FGFR1/PI3K/AKT SIGNALING PATHWAY IS A NOVEL TARGET FOR ANTI-ANGIOGENIC EFFECTS OF THE CANCER DRUG FUMAGILLIN (TNP-470)

A Dissertation

by

GREGORY J. CHEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Biochemistry

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Approved by:

Co-Chairs of Committee, Jerry Tsai
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ABSTRACT

FGFR1/PI3K/AKT Signaling Pathway is a Novel Target for Anti-angiogenic Effects of the Cancer Drug Fumagillin (TNP-470).

(May 2007)

Gregory J. Chen, B.S., University of California Santa Barbara;

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Fibroblast growth factor-1 (FGF1), a prototypic member of the FGF family, is a potent angiogenic factor. Although FGF-stimulated angiogenesis has been extensively studied, the molecular mechanisms regulating FGF1-induced angiogenesis are poorly understood *in vivo*. Fumagillin, an antiangiogenic fungal metabolite, has the ability to inhibit FGF-stimulated angiogenesis in the chicken chorioallantoic membrane (CAM). In the current study, chicken CAMs were transfected with a signal peptide-containing version of the FGF1 gene construct (sp-FGF1). Transfected CAMs were then analyzed in the presence and absence of fumagillin treatment with respect to the mRNA expression levels and protein activity of the FGF1 receptor protein (FGFR1), PI3K (phosphatidylinositol 3-kinase), and its immediate downstream target, AKT-1 (protein kinase B).

Results from the treatment of sp-FGF1-transfected CAMs with fumagillin showed downregulation of PI3K and AKT-1 proteins at the mRNA and protein levels. In contrast, no major alterations in FGFR1 mRNA expression level were observed. Similar patterns of mRNA expression for the above three proteins were observed when the

CAMs were treated with recombinant FGF1 protein in place of sp-FGF1 gene transfection. Investigation using biotin-labeled fumagillin showed that only the FGF1 receptor protein, containing the cytoplasmic domain, demonstrated binding to fumagillin. Furthermore, I demonstrated endothelial-specificity of the proposed antiangiogenic signaling cascade using an *in vitro* system. Based on these findings, I conclude that the binding of fumagillin to the cytoplasmic domain of the FGF1 receptor inhibited FGF1-stimulated angiogenesis *in vitro* and *in vivo*.

DEDICATION

I would like to dedicate this work to my parents, Edmund Chen and Julia Chen. They've supported me continuously through my entire life. Anytime I've needed anything or have had any trouble they have helped me. I cannot express how much this has meant to me, especially the last five years. Also, I like to thank my brother Joseph, sisters Mary and Lih for their unconditional emotional support. Without them, I never would have been able to get to this point.

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I like to sincerely thank Dr. Forough for giving me the opportunity to work in his lab as a graduate student. Without his support and understanding, this degree would not have been possible.

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It has been a long road to finally reach the point of obtaining my doctoral degree. Along the way, I have met and learned from many people about science and life. There were a few that stood out from the rest and left a deep impression on me. I like to thank Dr. Dunker, Vilma, Yongrong, Randy, Eve, Suzanne, and Pavlina for supporting me emotionally and spiritually during some of the very difficult times. They had made my journey much more enjoyable and worthwhile and for that I will be forever grateful.

Finally, to my family: You have always been my safety net. I've always felt that whatever happened here, you would be there for me, and you have been – letting me vent the frustrations, cheering the successes, and sharing my ups and downs. It's been a long road and I've been a long way from home, but with you behind me I never doubted that I'd be here in the end. I love you all, and thank you.

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CHAPTER I

INTRODUCTION*

Cancer develops when cells in a part of the body begin to grow out of control. Although there are many kinds of cancer, they all start because of out-of-control growth of abnormal cells. Normal body cells grow, divide, and die in an orderly fashion. During the early years of a person's life, normal cells divide more rapidly until the person becomes an adult. After that, cells in most parts of the body divide only to replace worn-out or dying cells and to repair injuries. Because cancer cells continue to grow and divide, they are different from normal cells. Instead of dying, they outlive normal cells and continue to form new abnormal cells. Cancer cells often travel to other parts of the body where they begin to grow and replace normal tissue. This process, called metastasis, occurs as the cancer cells get into the bloodstream or lymph vessels of our body. When cells from a cancer like breast cancer spread to another organ like the liver, the cancer is still called breast cancer, not liver cancer. Cancer cells develop because of damage to DNA. This substance is in every cell and directs all its activities. Most of the time when DNA becomes damaged the body is able to repair it. In cancer cells, the damaged DNA is not repaired. People can inherit damaged DNA, which accounts for inherited cancers.

This dissertation follows the style and format of *Analytical Biochemistry*.

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Many times though, a person's DNA becomes damaged by exposure to something in the environment, like smoking. Cancer usually forms as a solid tumor. Some cancers, like leukemia, do not form tumors. Instead, these cancer cells involve the blood and blood-forming organs and circulate through other tissues where they grow.

Cancer is the second leading cause of death in the United States (Table 1). Half of all men and one-third of all women in the US will develop cancer during their lifetimes. Today, millions of people are living with cancer or have had cancer. The risk of developing most types of cancer can be reduced by changes in a person's lifestyle, for example, by quitting smoking and eating a better diet. The sooner a cancer is found and treatment begins, the better are the chances for living for many years.

Angiogenesis

Tissue homeostasis is dependent upon an adequate supply of oxygen and nutrients delivered through blood vessels. Since the cells must be located within 100-200 μm of blood vessels, the diffusion limit for oxygen, to survive and properly function, the maintenance of the vascular network is critical for survival of tissues. Angiogenesis, derived from the Greek word angeion meaning vase, and genesis meaning birth, is the name given to the outgrowth of new capillaries from pre-existing primary plexus and is crucial in responding to tissue demands both in physiological and pathological conditions.

Table 1. Causes of death among US population in the year 2006.

US Mortality, 2006

Rank	Cause of Death	No. of deaths	% of all death
1.	Heart Diseases	696,947	28.5
2.	**Cancer**	**557,271**	**22.8**
3.	Cerebrovascular diseases	162,672	6.7
4.	Chronic lower respiratory diseases	124,816	5.1
5.	Accidents (Unintentional injuries)	106,742	4.4
6.	Diabetes mellitus	73,249	3.0
7.	Influenza and pneumonia	65,681	2.7
8.	Alzheimer disease	58,866	2.4
9.	Nephritis	40,974	1.7
10.	Septicemia	33,865	1.4

Source: US mortality public use data tape 2006, National Center for Health Statistics, Centers for Disease Control and Prevention, 2006.

The term angiogenesis was coined in 1794 by the British surgeon John Hunter to describe blood vessel growth in reindeer antlers as a result of long lasting exposure to cold [1], a situation that can be interpreted today as a response to vasoconstriction, and therefore increased luminal shear stress [2]. In 1935, the term angiogenesis was applied to medicine by the pathologist Arthur Hertig to describe the formation of new vessels in the placenta. Angiogenesis became a common word in the biomedical community after surgeon Judah Folkman proposed that tumor growth is dependent upon neovascularization [3] (Figure 1). Currently, dysregulated angiogenesis is considered a common denominator in most frequent diseases, including cancer, ischemic heart disease, blindness, psoriasis, and arthritis [4]. Consequently, understanding how blood vessels form has become a principal, yet challenging, objective of studies over the last decade.

The protagonist in angiogenesis is the endothelial cell and co-stars are mural cells as well as extra-cellular matrix (ECM) components. The endothelium is a dynamic, highly heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic and immunologic functions [5] (Figure 2). Endothelial cells are among the longest-lived cells outside of the nervous system. In fact, physiological endothelial cell turnover is reportedly measured in years in tissues that do not require angiogenesis. In a normal adult vessel, only 1 in every 10,000 endothelial cells (0.01%) is in the cell division cycle at any given time [6]. Mural cells, i.e. the smooth muscle cell in arterioles and arteries, or pericytes in the capillaries, are associated with the abluminal surface of the vessel. Pericytes are required for normal microvascular structure and function because they provide structural support, protect endothelial cells from apoptosis, and actively control the stability of the vessels [7, 8]. Smooth muscle cells endow blood

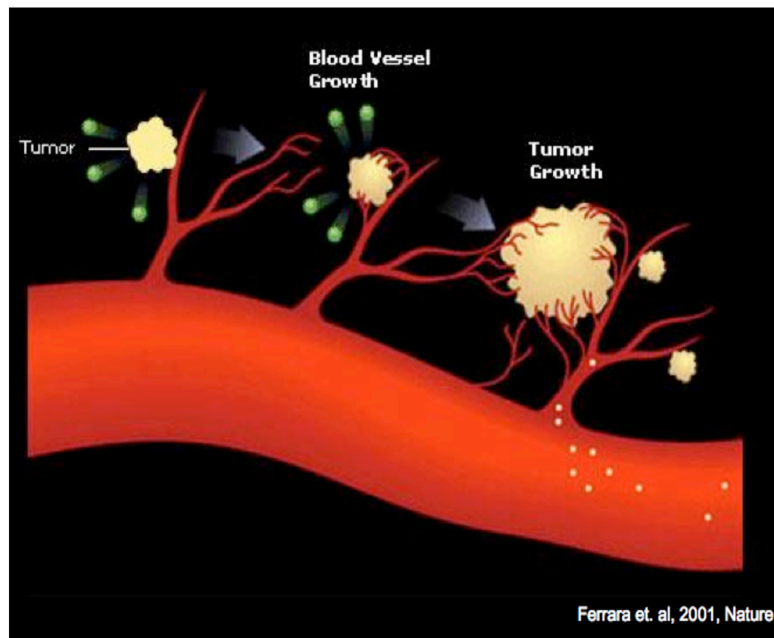

Figure 1. Tumor growth and survival requires a constant supply of oxygen and nutrients from the blood vessels.

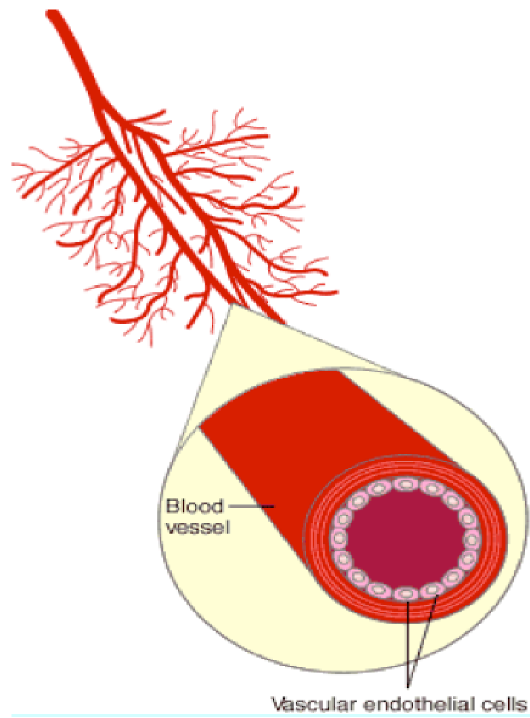

Figure 2. Endothelium inside a blood vessel.

vessels with vasomotor properties, which are necessary to accommodate the changing needs in tissue perfusion.

During embryonic development, blood vessels develop from endothelial precursors called angioblasts which assemble into a primary capillary plexus. This process is referred to as vasculogenesis. The primitive network is then remodeled by angiogenesis, so that new blood vessels sprout and branch from pre-existing capillaries in order to complete the circulatory system [9]. Later in development, endothelial heterogeneity arises due to micro-environmental signals to the endothelial cells. These signals involve a variety of angiogenic growth factors and angiogenesis inhibitors. Normal, controlled angiogenesis results from the balance between pro- and antiangiogenic factors. The last step is the recruitment and organization of mesenchymal cells into layers, generating mature, functional blood vessels.

In the adult, new vessels are produced mainly through angiogenesis. The few adult tissues that require ongoing angiogenesis are the female reproductive organs. Angiogenesis occurs during the monthly reproductive cycle to rebuild the uterus lining and to mature the egg during ovulation, and during pregnancy to build the placenta, allowing circulation between mother and fetus. Angiogenesis is also necessary for the repair or regeneration of tissues during wound healing. These normal processes lead to the formation of a stable, operational vascular bed through a tightly regulated balance between pro- and anti-angiogenic signals. An alteration of this equilibrium promotes dysregulated vessel growth with a consequent major impact on health [10]. Such uncontrolled angiogenic events can lead to health complications such as rheumatoid arthritis, retinopathy, psoriasis, and obesity, just to name a few.

History of FGF

Fibroblast growth factors (FGFs) are a family of heparin-binding growth factors. Historically, it was discovered that brain homogenates contain activities capable of stimulating the growth of fibroblasts in culture, hence the isolated factor was named FGF. They exert multiple functions on various cell types of mesodermal and neuroectodermal origin, affecting cell proliferation, motility, survival, and differentiation. FGFs exert their activity mainly by binding and activating their high-affinity tyrosine kinase receptors (FGFRs) present on the cell surface. Additional membrane-bound low-affinity FGF receptors known as heparan sulfate proteoglycans are also required for enhancing FGF interaction with its cognate high-affinity receptor and subsequent cell signaling.

Members of the FGF family are structurally related and exhibit high degree of sequence identity at the amino acid level reaching approximately 55%. FGFs are single polypeptide chains and range in size between 17-30 kDa. A feature of the FGF's gene structure is that they are formatted in three exons separated by two introns [11]. While the structural similarity is conserved within the central fragment of their coding frame regions, structural diversity is more frequent in the segments encoded for more amino and carboxyl ends.

The two precursors of the FGF family (acidic and basic FGF, now named FGF1 and FGF2, respectively) were isolated approximately three decades ago using heparin-affinity column [12, 13]. Since then, the FGF family has continued to grow and currently contains 23 established members plus two newly identified members in zebra fish.

More than half of the 25 known mammalian FGFs have been investigated by targeting the genes of individual FGFs by homologous recombination. The data presented in Table 2 summarizes the phenotypes caused by targeted disruption of 15 out of the 25 FGFs.

FGF1 and FGF2

FGF1 (acidic FGF) is a 17 kDa, 154-amino acid protein, first identified as a mitogen in neural tissue extracts in bovine brain. It was named acidic FGF because of its acidic pI value of 5 [14]. FGF1 lacks a signal peptide for secretion, however, it is found in the extracellular matrix and body fluids. It has been reported that FGF1 is not secreted outside the cell through the classic secretory pathway facilitated with the help of a signal peptide. In other words, FGF1 and for the same matter FGF2, the other precursor member of FGF family, use a non-conventional pathway mediated by a piggyback ride from a binding protein. Several different proteins have been identified to act as a chaperone by forming a complex with FGF1 or FGF2 in order to facilitate their secretion [15, 16, 17]. FGF1 is found in the nucleus of many cells, and a putative nuclear localization signal has been located at amino acids 21-27 [18]. FGF1 is a potent inducer of DNA synthesis, proliferation, cell survival, differentiation, and cell migration in a large number of different cell types including endothelial, fibroblast, and smooth muscle cells [19, 20].

FGF2 (basic FGF) was initially purified from bovine pituitary gland; and based on the pI of 9.6, it was named basic FGF. Subsequently it was detected and purified from

Table 2. Phenotypes of FGF knockouts.

Gene	**Phenotype**
FGF1	No phenotype change
FGF2	Neuronal, skeletal and skin changes
FGF3	Affects inner ear development and tail outgrowth
FGF4	Inner cell mass proliferation
FGF5	"Angora" phenotype
FGF6	Muscle formation
FGF7	Deficiency in hair follicle and kidney development
FGF8	Affects gastrulation, brain, heart and craniofacial development
FGF9	Lung development and xy sex reversal
FGF10	Affects limbs, lungs, and kidneys development
FGF14	Neurological development such as ataxia and paroxysmal hyperkinetic movement disorder
FGF15	Poor survival ability
FGF17	Affects midline cerebral development
FGF18	Causes ossification delay and increased chondrocyte development; decreased alveolar spaces in the lung
FGF23	Causes hyperphosphatemia, hypoglycemia, reduced bone density and infertility

many other tissues. FGF2 is a 18-kDa, 155-amino acid protein. FGF2, like FGF1, lacks a signal sequence for secretion [20, 21]. In contrast to FGF1 which has one isoform, FGF2 has multiple isoforms resulting from the protein translation from different upstream initiation codons. More specifically, N-terminal extended isoforms of approximately 22, 23, and 25 kDa have been described. The most abundant 18 kDa isoform of FGF2 is localized to the cytosol, but the larger isoforms are predominantly intranuclear and presumably translocate with the aid of a nuclear localization signal present in their N-terminal domain [21, 22]. FGF2 exhibits a higher affinity for heparin than FGF1; however, unlike FGF1, heparin does not appreciably stimulate the mitogenic activity of the FGF2 protein.

In summary, the two prototypic members, FGF1 and FGF2, are critical polypeptide growth factors that stimulate cell growth, differentiation, and perhaps best known as powerful angiogenic factors because of their effects on endothelial cells. In vivo, these FGFs are active in angiogenesis, nerve regeneration, cartilage repair, and wound healing and are thought to play normal roles in tissue repair and homeostasis. The observation that an inappropriate expression of FGFs can result in tumor growth and development also suggests that they participate in the production of a large variety of pathogenic conditions that result in cell proliferation or are angiogenesis-dependent.

FGF Receptors

FGFs mediate their cellular responses by binding to and activating a family of four receptor tyrosine kinases (RTKs) designated the high-affinity FGF-receptors FGFR1-FGFR4 [23]. FGFs also bind to heparin or heparan sulfate proteoglycans (HSPG), the so-called low affinity receptors that do not transmit a biological signal but rather function as an accessory molecule that regulate FGF-binding and the activation of the occupied high affinity FGF RTKs [24, 25]. The core structures of FGFR1-FGFR4 consist of an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain containing the catalytic protein tyrosine kinase (Figure 3). The extracellular ligand-binding domain of FGFR is composed of three immunoglobulin (Ig)-like domains, designated Loops 1 to 3 (L1-L3). Based on deletion/mutation studies, L2 and L3 domains appear to be the regions that the FGF ligands bind to and activate the receptor leading to stimulation of signal transduction. Two structural features of the extracellular portion of the FGFRs are: 1) a stretch of seven to eight acidic residues, referred to as the "acid box" located in the region connecting L1 and L2, 2) a conserved positively charged domain in L2 for heparin binding.

It is important to note that while there are four distinct FGFRs each located on a different chromosome, there are a large number of FGFR isoforms including two or three loop-containing domains or truncated intracellular domains, generated as a result of alternative splicing of FGFR transcripts [26]. For example, no fewer than six different classes of FGFR1s that can be produced from the FGFR1 gene. They either have no secretory signal sequence, deletion of the first Ig loop, alternative splicing into the third

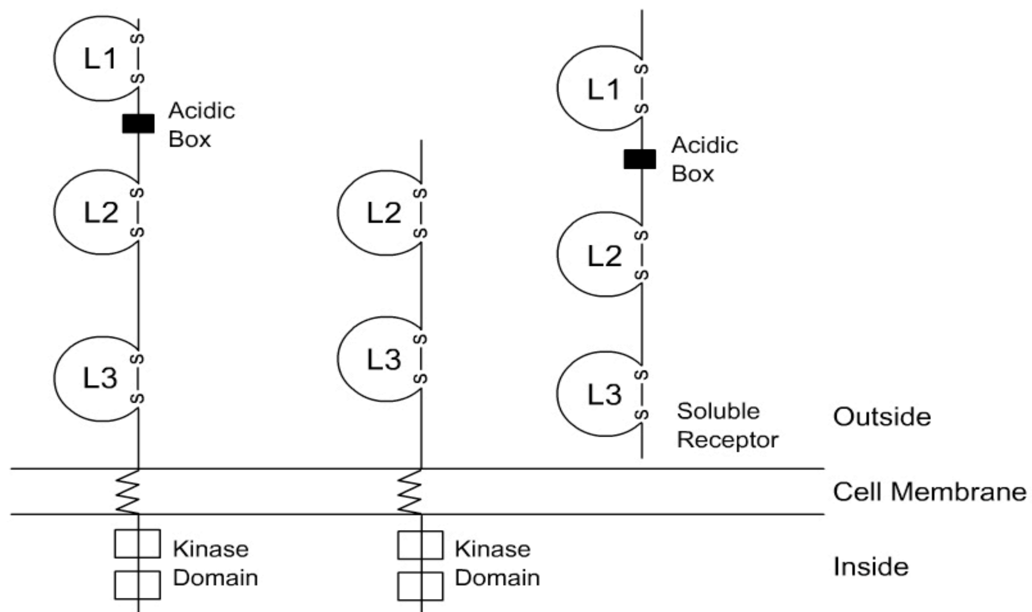

Figure 3. **Splice variants of fibroblast growth factor receptor-1 (FGFR1)**. The structural features include: Ig-like domains (loops) defined by the disulfide bonds, acidic box (a stretch of about 7 amino acids), the split kinase domain.

Ig loop, deletions prior to the third Ig loop, or truncation of the transmembrane domain to generate a soluble FGFR1. Together with the isoforms generated by FGFR2 and FGFR3 genes, there are over 100 different combinations possible at the cell surface. The variety of FGFR isoforms give rise to the multitude of FGF ligands possible for binding and the initiation of signal transduction cascades. Given that 25 different FGFs have been discovered so far, the list for more diverse ligands is sure to increase.

Similar to the FGF ligands, unregulated expression or mutations of FGFRs have been shown to be the cause of a variety of diseases. For example, the genes for FGFR1 and 2 were found to be amplified and over-expressed in a subset of breast cancers [27]. In these cases, amplification of FGFR2 significantly correlated with amplification of the gene for c-myc. Mutations within the FGFR2 gene is implicated as the cause of Apert syndrome whereby the individual is born with fused fingers and toes and well as an enlarged cranium [28]. Mutations within the FGFR3 gene have been shown to be linked to dwarfism whereby the individual has a shrunken stature [29]. Also, different labs have shown a positive correlation between elevated levels of FGFs and their receptors in a wide range of sarcomas and carcinomas [28, 29, 30]. Moreover, several human skeletal dysplasias have also been linked to specific point mutations in three members of the FGFR family. Table 3 summarizes the phenotypes caused by targeted disruption of FGFR1-FGFR4.

Table 3. Phenotypes of FGFR knockouts.

Receptor	Phenotype
FGFR1	Impaired cell migration and posterior axis defects
FGFR2	Defects in placenta and limb bud development
FGFR3	Causes bone over growth and inner ear defect
FGFR4	No phenotype change. Can cause growth retardation and lung defects in FGFR3 null background

FGF/FGFR Signaling

Signaling via FGFRs is initiated by the FGF ligand binding to its cognate high-affinity receptor, followed by dimerization of these receptors at the cell membrane leading to both tyrosine auto- and trans- phosphorylation of the dimerized FGF receptors [31]. The role of low-affinity FGF receptors, soluble or cell surface-bound HSPGs, is to facilitate FGF/ high-affinity FGFR interaction and further promote FGFR dimerization and activation. Activation following FGFR dimerization is mainly the dimerized receptor auto- as well as trans- phosphorylation of the intracellular tyrosine kinase domains of the receptor [32]. The cytoplasmic domain of FGFR contains in addition to the catalytic phospho-tyrosine kinase core, several regulatory sequences. This domain of FGFRs is considerably longer than that of other receptor tyrosine kinases. Mutations within this region have been shown to severely affect downstream signaling cascades that are FGFR-dependent [31]. Phosphorylated tyrosine residues on the FGFR also serve as docking sites for the complex formation of downstream signal transduction molecules containing either Src-homology 2 (SH2) or phosphotyrosine-binding (PTB) domains, leading to the activation of various intracellular signaling pathways responsible for growth, survival, motility, and differentiation of the cell, to list a few [33-37]. The multiple signal transduction pathways activated by FGFRs include, among others, the ras pathway [38], Src family protein tyrosine kinases [39], phosphoinositide 3-kinase (PI3K) [40-43], and the phospholipase C-gamma (PLC-gamma) pathway [40]. Of particular interest to our research focus is the PI3K signaling pathway which is involved in angiogenesis. Figure 4 depicts a comprehensive summary of the FGF/FGFR signaling pathways.

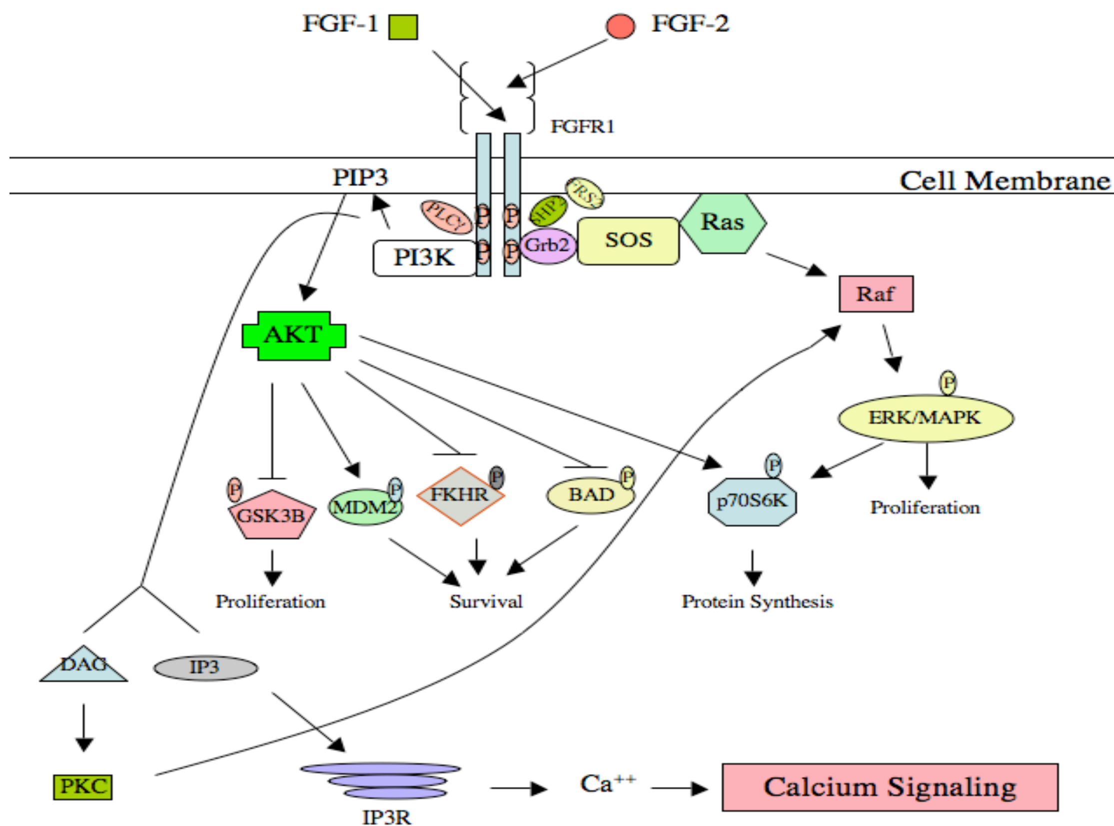

Figure 4. Schematic representing signaling cascades induced by fibroblast growth factors (FGFs). Receptor tyrosine kinases including FGFR generally activate one or more of four main signaling pathways: PI3K/AKT, Ras/MAPK, Src, and PLC-gamma.

Utilizing the CAM Assay to Study Angiogenesis

The study of the angiogenic process and the search for novel therapeutic agents to inhibit, or stimulate angiogenesis has employed a wide range of *in vivo* 'angiogenesis' assays [44]. They differ greatly in their difficulty, quantitative nature, rapidity, and cost. The classical in vivo models include the rabbit ear chamber, hamster cheek pouch, dorsal skin chamber, dorsal skin and air-sac model, anterior chamber/iris and avascular corneal pocket assay, and the chick embryo chorioallantoic membrane (CAM) assay. More recent methods involve the implantation of preloaded Matrigel or alginate plug, or collagen or polyvinyl sponges [45]. Largely owing to its simplicity and low cost, the CAM is the most widely used in vivo model for the study of both angiogenesis and antiangiogenesis [46].

The CAM develops over the first 11 day of the 21-day gestation period. At about 24 hr the development of blood islands around the embryo can be seen. Capillaries appear in the yolk sac of fertilized hens' egg at 48 hr and grow rapidly over the next 6-8 days. The embryonic yolk sac itself consists of four layers: ectoderm (bilayered vitelline membrane), somatic mesoderm, visceral mesoderm (including blood vessels), and endoderm [47]. The circulatory system originates from the mesoderm and develops as three distinct divisions, in the following sequence: the extraembryonic (for nutrition), the accessory or allantoic (for respiration and excretion), and the embryonic (for distribution within the embryo). By day 4, the yolk sac membrane is well vascularized and continues to develop until the chorioallantoic membrane supersedes it, about day 7 (Figure 5). The endothelial cells of this developing vasculature have an abundance of secretory

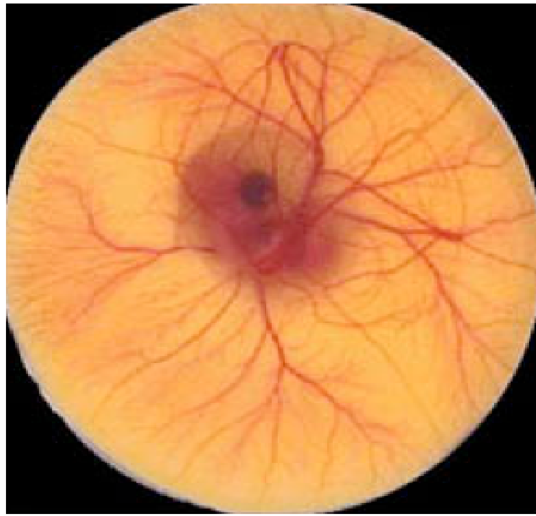

Figure 5. The developing vasculature of the chick embryo during gestation.

organelles, most likely related to the synthesis of the extra cellular matrix and basement membrane [48]. After day 11, proliferation of endothelial cells in existing capillaries is complete, and they appear to have acquired the morphological characteristics of differentiated cells [48, 49].

Studies conducted on growth factors in the chicken CAM have yielded promising results and indicated that the chicken CAM is a useful system to elucidate signaling and other molecular pathways [50, 51]. Essential to chick development, the CAM provides a medium for oxygen and nutrient exchange for the developing chick embryo. The chicken CAM is an ideal system in which to study different angiogenic molecules and their effects (Figure 6). Since the CAM undergoes rapid neovascularization in the developing embryo, researchers are provided with a living model with which to study angiogenesis. Utilizing the chicken CAM allows the examination of factors such as toxicity, blood vessel maturity, and quantity of blood vessels in a single assay. These factors are important for studying the therapeutic effects of angiogenesis and the effect of each growth factor on an *in vivo* system. More importantly, chicken FGF1 receptor is highly homologous to its human counterpart (98% amino acid sequence identity in the kinase region, 86% in the transmembrane region, 92% in the juxtamembrane region, and 80% in the carboxyterminus region) [52] and can be activated when treated with a small dose of human FGF1 ligand [53], which makes chicken CAM an ideal system in which to study angiogenesis for human therapy.

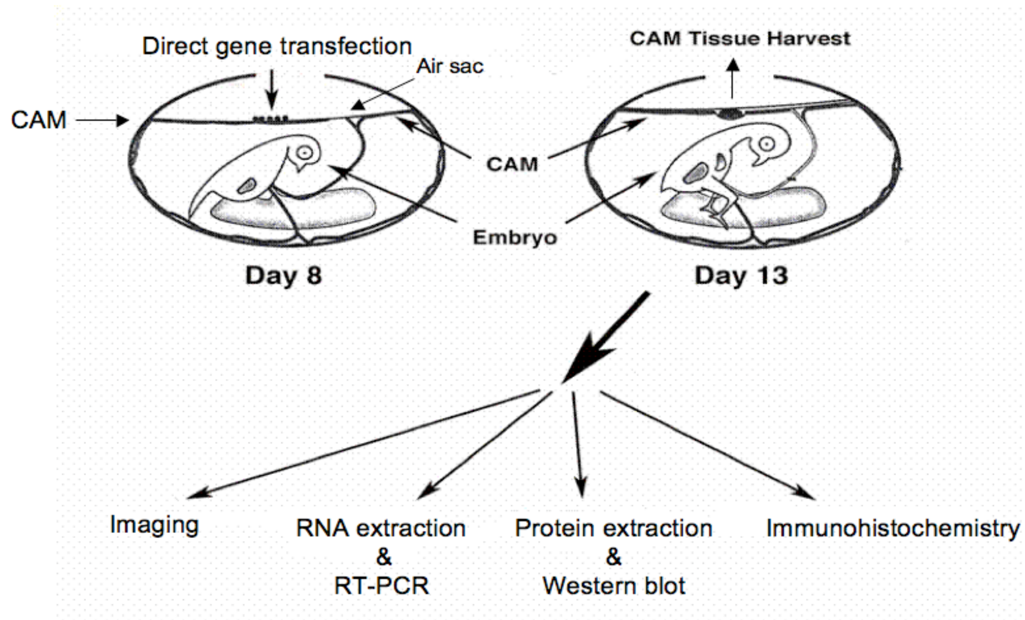

Figure 6. The chicken chorioallantoic membrane assay, commonly referred to as the CAM assay.

Sp-FGF1-stimulated Angiogenesis in the CAM

To study the effects of angiogenic stimulators and inhibitors on vessel growth, we've established a model for studying angiogenesis in the developing chick egg using gene transfer of naked DNA encoding a secreted version of the angiogenic FGF1 [54]. FGF1 has the ability to induce metabolic and phenotypic changes in endothelial cells (ECs) required to stimulate angiogenesis in both *in vivo* and *in vitro* models. Unlike other members of the FGF family, FGF1 lacks a signal peptide for secretion [55]. The addition of known signal peptide sequences to FGF1 has resulted in the generation of a modified version of the growth factor that has potent transforming activities [56, 57, 58]. Presumably, this transforming activity is the result of a constitutive interaction between the modified growth factor and its cognate receptor because of establishment of an external loop [59, 60, 61]. The pleiotropic effects of most of the members of the FGF family have been studied in the angiogenic model where exogenously added growth factor has been the sole angiogenic stimulus [62, 63]. Conversely, we have used an over-expression system of the secreted version of FGF1 (sp-FGF1) whereby a constant supply of the growth factor is synthesized and secreted by the transfectants. A retroviral-based plasmid vector (pMEXneo) was used for introducing the sp-FGF1 gene into ECs because of its high efficiency and the persistence of gene expression (Figure 7).

The signal-peptide used corresponds to the published sequence of one of the secretory members of the FGF family known as FGF4 or human stomach tumor [64] or Kaposi sarcoma (KS3) [65] growth factor. We chose the FGF4 signal peptide sequence

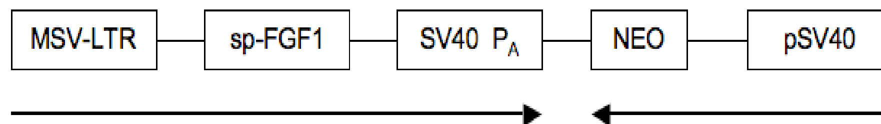

Figure 7. Structures of the secretory version of fibroblast growth factor-1 (sp-FGF1) expression plasmid. Signal-bearing FGF1 is expressed from the long terminal repeat (LTR) of the murine sarcoma virus (MSV) promoter. G418 resistance (NEO) is expressed from the SV40 promoter.

for our construct because the FGF4 oncogene in its native form encodes a secreted growth factor with 30-40% homology to FGF1. In addition, the polymerase chain reaction (PCR) was used to add a Kozak sequence (CCACCATGG) to the final construct for maximal eukaryotic translation efficiency [66]. The expression vector pMEXneo containing murine sarcoma virus long terminal repeat and the SV40 polyadenylation site [67] was chosen for the stable transfection of the sp-FGF1 into microvascular ECs.

Although existing CAM models of angiogenesis using delivery of growth factor proteins have provided important information, gene transfer approaches have several unique advantages. Although viral vectors have been successfully used to introduce foreign genes into the chick CAM [67], the major advantage of gene transfer using naked DNA vs. viral vectors are the lack of toxicity and technical simplicity. Also, the use of genes encoding these proteins and a constitutive expression of the growth factor through gene transfer can overcome the inherent instability of pure angiogenic proteins. Our introduced direct gene transfer model establishes a powerful approach for studying different stages of angiogenesis as well as vasculogenesis during the embryonic development of chick embryo (Figure 8).

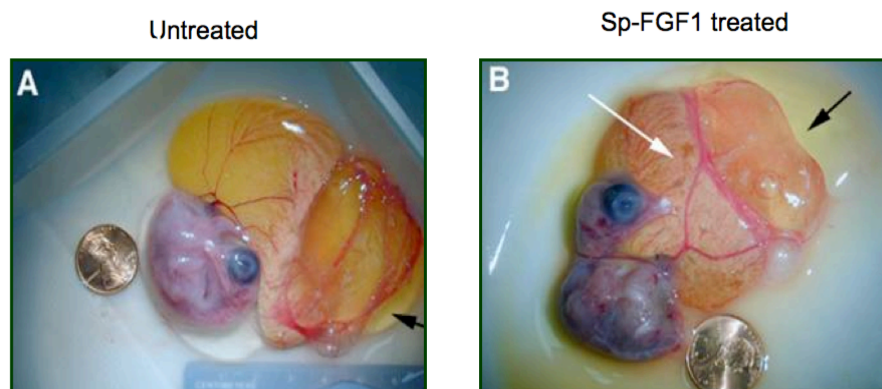

Figure 8. A global view of day 13 CAM in direct gene transfer. A: untreated CAM. B: sp-FGF1-transfected CAM. White arrow indicates vascularization at CAM locations distant to the DNA transfection site. Black arrow point to approximate positions of the airsacs where plasmid DNAs were introduced.

PI3K/AKT Signaling Pathway

Phosphoinositide 3-kinase (PI3K) is a major signaling component downstream of growth factor receptor tyrosine kinases (RTKs) [68] (Figure 9). Growth factor RTKs engage the class-Ia PI3K, which is a heterodimer comprised of the p85 regulatory and p110 catalytic subunits [68]. Specific phospho-tyrosine residues on the activated receptor or on associated adaptor proteins bind the Src-homology 2 (SH2) domains of p85 and recruit the enzyme to the membrane. The small GTPase Ras can also recruit and activate PI3K through direct binding to p110. At the membrane, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) at the 3′ position on its inositol ring and converts PIP2 to PIP3. Subsequently, PIP3 recruits other downstream molecules—particularly the serine-threonine kinases Akt and PDK1—via binding to their pleckstrin-homology (PH) domains. At the membrane, Akt is partially activated through phosphorylation at threonine 308 in its activation loop by PDK1. Additional phosphorylation at serine 473 in the C terminus of AKT results in its full activation. AKT in turn regulates a wide range of target proteins that control cell proliferation, survival, growth, and other processes.

The PI3K-Akt signaling pathway regulates many normal cellular processes including cell proliferation, survival, growth, and motility—processes that are critical for angiogenesis. Aberrant activation of the PI3K-Akt pathway has been widely implicated in many cancers. PI3K was first identified as an enzymatic activity associated with the Rous sarcoma pp60 v-src protein and the polyoma middle T antigen that is essential for

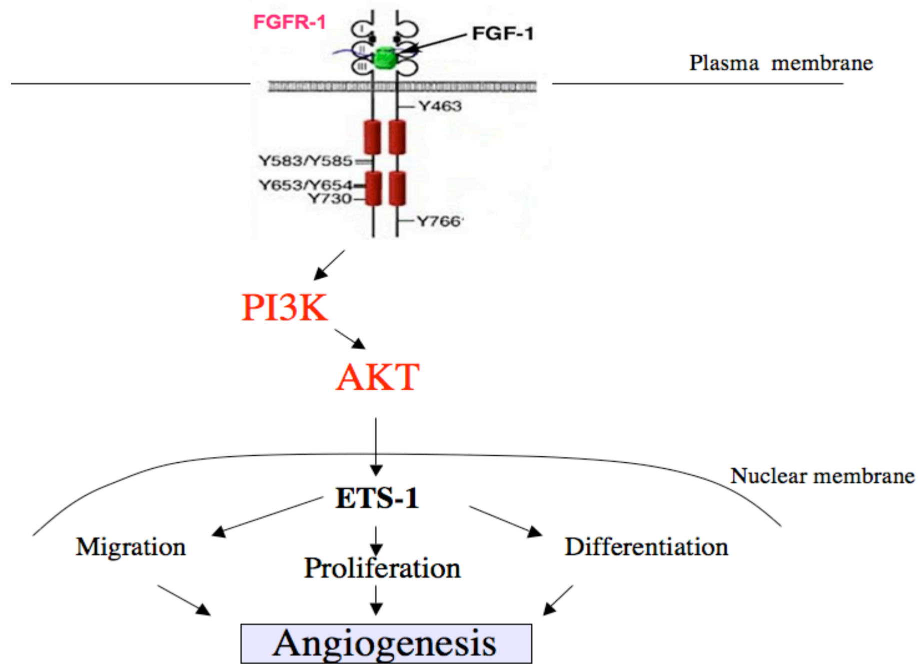

Figure 9. The PI3K/AKT pathway located below the FGFR1 receptor protein.

the transforming activity of these oncogenes [69, 70, 71], and Akt was also found to be a viral oncogene [72]. Gene amplification of p110 occurs in some cases of human ovarian cancer, and amplification of Akt is found in ovarian, breast, and colon cancer. In addition, activating mutations in p85 have been identified in ovarian and colon cancer [73]. Hyperactivation of the PI3K-Akt pathway is therefore often genetically selected during tumorigenesis, and the normal cellular functions regulated by this pathway are recruited to promote proliferation and survival of cancer cells.

Fumagillin (TNP-470)

In 1985, Donald Ingber, while working as a postdoctoral fellow in Folkman's lab, observed a unique phenomenon in endothelial cell cultures contaminated with fungus [74]. While most fungal contaminants induced complete cell detachment and death in endothelial cells, one variety of fungus appeared to induce a gradient of cell retraction and rounding in the adjacent endothelial monolayer [75]. Endothelial cells along the edge of the fungal colony appeared completely spherical, while cells only a few diameters away exhibited a normal morphology. The therapeutic potential of this discovery lay in previous observations that endothelial cells induced to round filed to grow when stimulated by any angiogenic factor [76]. The fungus was identified as *Asperfillus fumigatus fresenius*, which secreted a soluble factor that induced endothelial cell rounding in culture. A collaboration between Takeda Chemical Industries Limited, Japan, and Folkman's lab led to isolation of the active compound secreted by the fungus,

which was identified as fumagillin (Figure 10), a known antibiotic that had previously been used to treat amebiasis in humans [77].

Purified fumagillin was shown to completely inhibit endothelial cell proliferation in the presence of saturating levels of basic fibroblast growth factor (half maximal inhibition of human umbilical vein endothelial cells at 0.5 ng/mL) [78]. Fumagillin also suppressed tumor-induced neovascularization in the mouse dorsal air sac model, but its effectiveness as an antitumor agent was limited by severe weight loss in mice (>15% of the starting weight). This led to the search for fumagillin analogues that would retain the potent antiangiogenic activity of fumagillin without its side effects. Alkaline hydrolysis of fumagillin yielded fumagillol, from which over a hundred derivatives were synthesized and tested. Among these analogues, a new subset of angiostatic antibiotics called angioinhibins was identified; one of the most potent of these was O-(chloroacetylcarbamoy) fumagillol, or AGM-1470 (later termed TNP-470) (Figure 11).

Because of evidence of antitumor activity coupled with a favorable toxicity profile in animal experiments, TNP-470 was chosen for clinical development as an anticancer agent.

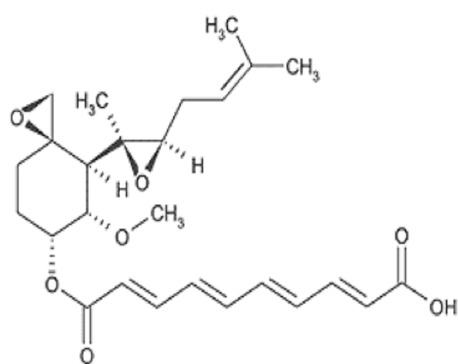

Figure 10. Structure of fumagillin.

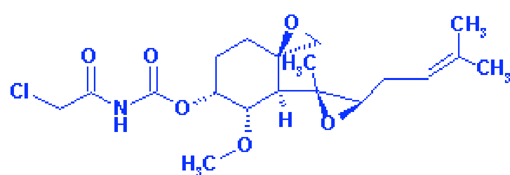

Figure 11. Structure of TNP-470.

Research Aim

Tumor metastasis is dependent upon angiogenesis and large efforts have been made to develop specific antiangiogenic drugs where the objective is to starve and thereby shrink tumor mass cells by eliminating their blood supplies. These efforts have resulted in the discovery of a number of highly profile compounds such as fumagillin (TNP-470), endostatin, and angiostatin to name a few. We have been studying the molecular mechanisms regulating FGF1-induced angiogenesis and have been interested in understanding how fumagillin inhibits FGF1-induced angiogenic activity. Specifically, we are investigating the antiangiogenic activity of the fumagillin on FGF1-stimulated angiogenesis in the chicken CAM. Using a cDNA encoding a secreted form of FGF1 (sp-FGF1) that had been previously shown to stimulate an exaggerated angiogenic response through activating the PI3K/AKT signaling cascade, we sought to investigate whether the disruption of the FGF1/FGFR1/PI3K/AKT signaling pathway was responsible for the antiangiogenic mechanism of fumagillin.

CHAPTER II

FUMAGILLIN REDUCES VESSEL NUMBER AND mRNA EXPRESSION LEVELS OF PI3K AND AKT-1 GENES BUT NOT THAT OF FGFR1*

Introduction

Despite the known role of FGF1 as an inducer of angiogenesis, its signaling through the PI3K/AKT signaling pathway has not been well examined as a potential target for therapeutic angiogenesis. Knowing that the FGF1 receptor is a member of the receptor tyrosine kinase (RTK) family, we are focusing on identifying the potential pathways through which FGF1 could exert its effects. Of the known RTK downstream signaling pathways, PI3K is recognized as a key intermediate molecular signaling pathway in stimulating physiological and pathological angiogenesis via a wide range of angiogenic growth factors including vascular endothelial growth factor (VEGF), epidermal growth factor, and interleukin-8 [79]. In contrast, little is known about the importance of the PI3K signaling pathway in mediating FGF1-triggered angiogenesis.

Fumagillin's antiangiogenic effect maybe closely linked to inhibiting signaling molecules within the PI3K signaling pathway although several published reports have

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shown that it interferes with endothelial cell proliferation by inhibiting cell cycle progression [80]. Others have demonstrated its inhibitory effects occur via inhibition of methionine aminopeptidase 2 (MetAP-2), a cytosolic enzyme involved in maturation of nascent proteins inside the cell by acting on their amino-terminal methionine [81, 82]. We believe that the broad nature of substrates for MetAP-2 –virtually any nascent protein in a cell- argues for the existence of a more specific target molecule/pathway for the antiangiogenic effects of fumagillin.

To determine the effect of fumagillin on blood vessels and to determine the PI3K signaling pathway molecules involved, we've performed experiments utilizing our angiogenesis assay with the CAMs. By direct gene transfection using the sp-FGF1 construct and the direct application of recombinant FGF1 protein on the developing CAM vasculature, we sought to investigate the antiangioenic effects of fumagillin.

Materials and Methods

Chicken embryos for Angiogenesis assay

Single-comb-white-Leghorn fertilized chicken eggs (Poultry Sciences, Texas A&M University, College Station, Texas) were incubated at 37°C under an ambient atmosphere with constant humidity for 8 days. A rotary tool was used to create a circular opening of 6-8 mm in diameter in the shell over the air sac to expose the CAM.

Direct gene transfer of sp-FGF1 with and without fumagillin

A 50 μL mixture containing 10 μL (10 μg) of the sp-FGF1 expression plasmid or empty vector pMEXneo (for control) in methanol solvent; 10 μL (10μg) of fumagillin

(A.G. Scientific, Inc., San Diego, CA) in methanol solvent, 20 μL of Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies, Carlsbad, CA); and 10 μL of sterile PBS solution was pipetted onto the exposed CAM. The opening was sealed with parafilm and incubated at 37° C under constant humidity. CAMs were harvested 5 days post-transfection and digital images were prepared. For blood vessel counts, a 'point counting' method was used [83]. After digital images of the CAMs were produced, each group was transferred to a 1.5 ml microcentrifuge tube and stored at –20°C for further analysis.

For those experiments where rFGF1 was substituted for sp-FGF1 gene transfection, three doses of rFGF1 (50 ng at each time point/CAM; Biosource International Inc.) were added directly to each CAM. Time points were days 8, 10, and 12.

CAM vessel count

CAMs were transfected with 10 μg of sp-FGF1 cDNA construct or 10 μg of sp-FGF1 construct in the presence of 10 μg fumagillin. All samples were incubated for five days at 37°C under ambient atmosphere. Control samples were either un-transfected or transfected with the empty vector pMEX neo. Eggs were sacrificed 5 days post-treatment and the CAM tissues removed to collect digital images (Figure 12) and to count vessels.

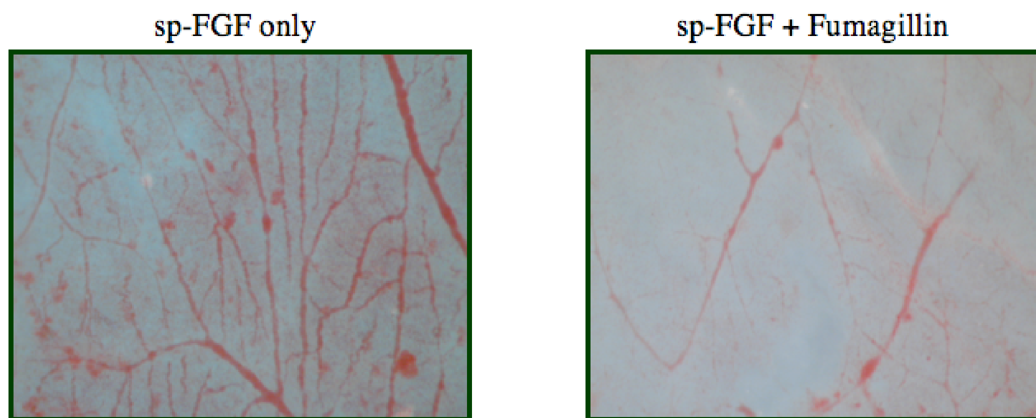

Figure 12. Images of chick vasculature pre and post treatment with fumagillin.

For those experiments where rFGF1 was substituted for sp-FGF1 gene transfection, CAMs were applied with 50 ng/CAM rFGF1 plus 10 μg/CAM fumagillin and incubated for 5 days at 37°C under ambient atmosphere. Starting on day 5, eggs were sacrificed and the CAM tissues removed to collect digital images and to count the vessels.

CAM RNA extraction

For RNA extraction from CAM tissue, we followed the protocol for Animal Tissue Samples included with the Totally RNA kit (Ambion, Inc., Austin, TX), a modified guanidinium thiocyanate-based method.

Real-Time PCR

For reverse transcription, 1 μg of total CAM RNA was incubated with 0.5 μg of random primers and 10 mM each dATP, dGTP, dCTP, dTTP in the presence of M-MLV RT, following the manufacturer's protocol (Invitrogen Life Technologies). For real-time PCR, each well contained a 50 μl mixture consisting of 3 μl of the CAM cDNA, 5 μl each of 300 nM primers specific to PI3K (forward: 5'-GGAATGAATGGCTGT CGTATGAC-3' and reverse: 5'CCAATGGACAGTGCTCCTCT-TTA-3'), 25 μl Sybr Green 1 Mix (Eurogentec North America Inc., Philadelphia, PA), and 12 μl dH_2O. Background controls substituted dH_2O for CAM cDNA. Baseline controls used chicken β-actin specific primers (forward: 5'-CTGATGGTCAGGTCATCACCATT-3' and reverse: 5'-TACCCAAGAAAGATGGCTGG-AA-3') in substitution for PI3K, AKT-1 (forward: 5'-AAGGAAGGATGGCTCCACAAA-3' and reverse: 5'-CGTTCCTTGTAG

CCAATGAATGT-3'), and FGFR1 primers (forward: 5'-GAGACCACCTACTT CTCCGTCAAC-3' and reverse: 5'-GGGATAGGT-CCAGTAAGGAGCTACA-3'). Differences in mRNA levels were measured between sp-FGF1 with and without fumagillin treatment by quantitative amplification using ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative quantification of gene expression was done using the Comparative Ct Method [84].

Results and Discussion

Fumagillin inhibits vascularization in sp-FGF-1 CAMS

Based on our initial dose response curve study (n = 3 eggs per drug concentration), an ideal dose of 10 μg fumagillin was determined to inhibit sp-FGF1-mediated angiogenesis without toxicity in CAMs (data not shown). The degree of angiogenesis was measured between sp-FGF1 (10 μg) and sp-FGF1 plus fumagillin (10 μg) treated CAMs (Figure 13). Based on these studies, we observed a 30% reduction in angiogenesis in sp-FGF1 cDNA transfected CAMs five days after treatment with fumagillin.

Fumagillin alters mRNA levels of PI3K in sp-FGF1 CAMs

We next sought to evaluate fumagillin's effect on the PI3K pathway due to our recent data supporting the importance of this pathway in FGF1-stimulated angiogenesis in chicken CAM. To determine whether fumagillin influenced its antiangiogenic activity via the PI3K signaling pathway, we assessed alterations in mRNA levels of p85α, the

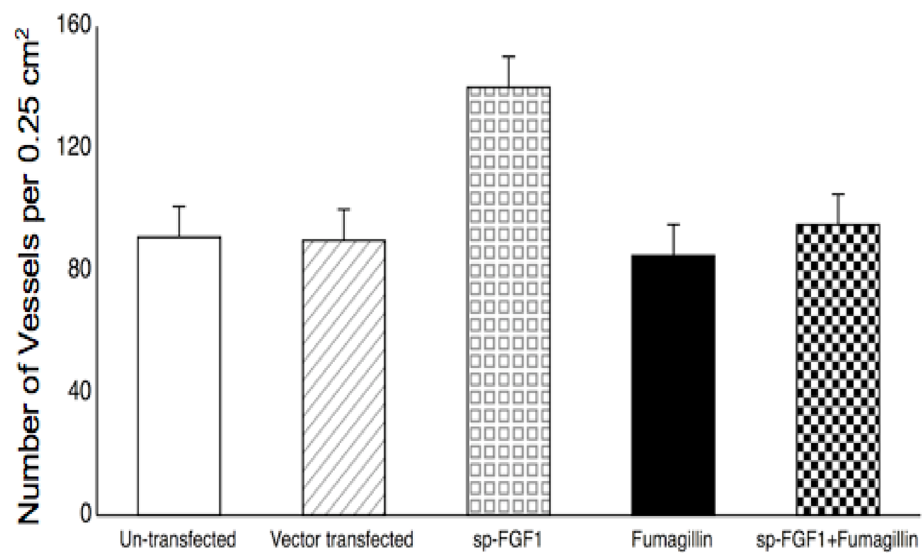

Figure 13. Number of CAM vessels counted per 0.25 cm^2 in day 5 post gene transfection. Eggs were sacrificed 5 days post-treatment and the CAM tissues removed to collect digital images and to count vessels. (n=5 eggs per group, *p<0.05, Student t-test for unpaired samples).

regulatory subunit of PI3K Class IA. Class IA PI3K is known to mediate downstream signaling of the activated receptor tyrosine kinases [85]. Using RT-realtime PCR, we showed over two-fold increase in PI3K p85α mRNA level in sp-FGF1 cDNA transfected CAMs at day 5 post-transfection (Figure 14). Treatment with fumagillin inhibits sp-FGF1-stimulated p85α mRNA synthesis ($p < 0.05$).

Fumagillin alters mRNA levels of AKT-1 in sp-FGF1 CAMs

Since it has been documented that PI3K often partners with AKT-1 in signaling cascades [86], we assessed whether fumagillin treatment alters mRNA synthesis of AKT-1 in our chicken CAM model of angiogenesis. We chose to focus on day 5 CAMs only since we had established significant reductions in both vessel numbers (Figure 14) and PI3K mRNA synthesis in response to fumagillin treatment for this time point. Our RT-realtime PCR evaluation of AKT-1 revealed a statistically significant decrease (>2 fold) in AKT-1 mRNA level in sp-FGF1 plus fumagillin compared to sp-FGF1 only treated CAMs (Figure 11).

Fumagillin does not alter mRNA level of FGF1 receptor in sp-FGF1 CAMs

Since FGF1 exerts its biological effects through its high affinity receptor FGFR1, and because FGFR1 is a likely target for fumagillin binding and antagonism, we evaluated the effect of fumagillin on FGFR1 mRNA levels. RT-realtime PCR was employed to assess the FGFR1 mRNA levels. We observed no significant differences when comparing the level of FGFR1 mRNA expression between sp-FGF1-treated and sp-FGF1+fumagillin treated CAMs (Figure 14). These data show that fumagillin doesn't

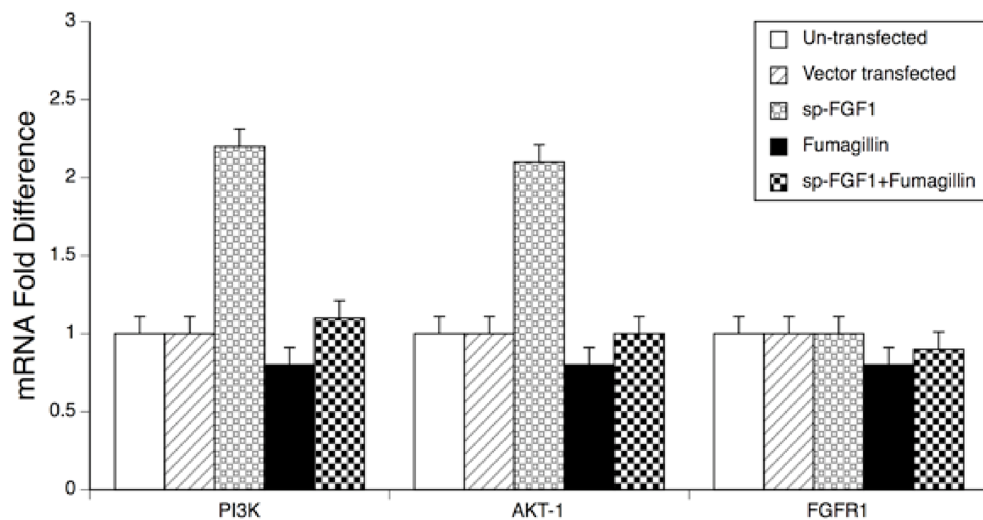

Figure 14. Quantatative PCR of mRNA levels in day 5 CAMs. According to this data, sp-FGF1 alone-treated samples exhibit a two-fold increase in mRNA expression levels of PI3K and AKT-1. However, FGFR1 mRNA expression level remains the same in tested conditions. Chicken β-actin served as internal control. Experiments were performed in triplicate and at least two independent experiments were performed. (*$p<0.05$, Student t-test for unpaired samples).

downregulate the FGFR1 gene expression when suppressing angiogenesis. One interpretation of this observation is that fumagillin might have the ability to bind the FGFR1 protein and in the process downregulate the downstream PI3K/AKT signaling required for angiogenesis in the CAM.

To confirm that irrespective of the delivery method of FGF1 into the chick CAM and that fumagillin inhibits angiogenesis via the FGFR1/PI3K/AKT signaling pathway, we assessed alterations in mRNA levels of PI3K p85α, AKT-1, and FGFR1 in CAM tissues in response to rFGF1 treatment.

Fumagillin inhibits vascularization in rFGF1 CAMS

We substituted sp-FGF1 gene transfection in our CAM assay with pure recombinant FGF1 protein (rFGF1) in order to obtain a better estimation of: 1) the concentration of FGF1 protein required to stimulate angiogenesis, 2) fumagillin's antiangiogenic effect via pure FGF1-stimulated angiogenesis. Similar to the previously described experiment with sp-FGF gene transfection, we observed a reduction in CAM angiogenesis albeit to a lesser extent (Figure 15). This may be due to the inherent instability of pure angiogenic compounds. The data demonstrate that fumagillin exerts its antiangiogenic activity on FGF1-stimulated angiogenesis regardless of whether FGF1 is provided as a transgene or pure protein.

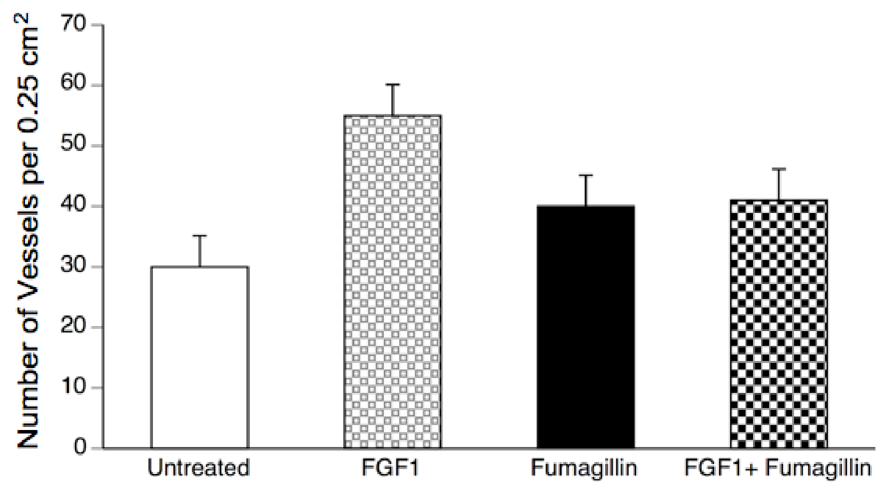

Figure 15. Number of CAM vessels counted per 0.25 cm^2 in day 5 post recombinant FGF1 application. Starting on day 5, eggs were sacrificed and the CAM tissues removed to collect digital images and to count the vessels. (n=5 per group, *p<0.05, Student t-test for unpaired samples).

Fumagillin alters mRNA levels of PI3K and AKT-1 but not FGFR1 in rFGF1 CAMs

We detected similar patterns of gene expression for these molecules when comparing to sp-FGF1-treated CAMs. In other words, there is a statistically significant decrease in PI3K (two-fold) and AKT-1 (three-fold) but not FGFR1 mRNA levels in rFGF1 protein plus fumagillin compared to rFGF1 alone treatments using RT-realtime PCR (Figure 16). Taken together, these data suggest: i) a sustained supply of FGF1 is required to stimulate angiogenesis as shown by the sp-FGF1 gene transfection approach which resulted in constitutive generation of the FGF1 protein; and also the application of pure rFGF1 protein in three deliveries that span the course of the study, ii) the effect of fumagillin on the FGFR1/PI3K/AKT pathway is long-lasting and can be detected at least 5 days following exposure.

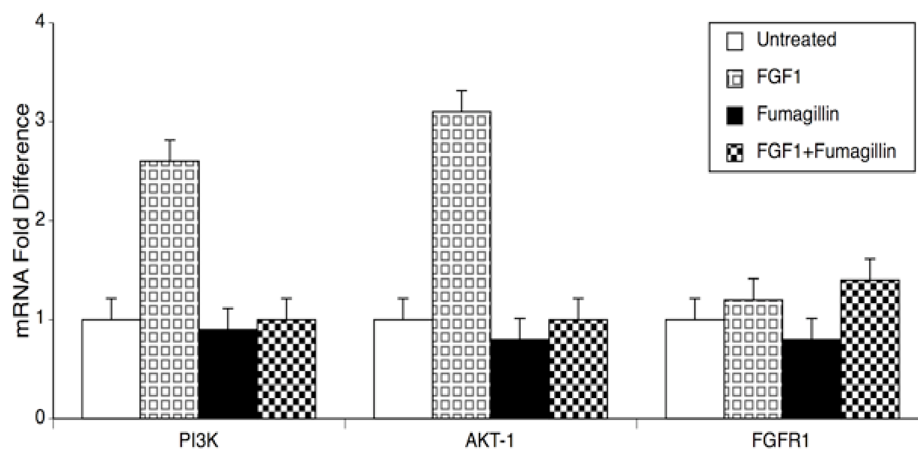

Figure 16. RT-real-time PCR of rFGF1-treated CAMs show elevated mRNA levels of PI3K, AKT-1, but not FGFR1. The evaluated mRNA levels were normalized to actin mRNA levels in each condition. Experiments were performed in triplicates and at least two independent experiments were performed. (*$p<0.05$, Student t-test for unpaired samples).

CHAPTER III

FUMAGILLIN INHIBITS PI3K ACTIVITY BY REDUCING AKT-1 PHOSPHORYLATION*

Introduction

Currently, much is known regarding the PI3K/AKT pathway and its functions in various forms of solid metastatic cancers such as breast and colorectal cancer [87]. Only recently has the pathway received attention for its role in the maintenance and development of blood vessels [88]. To evalulate the role of PI3K signaling in sp-FGF1-induced angiogenesis, we analyzed mRNA expression levels and protein activity through its immediate downstream target, AKT-1. The AKT signaling pathway has been implicated in regulation of cell survival/anti-apoptosis and proliferation in response to cytokines and cell adhesion molecules [89]. The membrane-associated AKT undergoes activation after undergoing phosphorylations on specific serine and threonine residues. Activated AKT dissociates from the membrane and continues the signaling processes initiated by extra cellular growth factor(s) through phosphorylation of the downstream cytosolic substrates [90]. Among the multiple signaling pathways downstream of AKT, cascades leading to angiogenesis are of major importance [91].

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To elucidate the effects of the PI3K signaling pathway in angiogenesis, we directly examined the activity of PI3K through the presence of AKT-1 phosphorylation. This was made necessary due to the lack of commercial antibodies that specifically detect PI3K activity. Since AKT-1 is a primary downstream target of PI3K in many important cellular pathways [92, 93], we focused our activity study on AKT-1, of which specific commercial antiphospho-AKT-1 antibodies for detecting its active form are available.

Since our sp-FGF1 gene transfection model is designed to constitutively synthesize and release recombinant FGF1 protein in the CAM, we decided to examine AKT-1 activity at day 5 after transfection due to the high degree of vascularization observed day 5 sp-FGF1 versus day 5 pMEX CAMs.

Materials and Methods

CAM tissue protein extraction

CAMs were thawed at room temperature and treated with 60 µL of Lysis buffer (50 mM Tris HCl pH 7.5, 0.2% Tx-100, 10 mM $CaCl_2$), 10 µl Aprotinin, and 10 µl of 100 mM PMSF. Each sample was vortexed for about 10 seconds and kept on ice for 20 minutes. The samples were then centrifuged at 13,000x g at 4°C for 10 minutes. The supernatant was then transferred to a fresh tube and the protein concentration determined using the BCA Protein Assay Kit (Pierce Inc., Rockford, IL).

Western blot analysis of phospho-AKT-1 and total AKT-1

CAM protein was extracted from sp-FGF1, sp-FGF1 with methanol, pMEXneo vector, pMEXneo vector with methanol, sp-FGF1 with fumagillin, pMEXneo vector with fumagillin and fumagillin only samples. A volume containing 40 μg of protein from each sample was subjected to 10% SDS-PAGE and run at 200 volts for approximately 45 minutes. The gel was then transferred to a nitrocellulose membrane (BioRad, Hercules, CA) and blocked in 5% BSA in TBST solution (10 mM Tris. HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 40 minutes with gentle shaking at room temperature. After blocking, the membrane was probed with a 1:1000 dilution of the primary antibody (phospho-AKT Ser 473, catalog #9271S; Cell Signaling Technology, Inc., Beverly, MA) overnight with shaking at 4°C. The membrane was washed 3 times for 5 minutes each in TBST to remove non-specific primary antibody binding and then probed with a 1:25,000 dilution of HRP-conjugated donkey anti-rabbit IgG secondary antibody (Pierce) made in TBST containing 5% BSA for one hour at room temperature. The membrane was washed three times with TBST for 10 minutes and processed for development using SuperSignal West Pico Chemiluminescent kit (Pierce) according to the manufacturer's recommendations. The membrane was then exposed to film, the luminescent image scanned into a computer, and analyzed for optical density using the Multianalyst software program (BioRad, Philadelphia, PA). After the signals on the membranes were quantified, they were stripped of the phospho-AKT antibody by incubating for 30 minutes at 70°C with shaking in a stripping buffer consisting of 2% (w/v) SDS, 62.5 mM Tris.HCl, pH 6.8, 100 mM β-mercaptoethanol. The membrane was washed in TBST

wash buffer three times for 5 minutes each and re-probed with total AKT-1 antibody (Catalog #9272; Cell Signaling Technology, Inc.) for loading control.

Results and Discussion

Fumagillin alters AKT-1 protein activity in sp-FGF1 treated CAMs

Our data showed that sp-FGF1-stimulated AKT-1 phosphorylation decreased 3-fold compared to sp-FGF1 only treated CAMs (Figure 17). These results suggest that fumagillin exerts its antiangiogenic activity by downregulating FGF-triggered PI3K/AKT signal transduction.

Previous work from our lab showed upregulation of both PI3K and AKT mRNA expression levels in sp-FGF1-treated versus control vector-transfected CAMs. Furthermore, by blocking PI3K phosphorylation using a specific inhibitor LY294002, we found that downstream phosphorylation of AKT-1 was inhibited. More importantly, the blockade of the PI3K pathway via LY294002 in sp-FGF1-transfected CAMs significantly inhibited angiogenesis. These results further elucidate the molecular mechanism of the sp-FGF1-signaling pathway and it underscores the importance of PI3K signaling in FGF1-stumulated angiogenesis *in vivo*.

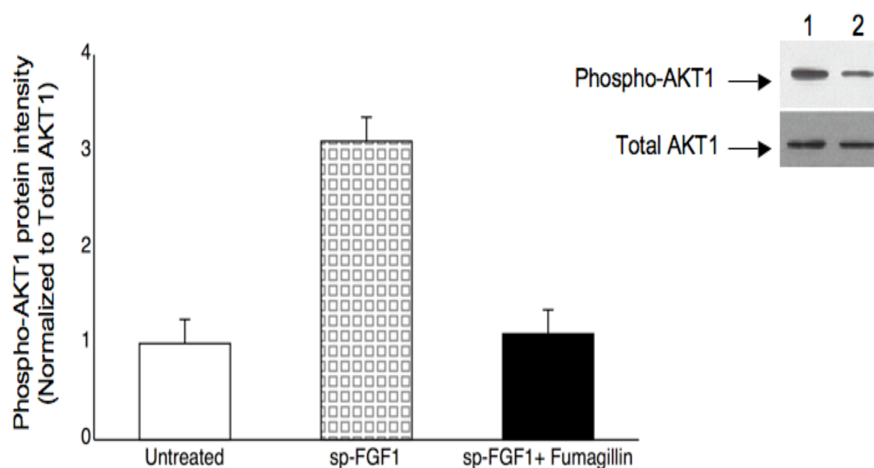

Figure 17. Western blot protein analysis using a phospho-specific AKT-1 antibody to detect PI3K phosphorylated AKT-1 in sp-FGF1 and sp-FGF1 plus fumagillin treated CAMs. There is a significant decrease in the amount of phospho-AKT-1 in sp-FGF1 plus fumagillin (Lane 2 top) versus sp-FGF1 only (Lane 1 top) samples. Total AKT-1 antibody was used to quantify total AKT-1 protein in each sample (n=3 egg for each sample). Three independent experiments were performed, $*p < 0.05$, Student t-test for unpaired samples.

CHAPTER IV

FUMAGILLIN BINDS TO THE CYTOSOLIC DOMAIN OF RECMOMBINANT FGFR1 IN A CELL-FREE SYSTEM*

Introduction

TNP-470, an analogue of fumagillin, is in a number of phase II clinical trials for the treatment of solid cancers [94]. The current available data indicate that fumagillin exerts its antiangiogenic activity mainly by inhibiting the biological activity of methionine aminopeptidase (MetAP-2), a cytosolic enzyme that indiscriminately modifies newly synthesized cellular proteins by removal of the amino acid methionine positioned at their amino-termini [95]. However, we believe fumagillin has more than one target within the cell in which it can bind to and exert its antiangiogenic effect. Given the apparent role of the FGF receptor in fumagillin action, it is important to establish whether fumagillin was acting directly or indirectly to alter FGF receptor-mediated responses. To determine if fumagillin binds to FGFR1, we designed a cell-free system where we assessed the ability of a pure recombinant fusion protein consisting of the FGFR1 intracellular domain encompassing amino acids 456-765 linked to a GST tag to bind to biotin-labeled fumagillin. The biotinylated fumagillin is then incubated with

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FGFR1 to test whether a complex of fumagillin-FGFR1 can be extracted from solution using avidin cross-linked to agarose beads. The bead-bound materials are subjected to Western blot analysis using antibody specific to the GST moiety.

Although one cannot predict the exact location on FGFR1 where fumagillin might bind to in order to exert its antiangiogenic action, we chose to assess fumagillin's binding ability to the cytoplasmic domain of the FGFR1 protein. This decision was based on two reported modes of action for fumagillin, including the targeting of the MetAP-2 enzyme and a cell cycle control protein, both of which are cytosolic proteins [80, 95].

Materials and Methods

Fumagillin biotinylation

1.0 mg of fumagillin (Catalog # F-1028, A.G. Scientific, Inc., San Diego, CA) was first dissolved in 500 μl of methanol as recommended by the manufacturer. Separately, biotin (EZ-Link Biotin-PEO-Amine, Pierce Inc.) was dissolved in MES buffer (2-N-morpholino ethanesulfonic acid, pH 5.0) at a concentration of 50 mM. Equal volumes of the biotin solution was then added to the diluted fumagillin solution and mixed. 50 μl of EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride], Pierce Inc.) in MES buffer at 20 mg/ml was then added to the solution and mixed. The resulting mixture was incubated for 2 hours at room temperature with stirring. Non-reacted biotinylation reagent and EDC by-product from the biotinylated fumagillin was removed by dialysis overnight at 4°C in 10 mM Tris buffer pH 7.

Immunoprecipitation of biotin-fumagillin with FGF1 receptor protein

In a microcentrifuge tube, 2.5 μg recombinant human FGFR1 protein containing amino acids 456-765 (Catalog #14-582, Upstate Cell Signaling Solutions Inc., Lake Placid, NY) was solubilized in PBS buffer (0.1 M phosphate, 0.15 M NaCl; pH 7.0). Dialyzed fumagillin-biotin was added and the mixture incubated overnight at 4°C with shaking. Re-suspended beaded agarose cross-linked with avidin (Immobilized NeutrAvidin, Pierce Inc.) was combined with the protein/biotinylated fumagillin mixture. Sample was incubated with mixing for 1 hour at room temperature (RT). After incubation, sample was washed 4 times with 0.5 ml of PBS buffer and centrifuged for 1-2 minutes at ~2,500x g at RT. Supernatant was removed from the final wash and the sample boiled in SDS-PAGE sample buffer prior to electrophoresis.

Results and Discussion

Fumagillin exhibits binding to FGF1 receptor using a cell-free system

A higher molecular weight complex is observed (Figure 18) when a biotinylated fumagillin substrate was incubated with pure recombinant FGFR1 protein (1/12 ratio of bound protein to total protein in sample, as determined by density analysis using the Multianalyst software program). Precipitation of the bound complex using avidin-conjugated agarose beads was followed by Western blotting using antibody to the GST tag linked to the FGFR1 protein (Lane 2). Pure recombinant FGFR1/GST fusion protein was Western blotted to show the shift in molecular weight of fumagillin complex and serves as a positive Western blot control (Lane 1). Specificity of fumagillin for FGFR1

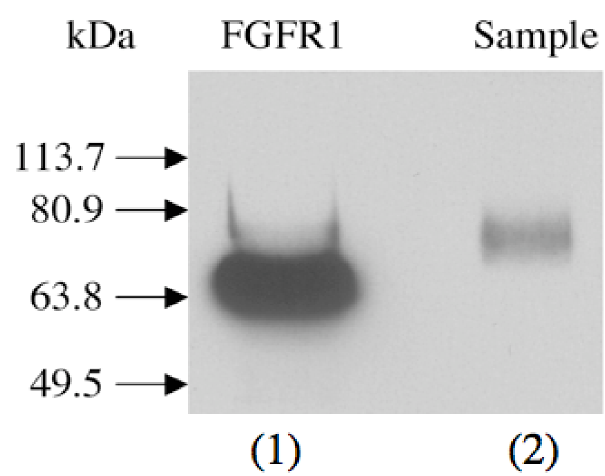

Figure 18. ***In vitro*** **binding of biotinylated fumagillin to a human recombinant FGFR1 protein**.

was confirmed as biotin-labeled fumagillin did not exhibit binding to pure recombinant proteins PI3K, AKT-1, or GST when tested in a similar immuno-precipitation/Western assay (data not shown). The gel is representative of three independent experiments.

Precipitation of the bound protein using avidin-conjugated agarose beads followed by Western blotting to detect the GST tag, revealed a protein of ~72 kDA (Figure 18). The increase in size can be accounted for by the aggregate molecular weights of fumagillin, biotin, and the cross-linking agent EDC. Furthermore, the specificity of fumagillin for FGFR1 was substantiated as the biotinylated fumagillin failed to bind and co-immunoprecipitate with pure recombinant proteins PI3K, AKT-1, and GST (data not shown). These data support the hypothesis that fumagillin can bind to the intracellular domain of the FGFR1 protein.

Conclusion

Our finding shows the presence of a higher molecular complex that has been shifted relative to the control sample containing purified FGFR1/GST fusion protein (Figure 15). We believe that this higher molecular weight complex is the result of the binding of the biotin-fumagillin moiety to the FGFR1 protein. The prediction from our study is that fumagillin binds to the cytoplasmic domain of the FGF1 receptor protein thereby blocking the phosphorylation of the tyrosine residues, positioned on the FGFR1 within the kinase domain, that are necessary to initiate the downstream signaling events. The purified, recombinant human FGFR1/GST fusion protein employed in this study contained only amino acid residues 456-765 which is located within the C-terminal

domain of the receptor. Within this domain are the five key tyrosine residues 583, 585, 653, 654, and 730 that are required to initiate a signaling event when phosphorylated. Activation of the PI3K protein requires a phosophorylated tyrosine residue on the receptor which serves as a docking site for the p85 regulatory subunit of PI3K [96]. This then recruits the catalytic subunit of PI3K, p110, to this complex. However, if the site containing the tyrosine residues were blocked, such as by fumagillin, then PI3K binding cannot occur and no signaling event can be initiated. We believe that fumagillin selectively binds to the cytoplasmic domain of the FGFR1 protein and thus "shut off" upstream signaling to the PI3K/AKT signaling pathway. Fumagillin is a relatively small molecule (459 daltons) that can readily diffuse across the cytoplasmic membrane and bind to intra-cellular proteins.

Our data correlates well with our conclusion that by inhibiting early signaling at the cell surface, activation of all downstream intermediates within the signaling cascade will be affected. This explains the down-regulation of mRNA and protein expression levels of PI3K and AKT-1 without affecting the FGFR1 mRNA expression.

CHAPTER V

FUMAGILLIN INHIBITS PROLIFERATION OF CULTURED ECs AND REDUCES EC mRNA EXPRESSION LEVELS OF PI3K AND AKT-1 BUT NOT FGFR1 GENES*

Introduction

Blood vessels consist of endothelial cells (ECs) that are in direct contact with the blood. The endothelial cells form a monolayer in every single blood vessel in the circulation and are actively involved in various regulatory processes in the body. Endothelial cell activation is the first process to take place in all types of angiogenesis. Cytokines from various sources are released in response to hypoxia or ischemia. The angiogenesis process begins with the degradation of the basement membrane by proteases secreted by activated endothelial cells that will migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space. Then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane. Once the tracks of the blood vessels are established, endothelial cells must form tubes through which blood elements can flow [97].

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CAM tissues treated with fumagillin showed decreased levels of gene expression for PI3K, AKT-1, but not FGFR1. The tissues contain developing chick blood vessels as well as neighboring tissues that are part of the embryo. To confirm that fumagillin acts directly on endothelial cells that form the vessel vasculature, we investigated the growth-inhibitory effect of fumagillin on FGF1-stimulated bovine microvascular ECs grown in culture.

Materials and Methods

Cell culture study

Primary endothelial cells (ECs) isolated from 15 μm diameter post-capillary venules of the bovine heart were grown in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO/BRL; Grand Island, NY) containing high glucose and L-glutamine supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone; Logan, UT), 100 U/ml penicillin G and 100 μg/ml streptomycin (GIBCO/BRL; Grand Island, NY—Gift from Drs. H. Granger and C. Meininger of Texas A&M University Health Science Center). The authenticity of ECs was demonstrated by their unique ability to uptake acetylated low density lipoprotein (LDL) (data not shown). Approximately $1x10^4$ ECs were seeded per well of a 6-well tissue culture plate in triplicate in 10% FBS/DMEM and incubated at 37°C with 5% CO_2/95% air overnight. Next day, the medium was removed from wells and cells washed with PBS. Medium was replaced with fresh 0.5% FBS/DMEM and incubated for 48h. Following this incubation, the cells were treated with rFGF1 protein alone (10 ng/ml), fumagillin alone (10 μg/ml), or rFGF1 plus fumagillin. For cell number

determinations, cells were harvested by trypsinization at different time points and counted using a hemacytometer.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and the Student t-test. All results are expressed as mean+/-SD. $P<0.05$ was accepted as significant.

Results and Discussion

Fumagillin inhibits FGF1-stimulated growth of cultured endothelial cells (ECs)

A combination of fumagillin plus rFGF1 or fumagillin alone treatment exhibited approximately 5 to 10 fold decrease in cell number compared to rFGF1 alone-stimulated ECs at 3 and 7 days post-treatment, respectively (Figure 19). These data demonstrate that fumagillin directly targets ECs and inhibit cell proliferation in both FGF1-dependent and FGF1-independent fashion.

Untreated ECs proliferated in the absence of FGF1 stimulation although not to the extent as FGF1-treated samples. This observation may be due to the paracrine effect of fibroblast growth factors being released by neighboring cells to stimulate EC proliferation. Therefore, even in the absence of pure FGF1 addition into the cell culture, growing ECs naturally secrete fibroblast growth factors to stimulate the growth of surrounding cells.

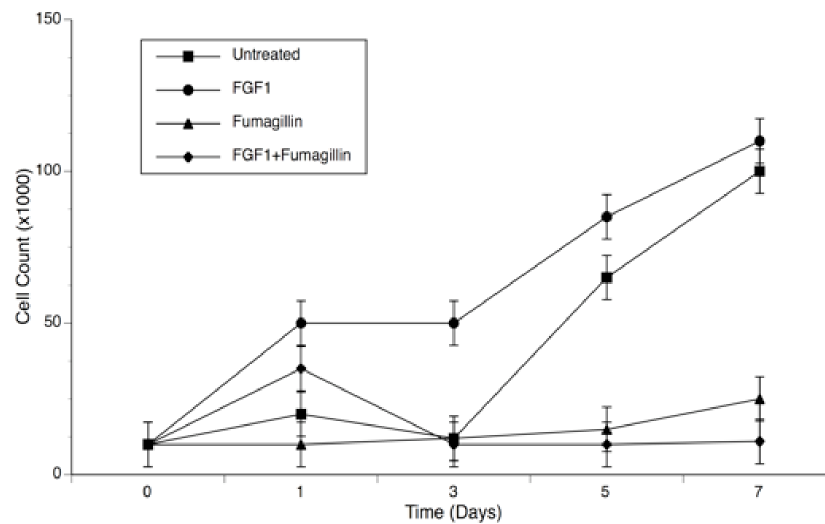

Figure 19. Inhibition of cultured endothelial cell proliferation in response to fumagillin treatment. At the designated time points, cells were trypsinized and counted. Results are shown as mean +/- SD for triplicate determinations. At least four independent experiments were performed.

Our findings show that fumagillin-alone treatment of ECs also demonstrated potential anti-proliferative effects. This observation does not rule out the possibility that fumagillin acts through the FGFR1/PI3K/AKT signaling pathway but also implies that fumagillin's inhibitory effect can be mediated by unrelated signaling cascades as proposed by others [98, 99].

Fumagillin alters mRNA levels of PI3K and AKT-1 but not FGFR1 in rFGF1-stimulated cultured ECs

To demonstrate that fumagillin also acts on the FGFR1/PI3K/AKT signaling pathway *in vitro*, we assessed mRNA levels of FGFR1, PI3K, and AKT-1 as before. Similar to our observations *in vivo*, we detected a statistically significant decrease in PI3K (13-fold) and AKT-1 (4-fold) but not FGFR1 mRNA levels in rFGF1 plus fumagillin samples compared to rFGF1 only treated ECs (Figure 20).

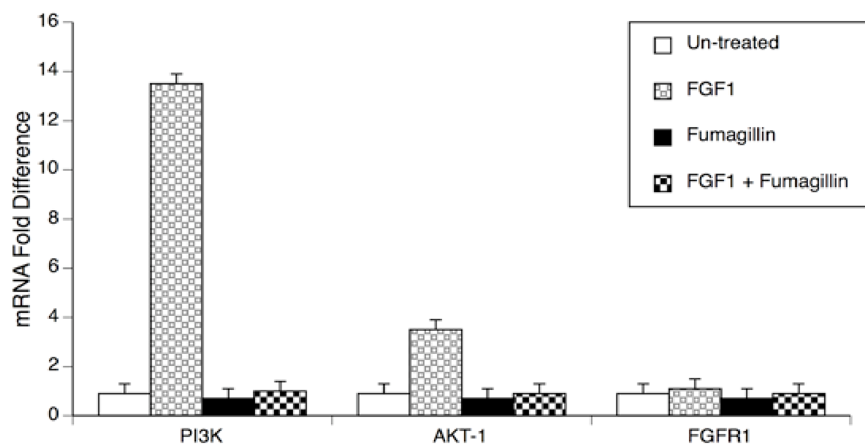

Figure 20. RT-real-time PCR of rFGF1-treated endothelial cells shows significant elevation of mRNA levels of PI3K, AKT-1, but not FGFR1. The evaluated mRNA levels were normalized to actin mRNA levels in each condition. Experiments were performed in triplicates and at least three independent experiments were performed

CHAPTER VI

CONCLUSIONS

Results of the present study reveal several important findings with broad implications in the field of therapeutic angiogenesis: 1) a novel molecular mechanism for the action of fumagillin in inhibition of angiogenesis is elucidated, 2) fumagillin and its less-toxic analogues may serve as excellent specific anticancer drugs where FGF-dependent tumor angiogenesis is the main cause of tumor progression, 3) fumagillin may be an effective anticancer drug for other receptor tyrosine kinase family members with close structural resemblance to FGFR1.

This study is a continuation of our previous efforts in demonstrating the importance of the PI3K/AKT signaling pathway in regulating FGF1-induced angiogenesis *in vivo* [84]. In the current study, we have demonstrated the novel action of fumagillin on the PI3K/AKT pathway leading to inhibition of FGF1-induced angiogenesis *in vivo* and inhibition of endothelial cell proliferation *in vitro*.

We also performed a computational search to determine whether there are other known proteins with strong sequence homology to the cytoplasmic domain of the FGFR1 protein used in our study. The rationale was that these cellular proteins may also serve as potential targets for interaction with fumagillin and perhaps play a role in fumagillin-mediated inhibition of angiogenesis. Remarkably, our search identified a number of proteins –all receptor tyrosine kinases- that demonstrated high sequence identity (> 70%) with a stretch of 20 amino acids ASKKCIHRDLAARNVLVTED located within the

intracellular domain of the FGFR1 protein. These RTKs include VEGFR-2, also known as Flk-1 (78% identity); human KIT protein (83% identity); VEGFR-1 or Flt-1 (73% identity); megakaryocyte-associated tyrosine-protein kinase, also known as MATK (78% identity); c-Src tyrosine kinase (86% identity); tyrosine kinase JAK3 (75% identity); and the LsK protein (73% identity). The results from this search raise two very exciting questions with major implications in the field of therapeutic angiogenesis. First, does fumagillin also have the ability to bind to the cytosolic domain of VEGFR1 & 2 and in the process inhibit VEGF-stimulated angiogenesis? Second, does fumagillin also have the ability to inhibit bone marrow-derived progenitor endothelial cells? The possibility of the latter is based on i) that some of the above protein with high sequence identity to FGFR1 are present in the bone marrow stem and progenitor cells, and ii) a report that fumagillin inhibits hematopoiesis [100].

There has been one published report documenting the binding of fumagillin to low affinity FGF receptors [101]. However, no reports so far have shown fumagillin binding to any of the four high affinity FGF receptors including FGFR1, FGFR2, FGFR3, FGFR4, or any of their splice variants. High affinity FGFR1 through 4 are the products of genes on different chromosomes [102] and serve as the main receptors for FGF-stimulated angiogenesis. We predict that the other three high affinity FGFRs also have the ability to bind to fumagillin since our BLAST search showed that the intracellular domain of FGFR1 used in this study has more than 85% homology to the three high affinity FGFRs.

Besides the PI3K/AKT signaling pathway, several other pathways can also be regulated by phosphorylation of the FGFR's cytoplasmic domain. Fumagillin's binding

to this region can potentially have an effect on the regulation of these downstream pathways. In fact, preliminary data suggest that the JNK pathway (involved in cell apoptosis) is negatively regulated (Figure 21) as a result of fumagillin binding while the ERK pathway (involved in cell growth and differentiation) is not affected. Our proposal is that fumagillin binds to the cytoplasmic domain of the FGF1 receptor and inhibit downstream signaling pathways leading to the inhibition of angiogenesis. We assessed phosphorylation levels of JNK and ERK proteins in the presence and absence of fumagillin.

CAMs treated with sp-FGF1 + fumagillin show downregulation of the phospho-SAPK/JNK protein (lane 2) but not that of the phospho-ERK1/2 protein when compared to samples treated only with the sp-FGF1 transgene. This suggests that the JNK pathway is affected by fumagillin and not the ERK pathway. Here, the same membrane was first probed with phospho-SAPK/JNK antibody (Catalog #9251, Cell Signaling Technology, Inc.) and then stripped and reprobed with phospho-ERK1/2 antibody (Catalog #9106, Cell Signaling Technology). Total SAPK/JNK and total ERK1/2 antibodies showed no changes in protein concentration (data not shown).

It is commonly believed that the inhibition of PI3K activity results in increased JNK activity [103, 104]. However, we believe that the negative regulation of JNK by fumagillin is due to inhibition of FGFR1's cytoplasmic domain. Having said that, a number of reports have confirmed the activation of JNK in response to FGF stimulation [105, 106].

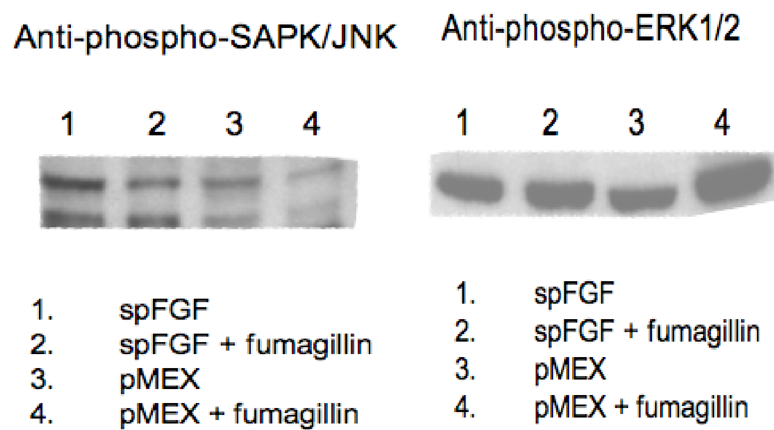

Figure 21. Western blot of anti-phospho-SAPK/JNK vs anti-phospho-ERK1/2.

In summary, we have introduced a new pathway for fumagillin's antiangiogenic function (Figure 22). Our future plan is to test whether fumagillin uses the same FGFR1/PI3K/AKT pathway to inhibit FGF1-induced angiogenesis in mammalian systems of angiogenesis and tumor angiogenesis.

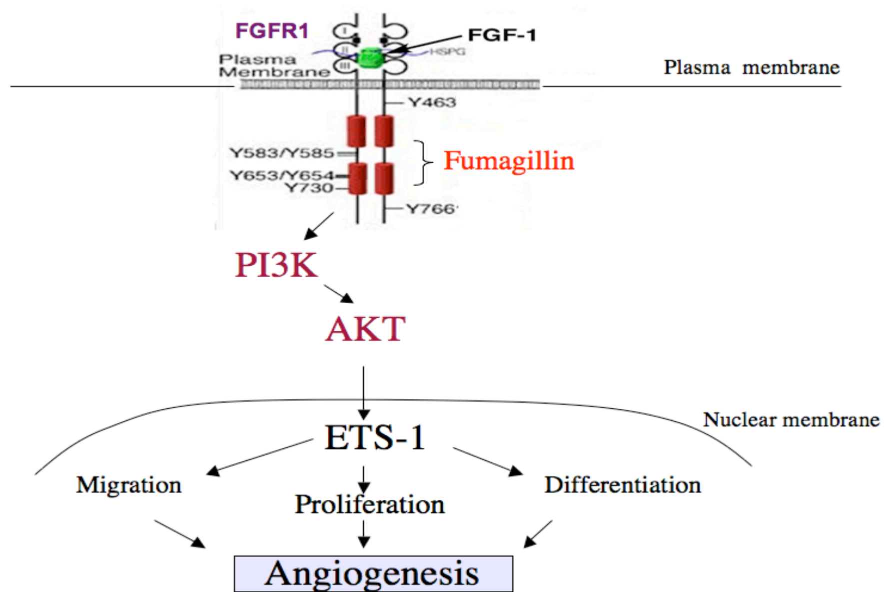

Figure 22. Fumagillin binds to the cytoplasmic domain of the FGF1 receptor protein to inhibit downstream PI3K/AKT signaling pathway.

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