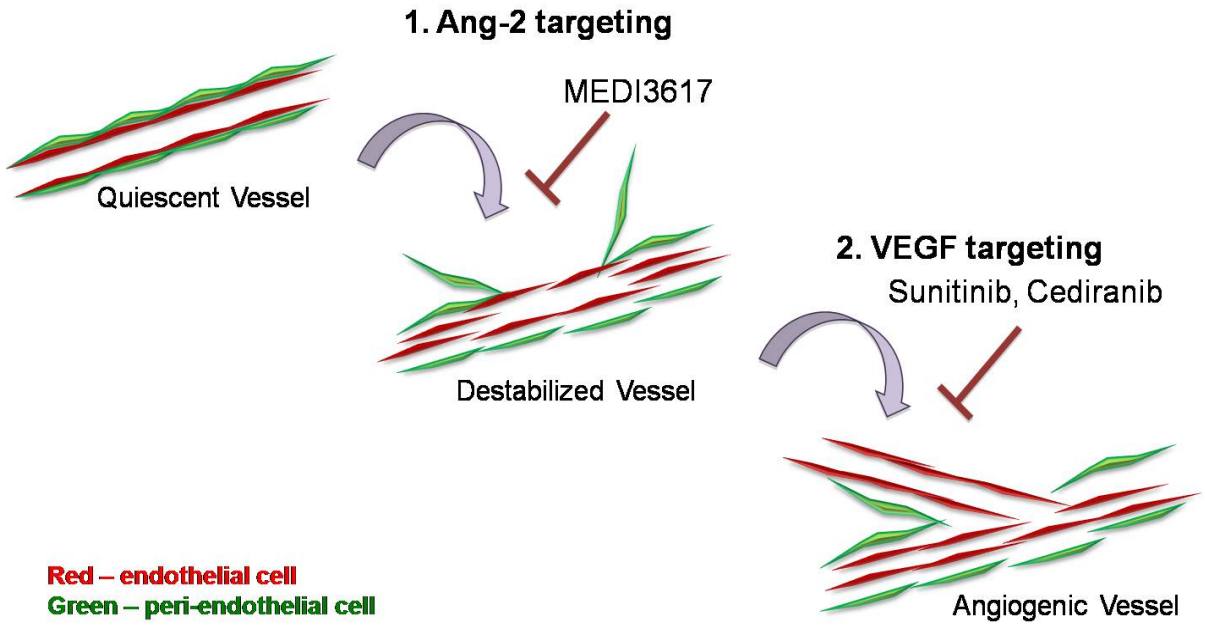

Figure 6-4. Targeting two key steps in angiogenesis. Vessel destabilization depends on the Ang-2/Tie2 axis which can be inhibited by the Ang-2 inhibitor, MEDI3617. Endothelial cell activation mediated significantly by the VEGF/VEGFR pathway can be inhibited by the multikinase inhibitor Sunitinib or the VEGFR2 selective small molecule inhibitor Cediranib.

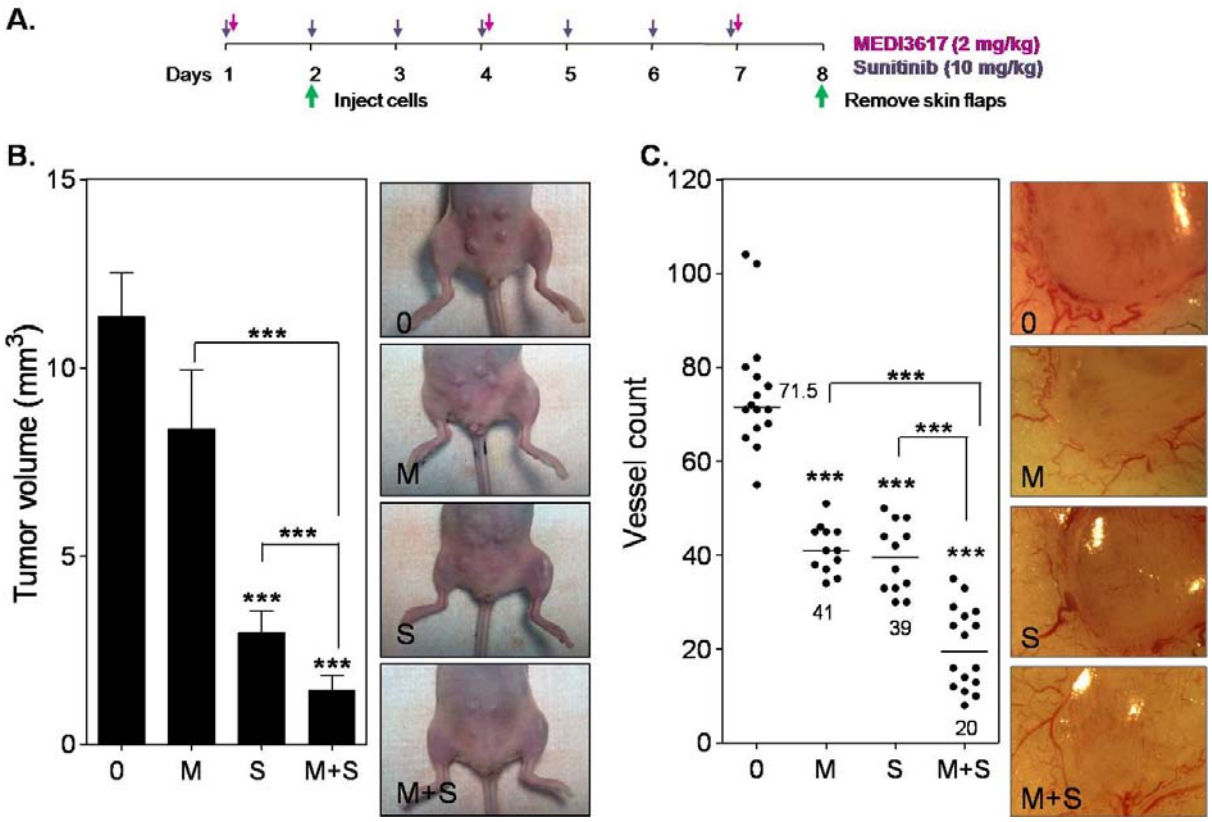

Figure 6-5. Ang-2 and VEGF combination therapy with Sunitinib reduce tumor volume and vessel number. Mice were injected intradermally with Caki-2 renal cell carcinoma cells and treated with MEDI3617 (2 mg/kg), Sunitinib (10 mg/kg) or both beginning the day prior to tumor cell inoculation. Tumor volume (A) and the number of tumor cell induced blood vessels (B) were determined at the end of a 7 day period. Bar, mean with SEM, control (0) and combination (M+S) (n=20), MEDI3617 (M) and Sunitinib (S) (n=16); line, median, control and combination (n=16), MEDI3617 and Sunitinib (n=12); ***, $p<0.0001$; Mann-Whitney U-Test.

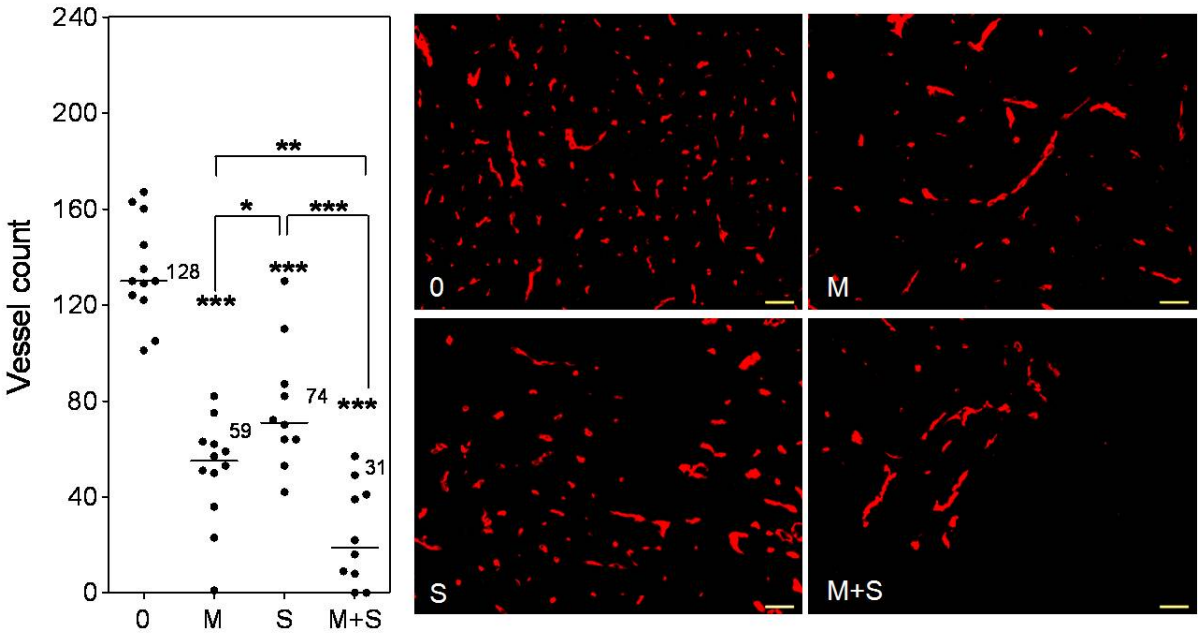

Figure 6-6. Ang-2 and VEGF combination therapy with Sunitinib impair tumor angiogenesis. The number of blood vessels within the tumor were evaluated using immunohistochemistry of fresh frozen tissue. Vasculature was stained with MECA-32. Line, median. Control (n=20), MEDI3617 (n=22), Sunitinib (n=16), combination (n=9). *, $p<0.05$; **, $p<0.01$; ***, $p<0.0001$; Mann-Whitney U-Test. Representative images of the median of each group. Red, MECA-32. Images taken with Zeiss Axioplan Imaging2.

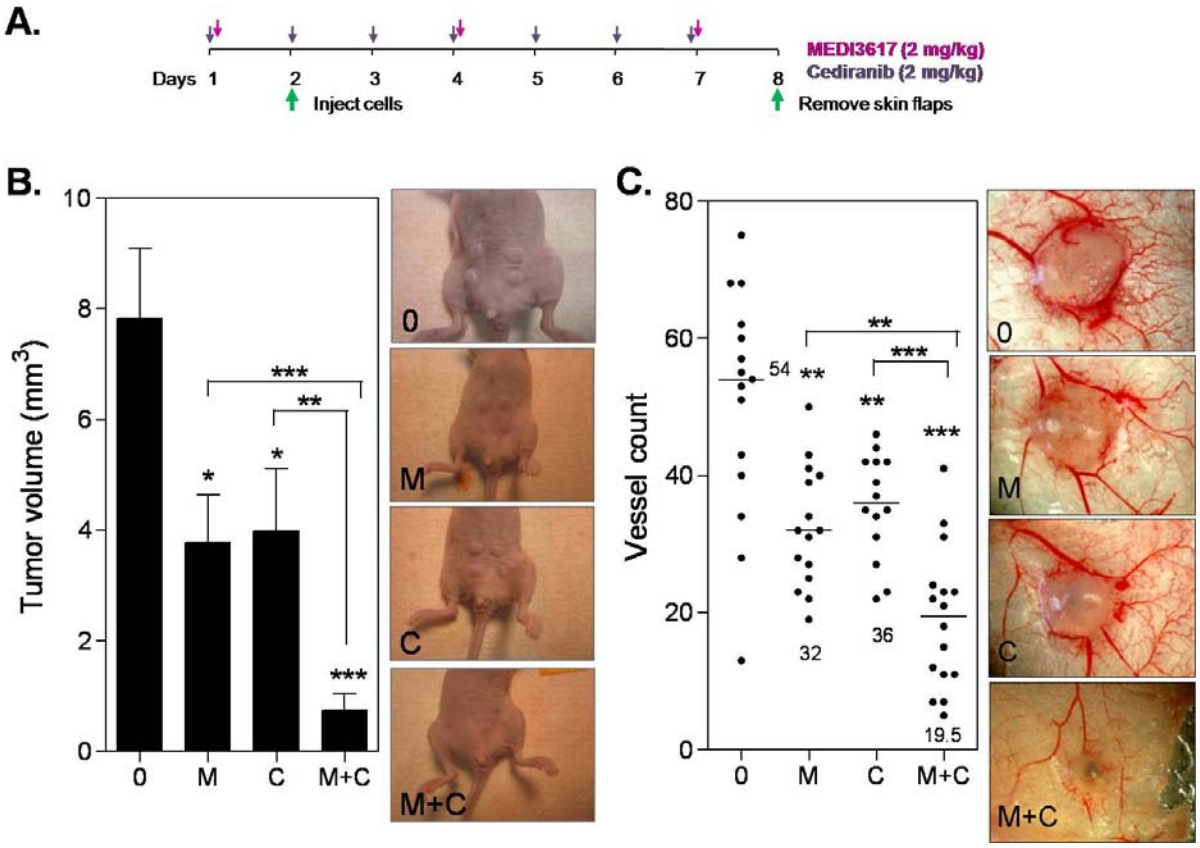

Figure 6-7. Ang-2 and VEGF combination therapy with Cediranib reduce tumor volume and vessel number. Mice were injected intradermally with Caki-2 renal cell carcinoma cells and treated with MEDI3617 (2 mg/kg), Cediranib (2 mg/kg) or both beginning the day prior to tumor cell inoculation. Tumor volume (A) and the number of tumor cell induced blood vessels (B) were determined at the end of a 7 day period. Bar, mean with SEM (n=16); line, median, (n=16); **, $p<0.01$; ***, $p<0.0001$; Mann-Whitney U-Test.

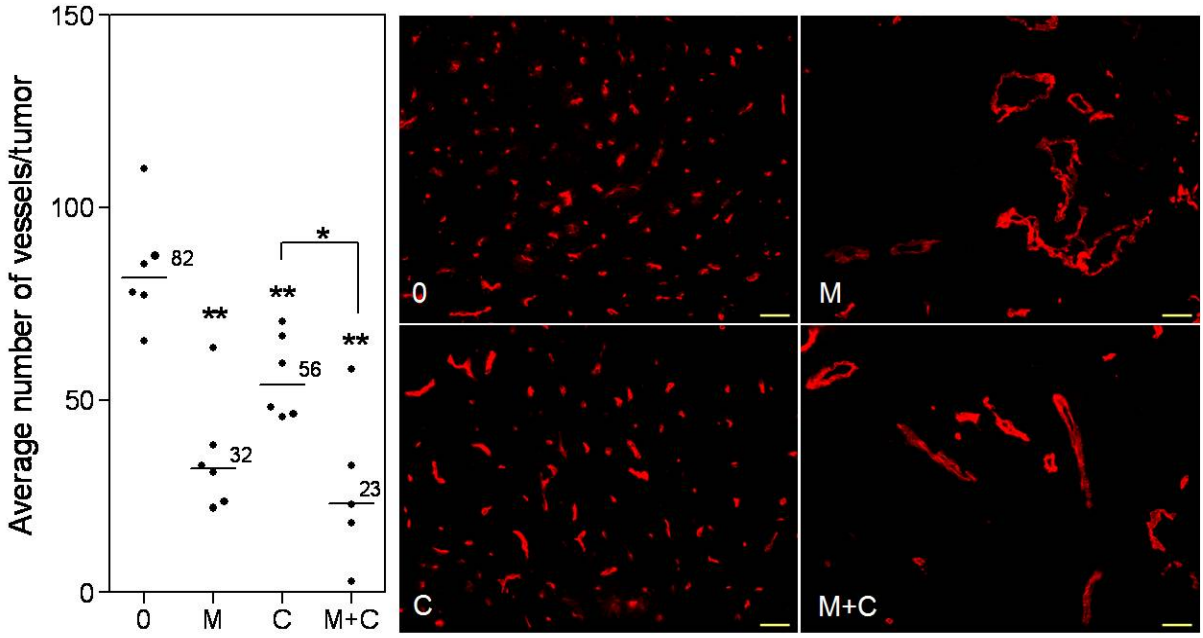

Figure 6-8. Ang-2 and VEGF combination therapy with Cediranib impair tumor angiogenesis. The number of blood vessels within the tumor were evaluated using immunohistochemistry of fresh frozen tissue. Vasculature was stained with MECA-32. Line, median. (n=6). *, $p<0.05$; **, $p<0.01$; Mann-Whitney U-Test. Representative images of the median of each group. Red, MECA-32. Images taken with Zeiss Axioplan Imaging2 microscope with 20x objective; scale bar = 140 µm.

CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

Summary

The research presented here explored the therapeutic potential of an Ang-2 targeted antibody MEDI3617. At the time these studies began this antibody was in preclinical development. It has now moved into phase I clinical trials to be evaluated for patient tolerability. The research presented here determined the effect of the Ang-2 inhibitor on the endothelial and smooth muscle cell contact destabilization, the first step in the angiogenic process. Studies showed that the presence of Ang-2 leads to cell-cell contact loss, which can be inhibited by the Ang-2 inhibitor. Furthermore, the involvement of the tumor microenvironment in the Ang-2 axis was explored. This area remains controversial and results here suggest that tumor cells do not significantly contribute Ang-2 to the tumor microenvironment; however, tumor cells do secrete factors that influence the behavior of endothelial cells. Therefore tumor cells have an indirect role in the initiation of angiogenesis through the Ang-2 pathway. The presence of the Ang-2 inhibitor impaired the tumor cell induced cell-cell destabilization both in normal and hypoxic conditions. Hypoxia did not seem to be a major factor in Ang-2 release from endothelial cells neither did it affect the tumor cell secreted factors in regards to stimulation of Ang-2 secretion. The possibility of VEGF as the tumor cell secreted factor to influence endothelial cells to release their Ang-2 contents was evaluated and results did not support such hypothesis.

In vivo the Ang-2 inhibitor significantly impaired tumor volume and vessel number and promoted a normal vascular phenotype with peri-endothelial cell coverage of vessels in an early tumor development model. However, the Ang-2 inhibitor did not elicit

significant effects when tumors were allowed to grow large and establish vasculature prior to treatment. No effects were seen in a metastatic model either. When evaluated in the murine dorsal skinfold window chamber model it became apparent that the model has limitations and that the natural wound healing and pro-inflammatory response through the Ang-2 axis skewed results when tumors were treated with the Ang-2 inhibitor. No such issues were seen when tumors were treated with a VEGF pathway inhibitor. Finally, results support the combination of Ang-2 with VEGF inhibition as been shown with two different VEGF pathway small molecule inhibitors.

Current Status Of Angiopoietin-2/Tie2 Inhibitors

In the last few years there has been tremendous interest in targeting the Ang-2/Tie2 axis in angiogenesis in hopes to circumvent the lack of response or adaptive resistance that has been seen in the clinic to VEGF targeted agents (Bergers and Hanahan, 2008; Ebos et al., 2009; Loges et al., 2010). Currently there are two small molecule inhibitors that could be considered Tie2 inhibitors in clinical development. CEP-11981 (Cephalon, Inc) is a pan-VEGFR and Tie2 inhibitor, although it has an IC_{50} 5-fold lower for VEGFR than Tie2 (Hudkins et al., 2012); Phase I clinical trial (NCT00875264) is complete and there are no other active trials at this time . ARRY-614 (Array BioPharma, Inc) is another small molecule inhibitor that targets Tie2 and p38; Phase I trial in myelodysplastic syndromes is currently ongoing. ACTB-1003 (ACT Biotech/Bayer AG) is an FGFR/PI3K inhibitor with comparable activity against Tie2, however there are no clinical trials with this agent at this moment (Burd et al., 2010). Several small molecule inhibitors are sometimes referred to as Tie2 inhibitors; however have very low activity toward this receptor. For example, Regorafenib (BAY 73-4506; Bayer AG) has 74-, 24-, 6.7- and 14-fold higher biochemical activity to VEGFR2, 1, 3

and PDGFR respectively than to Tie2 and it hardly affects the phosphorylation status of Tie2 while significantly reduces those of VEGFR2 and PDGFR (Wilhelm et al., 2011). Carbozantinib (XL-184/BMS-907351; Exelixis/BMS) is another such small molecule inhibitor with 408- and 7.9-fold greater activity to VEGFR2 and MET respectively than Tie2 (Zhang et al., 2010). Although MGCD-265 (MetylGene, Inc) and Foretinib (Exelixis/GSK) have smaller gaps in activity between VEGFR, MET and Tie2, these two compounds are being developed as VEGFR/MET inhibitors (Beaulieu et al., 2011).

There are two peptide-based agents, CVX-060 (Pfizer) and Trebananib (AMG386; Amgen) that are in phase II and III clinical trials respectively. Trebananib is the furthest in clinical development with over a dozen ongoing clinical trials in all phases and various disease settings in combination with either chemotherapeutic and/or anti-VEGF agents. Trebananib is an Ang-1/2 specific peptibody that has raised questions about this agent. Based on current knowledge about the basic biology of this axis, Ang-1/Tie2 interactions support and maintain a normal vascular phenotype while Ang-2/Tie2 interactions lead to vascular destabilization and angiogenesis in the presence of pro-angiogenic factors. Genetic data support the notion that Ang-1 is an agonist while Ang-2 is an antagonist of Tie2. The lack of Ang-1 or Tie2 correlates with the same vascular phenotype as over expression of Ang-2 (Maisonpierre et al., 1997). Therefore, one would assume that in this axis Ang-2 should be the sole target. However, Trebananib has yet to show any adverse effects other than the normal side effects seen with anti-angiogenic agents. Although there was an attempt to explain the benefit of targeting both Ang-1 and Ang-2 by Coxon and colleagues (Coxon et al., 2010) the article fell short of the real question which is the possible adverse effects of this agent on the

normal vasculature. Recently, Thomas and colleagues (Thomas et al., 2013) demonstrated that Ang-1 inhibition can have adverse effects on normal vascular phenotype. A provocative study by Daly and colleagues (Daly et al., 2013) complicates this issue further. In their study with the Ang-2 antibody (REGN910) they have demonstrated that the addition of Ang-1 to tumor bearing mice in the absence and presence of the Ang-2 inhibitor led to tumor growth comparable to control. Daly and colleagues interpreted this data suggesting that Ang-2 is a Tie2 agonist compensating for lack of Ang-1 in the microenvironment. However, they fail to discuss the physiological relevance of Ang-1 overexpression and a study by Suri and colleagues (Suri et al., 1998) showed that overexpression of Ang-1 leads to hypervascularization in mice attributing to initiation of vascular branching in the presence of VEGF, a process that is mediated by Ang-1/Tie2 in vascular development. Perhaps there is a fine balance of Ang-1 levels that are needed to be maintained in order to promote quiescent vascular phenotype and upsetting this balance could initiate vascular branching ultimately leading to increased vasculature especially in the presence of VEGF, an abundant factor in the tumor microenvironment. These results therefore could have been skewed by a non-physiological condition that would normally be seen. Clearly, the angiopoietin/Tie axis is complex and not fully understood.

There are a few agents that are Ang-2 specific; the modified antibody mentioned earlier CVX-060 that is currently in Phase I/II trial in combination with Sunitinib. A Phase II trial in combination with Axitinib was terminated due to adverse toxicities; new strategies for dosing and scheduling are being evaluated. Fully human monoclonal antibodies specific to Ang-2, MEDI3617 (MedImmune, LLC/AstraZeneca) and

REGN910 (Sanofi/Regeneron) are both in phase I clinical trials (Table 1) (Daly et al., 2013; Leow et al., 2012). Currently all the Ang/Tie2 pathway inhibitors are being evaluated in combination with chemotherapeutic agents and/or anti-VEGF targeted agents, such as Bevacizumab, Sorafenib, Sunitinib, that have over the years become part of the standard of care . While there is merit to these combinations there are also additional possibilities that have yet to be explored preclinically or clinically with these agents. Such considerations are the focus of the rest of this discussion.

Combination With Radiation Therapy

Conventional cancer therapies such as chemotherapy and radiation therapy target rapidly proliferating cells by inducing DNA damage. Radiation therapy uses ionizing radiation that creates reactive oxygen species and causes DNA damage resulting in tumor cell death (Bristow and Hill, 2005; Giaccia and Hall, 2006a; Giaccia and Hall, 2006b). Curative radiotherapy is used either as a single modality, before or after surgical resection of tumors. It can have detrimental effects on normal tissues and the therapeutic outcome can be greatly affected by the tumor microenvironment (Bristow and Hill, 2008). Hypoxic cells are resistant to radiation and pose a real problem in the treatment of cancer (Figure 7-1A) (Bristow and Hill, 2008; Giaccia and Hall, 2006b). Efforts to improve oxygenation in the tumor have shown some improvement in treatment outcome (Chapman and Whitmore, 1984; Coleman, 1985; Giaccia and Hall, 2006b; Overgaard and Horsman, 1996). Targeting the tumor vasculature offers a different approach and combinations with radiation therapy have shown that these treatments may be complementary (Figure 7-1A) (Dings et al., 2007; Horsman and Siemann, 2006; Jain et al., 2006; Siemann, 2006; Siemann, 2011) (Figure 7-1A).

There is some evidence that radiation of tumors leads to more aggressive phenotypes of cells that remain. One such reason for aggressive phenotype is the upregulation of pro-angiogenic factors in tumor cells thus the combination of radiation with anti-angiogenic therapy seems logical (Kargiotis et al., 2010). In general, the combination of radiation and anti-angiogenic therapies resulted in superior anti-tumor effects than either agent alone (Horsman and Siemann, 2006). However, it is now evident that timing of such combinations for maximal effect is important (Senan and Smit, 2007). Studies have suggested that treatment of tumors with anti-angiogenic agents may lead to a window of vascular “normalization” where the oxygenation of the tumor is improved (Jain, 2005b). Some speculate that the normalization window is responsible for the benefits seen in combination with chemotherapeutics and radiation treatments (Horsman and Siemann, 2006). Dings and colleagues (Dings et al., 2007) demonstrated that tumors experienced improved oxygenation post anti-angiogenic treatment and that it was crucial to irradiate tumors within this window for effective cell kill. Geng and colleagues (Geng et al., 2001) showed that the combination of radiation and anti-angiogenic agent at minimally effective low-dose resulted in the same damage to the tumor as either agent alone at higher doses, however the combination significantly reduced the normal tissue toxicities. Benefit of using anti-angiogenic agents as a maintenance therapy post radiation has also been shown (Schueneman et al., 2003).

Currently there are 41 open/ongoing trials combining anti-angiogenic agents with radiation alone or radiation and chemotherapy in several tumor settings such as brain metastasis, gliomas, glioblastomas, head and neck cancer, child glioma, colorectal

cancer, nasopharyngeal carcinoma, child CNS, rectal, non small cell lung cancer, colorectal liver metastasis, soft tissue sarcoma, hepatocellular carcinoma, prostate cancer, pelvic cancer. There are no reported preclinical or clinical data combining Ang-2 targeting agents with radiation therapy even though there is convincing evidence that the combination of a vascular targeted agent and radiation therapy complement each other. In the future, Ang-2 targeted agents should be pursued in this setting opening a new window of opportunities for this class of agents.

Combination With Vascular Disrupting Agents

Vascular disrupting agents (VDAs) are a class of vascular targeted agents that work distinctively different from anti-angiogenic agents. While anti-angiogenic agents prevent the formation of new vasculature VDAs destroy the existing tumor vasculature. VDAs exploit the different characteristics of normal and tumor endothelial cells and specifically target those associated with the tumor (Figure 7-1B). Normal endothelial cells are dormant unless physiological angiogenesis is required while tumor endothelial cells are continuously dividing, trying to keep up with the vascular demands of the growing tumor. VDAs can be separated into two classes (a) the flavanoids that induce hemorrhagic necrosis and (b) the tubulin depolymerizing/binding agents that disrupt the cytoskeleton of proliferating endothelial cells ultimately leading to vessel occlusion and necrosis (Siemann et al., 2005).

Preclinical evaluations show convincing data that the combination of VDAs and anti-angiogenic agents have complementary effects on tumor growth (Chen et al., 2012; Shaked et al., 2010; Siemann and Shi, 2004; Siemann and Shi, 2008). The different mechanisms of action of VDAs and anti-angiogenic agents allows for the destruction of existing tumor vasculature by the VDA and inhibition of vascular recovery normally seen

after VDA treatment by the anti-angiogenic agent (Figure 7-1B) (Siemann, 2006; Siemann et al., 2004). Even though VDAs are effective at destroying the tumor vasculature and have been shown to complement conventional therapies such as chemotherapy and radiation therapy they are not widely sought after in the clinic. Several clinical trials have been terminated due to toxicity issues. Recently two trials (Phase I/II) were conducted with Combretastatin and Bevacizumab with or without chemotherapy (Patterson et al., 2012). No other trials with VDAs are currently ongoing.

To date there are no reported studies combining VDAs and Ang-2 inhibition, however one study alludes to the possible benefits of this combination. Welford and colleagues (Welford et al., 2011) demonstrated that Tie2 expressing macrophages can hinder Combretastatin effects and depletion of these macrophages by CXCR4 inhibitor AMD3100 led to improved effects. Targeting Ang-2 has also been shown to decrease the amount of Tie2 expressing macrophages in the tumor microenvironment by hindering their recruitment through Ang-2 (Mazzieri et al., 2011).

Other Therapeutic Considerations

Ang-2 targeting as an anti-angiogenic agent could be beneficial in limiting the development and growth of distant metastases. Furthermore, Ang-2 targeting could be beneficial in targeting tumor associated macrophages (TAMs) especially the ones expressing Tie2 (TEMs). TEMs are recruited to the tumor microenvironment in the presence of Ang-2 and have been shown to aid tumor angiogenesis (De Palma et al., 2007; De Palma and Naldini, 2011). TAMs in general are known to aid tumor cells as well (De Palma et al., 2007; Solinas et al., 2009). Therefore this therapeutic modality could be useful not only to treat the microvasculature of the primary tumor but also eliminate tumor associated macrophages and impair the development of metastases.

Therapeutic Limitations Of Ang-2 Targeted Agents

There are possible limitations of Ang-2 targeted agents. For example, there are still some questions regarding the Ang-/Tie axis that remains to be answered. There is limited knowledge on the role of Tie1, however some studies speculate that a Tie1/2 heterodimeric interaction leads to the destabilizing effect of Ang-2 ligand (Seegar et al., 2010). Furthermore, the importance of Ang-1 in pathological conditions is still controversial. Based on the basic biology one would think that the presence of Ang-1 is important to establish a normal vascular phenotype and inhibition of this ligand would lead to impairment in the normal vasculature. However in the case of the Ang-1/2 peptibody, Trebananib, no overt toxicities have been noticed (Doi et al., 2013; Mita et al., 2010; Neal and Wakelee, 2010; Oliner et al., 2012; Rini et al., ; Rini et al., 2012; Robson and Ghatage, 2011). Also a study by Jeansson and colleagues (Jeansson et al., 2011) showed that the presence of Ang-1 is not crucial in maintenance of normal quiescent vasculature, however is essential for vascular repair upon injury. It has also been shown that Ang-2 can interact with integrins and aid in endothelial cell migration (Carlson et al., 2001; Hu et al., 2006; Imanishi et al., 2007); however currently we do not know whether Ang-2 binds integrins on the same site as it does Tie2 receptor and whether the currently available Ang-2 axis inhibitors would also impair the interaction of Ang-2 and integrins and how this would either positively or negatively affect the anti-angiogenic and anti-tumor effects of Ang-2 inhibitors.

In general, Ang-2 targeting will be beneficial in inhibition of angiogenesis that occurs from the normal surrounding vasculature. Since tumor microvasculature is disorganized and lack sufficient peri-endothelial cell coverage the inhibition of an endothelial-peri-endothelial cell destabilization agent would be out of context. However,

the combination of a VEGF and Ang-2 targeted agent would capitalize on two major pathways in this process and could be more superior.

Overall Conclusions

Vascular targeted therapies seldom have clinical efficacy alone. However, these agents can enhance benefits from conventional therapies. Combination therapies exploit targeting tumors in various ways and usually require lower dose of each agent limiting normal tissue toxicities, especially when toxicities do not overlap. There is merit to anti-angiogenic combination therapies with radiation therapy or vascular disrupting agents. However, a “killer” combination would be the combination of a conventional therapy e.g. chemotherapeutics or radiation that would target the rapidly proliferating tumor cells, a VDA that would destroy the existing tumor vasculature and lead to necrosis of endothelial and tumor cells and an anti-angiogenic agent that would prevent the recovery of the tumor vasculature. Based on current data the combination of Ang-2 and VEGF targeted modalities would be more effective anti-angiogenic agent than either alone. Furthermore, Ang-2 targeting would add an extra layer of tumor microenvironment effects by inhibiting the recruitment of tumor associated macrophages that have been shown to aid tumor and vascular growth. A combination modality of this magnitude would require rigorous preclinical and clinical evaluation for optimal dosing and timing of each agent to reach maximal tumor cell kill. This therapeutic modality would target major pathways involved in all solid tumor growth and development including metastasis and thus should be considered in the future for cancer treatment.

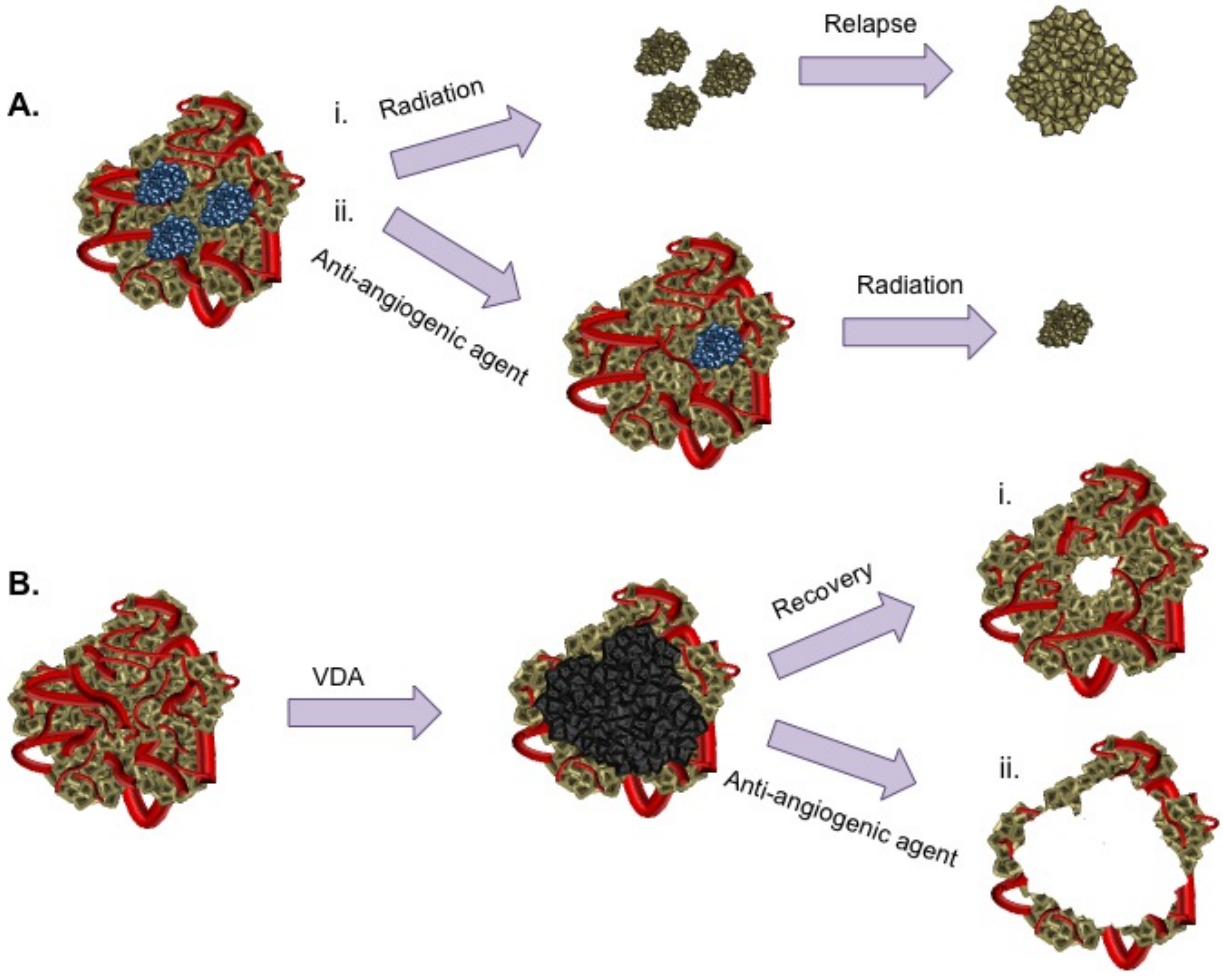

Figure 7-1. Radiation and VDA combination modalities. (A) Anti-angiogenic and radiation therapies combined. i) Areas of hypoxia within the tumor are common and cells at such areas are resistant to radiation and therefore survive while cells under normal oxygen conditions are killed. Surviving cells can lead to tumor relapse. ii) The treatment of tumors with anti-angiogenic agents could lead to a window of vascular normalization thus reducing the areas of hypoxia within the tumor. Decreased levels of hypoxia within the tumor lead to increased cell kill upon irradiation. (B) Anti-angiogenic and vascular disrupting agents combined. Treatment of tumors with vascular disrupting agents leads to the destruction of tumor vasculature and necrosis. i) A viable rim of tumor cells and vessels usually remain after treatment and angiogenesis is induced leading to regrowth of the tumor and its vasculature. ii) The treatment of tumors with anti-angiogenic agents after treatment with vascular disrupting agents inhibits the regrowth of the tumor and its vasculature.

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BIOGRAPHICAL SKETCH

Nikolett Molnar obtained her Bachelor of Science degree in biology from Gettysburg College, Pennsylvania (2003-2007) and joined the Interdisciplinary Biomedical Sciences Program at the University of Florida College of Medicine in 2008. Under the mentorship of Dr. Dietmar Siemann, Miss Molnar has presented her research in several international conferences such as the American Association for Cancer Research (AACR), Molecular Targets and Cancer Therapeutics, The International Workshop on the Tumor Microenvironment, and the International Symposium on Anti-Angiogenic Therapy as well as participated in the Shands Cancer Center Research Day and the College of Medicine Research Day. Throughout her training she has received honors and awards such as the NIH T32 Training Grant in Cancer Biology, best pre-doctoral poster award at the Shands Cancer Center Research Day and junior investigator award at the International Workshop on the Tumor Microenvironment. She has also successfully collaborated with a laboratory at the Department of Biomedical Sciences that led to co-authorship on poster presentations and a publication. She has shared her passion for cancer biology research by teaching a course entitled *"Cancer Biology and Therapeutics"* to honors high school students during the summer for three years as part of the University of Florida Student Science Training Program. Upon completion of her PhD program in Medical Sciences in August 2013, she aspires to remain in the cancer research field.