AKT/IKKα/VAV1 SIGNALING IN ENDOTHELIAL CELL SURVIVAL AND ANGIOGENESIS

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

May, 2008

Nashville, Tennessee

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ACKNOWLEDGMENTS

I wish to express my profound gratitude to so many people whose help made this dissertation work possible. First, and foremost I want to thank my mentor Dr. Charles Lin who has guided me from a research assistant through my graduate studies. None of this work would have been possible without his insight and passion for science. Not only is he a good mentor, Charles is simply a good person.

I also thank the members of my committee, Dr. Jin Chen, Dr. Dennis Hallahan, Dr. Ann Richmond and Dr. H. Scott Baldwin for their advice and encouragement.

I'd like to thank numerous people both here at Vanderbilt University that have collaborated and helped this work. The flow cytometry work described in Chapter III of this work was conducted by Cathy Allen and Jim Price of the Veteran's Administration FACS core. Dr. Ambra Pozzi taught me how to isolate primary endothelial cells from the lung. Dr. Dana Brantley-Sieders helped with issues related to the endothelial/tumor cell co-injection. The aortic ring assays were conducted by Dr. Jianhua Huang.

I am truely grateful to the past and present members of the Lin lab. I've been able to watch the lab grow from small lab containing only a PI and tech to a vibrant lab conducting numerous lines of research. Kimberly Boelte has been a close friend colleague. I truly value her friendship and scientific insight. I thank Hanako Kobayashi for her subtle humor, Candice Shaifer and Yongfen Min for their kindness, Angela Boutte for her constant help and John Mackert for his sweet quirkiness. All of these people are great scientists and I am lucky to work with them. I am also grateful to Toshi

Nishishita, Jennifer Veale, Ying Chen, Xiaoyang Bai, Brenda Green-Jarvis, Jianxiong Chen, Wenyu Lin, Masako Kamiyama, Kaori Fukuda, Andreas Rojas, Xiaobo Ren, Jingdong Li and Jianhua Huang. Concepts in this dissertation were improved and focused by discussions with all of these people. I also wish to thank Suneeti Sapatnekar who is truly wise in the way of science and a wonderful friend.

Finally, I am grateful to my family. My parents, Philip and Elaine DeBusk, and my brothers, Marshall and Joe, have always been supportive of me. Karen Caldwell, my partner, has taken this journey with me and given me love, strength, and humor along the way.

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LIST OF ABBREVIATIONS

Ab; antibody

Ang1: angiopoietin 1

AdExTek: adenovirus-mediated expression of extracellular Tie2 domain

AdFlk: adenovirus-mediated expression of extracellular VEGFR2 domain

Akt: AKR mouse thymoma

DAPI: 4',6-diamidino-2-phenylindole

dn-Akt: dominant negative Akt

EGM: Endothelial Growth Media

EMSA: Electromobility shift assay

EMT: Epithelial to Mesenchymal Transition

FACS: Fluorescence Activated Cell Sorting

GFP: Green Fluorescent Protein

HUVEC: Human Umbilical Veinous Endothelial Cells

IKK; IκB Kinase

IκB; Inhibitor of κB

Myr-Akt: myristylated-Akt

NF-κB: Nuclear Factor-kappa B

PI: Propidium Iodide

SDS-PAGE: sodium dodecyl sulfate

siRNA: small interfering RNA

TUNEL: Terminal deoxynucleotidyl Transferase Nick End Labeling

VEGF: Vascular Endothelial Growth Factor

CHAPTER I

INTRODUCTION

Angiogenesis

A functional vascular system is essential for embryonic development, as well as the progression of pathological disorders such as tumor growth and metastasis. Angiogenesis, the formation of blood vessels from preexisting vascular networks, is a multi-step process that includes endothelial cell migration, proliferation and assembly into vascular structures. In addition, endothelial cell survival is essential for the maintenance of proper vascular structure and function *in vivo*. Angiogenesis is regulated by many pro- and anti-angiogenic factors, as well as by interplay between endothelial cells and other tissues in the local environment. Among the interactions that regulate angiogenesis, receptor tyrosine kinases are known to be important mediators.

VEGF and Tie2

Of the molecular mechanisms identified to date, activation of endothelial receptor tyrosine kinases (RTKs) by polypeptide growth factors appears to play a pivotal role in angiogenesis (Yancopoulos et al 2000). Among them, RTKs for two families of angiogenic growth factors, the vascular endothelial growth factor family (VEGF) and the

angiopoietin (Ang) family, are expressed predominantly on vascular endothelial cells, making them attractive targets for both pro- and anti-angiogenic therapy (Yancopoulos et al., 2000). The Ang1/Tie2 and VEGF/VEGF receptor (VEGFR) pathways seem to work in a complementary and coordinated fashion during vascular development, with the VEGFR acting during the early stages of vessel development (Auerbach et al., 1997; Merenmies et al., 1997; Drake et al., 1998). Tie2 has also been shown to be critical to angiogenesis in both embryos and adults. In development, Ang1/Tie2 acts after VEGF/VEGFR signaling. A targeted disruption of Tie2 in mice results in death by E10.5 due to defects in vessel remodeling and recruitment of peri-endothelial support cells (Dumont, 1994; Sato, 1995). Later events such as angiogenic remodeling as well as vessel maturation and endothelial cell survival are promoted by Angiopoietin 1 (Ang1) (Asahara et al., 1998). Also, the Ang1/Tie2 pathway has been shown to be critical to angiogenesis and vascular maintenance in adult tissue (Wong et al., 1997).

Ang 1 is an agonist ligand of Tie2, which induces Tie2 phosphorylation (Davis et al., 1996; Suri et al., 1996). Ang1 regulates endothelial cell survival through a pathway involving the protein kinase, AKR mouse thymoma (Akt) (Kwak et al., 2000; Fujikawa et al., 1999; Papapetropoulos et al., 2000).

While Ang1- and VEGF-induced endothelial cell survival has been shown to act at least partially through Akt (Datta et al. 1999), the relative importance of Akt in both the Ang1/Tie2 and VEGF/VEGFR survival pathways has not been clear. In chapter III, we compare the role of Akt in Ang1- versus VEGF-mediated endothelial cell survival and endothelial sprouting.

Akt

Akt acts as a signaling node for cell survival and cell motility. The protein regulates survival by activating pro-survival factors and inhibiting mediators of apoptosis. For example, active Bad can induce cell death by inhibiting the pro-survival protein Bcl-X_L, but when Bad is phosphorylated by Akt on Ser-136, and possibly on Ser-112, it is inactivated (Datta, 1999). Caspase 9 activates both caspase 3 and 7 which induces apoptosis. Akt has been shown to inactivate caspase-9 by phosphorylating it on Ser-196, thus blocking cell death (Kim and Chung, 2002).

In addition to inhibiting apoptosis, Akt also promotes cell survival by activating pro-survival signaling. An example of this is Akt phosphorylation of forkhead family members FOXO1a, FOXO3a and FOXO4, which are responsible for the inhibition of apoptotic genes such as Bim and FasL (Datta, 1999) (Burgering and Kops, 2002). Most interesting to us are the pro-survival effects of Akt phosphorylation of IKKα on Thr-23. IKKα/NF-κB is critical for cell survival by inducing transcription of the pro-survival genes, Bcl-X_L and c-Myb (Piva et al., 2004). This evidence supports our finding that activation of Akt induces cell survival.

Akt actively induces cell migration in multiple ways such as activation of PAK1 and inhibition of GSK3b. Akt phosphorylates PAK1 on Thr579, which targets the protein to the cell membrane, where it interacts with both Rac1 and Cdc42 and promotes cell motility (Chung et al. 2001). Akt phosphorylates GSK3b on Ser-9, inhibiting its activity. Since, GSK3b inhibits cell survival and migration, inactivation of GSK3b by Akt indirectly promotes both cell survival and motility. GSK3b also inhibits cyclin D and c-myc, both involved in cell proliferation.

In addition, Akt can also regulate angiogenesis though mammalian Target of rapamycin (mTOR). mTOR regulates endothelial cell proliferation and survival via activation of translation regulators S6K1 and 4E-BP1**.** The protein also protects cells against autophagy. This is due, in part, to regulation of eEF-2 activation. mTOR inhibits eEF-2K which serves as an inhibitor of eEF-2. Thus, when mTOR is activated, eEF-2 is active and autophagy is prevented (Easton and Houghton, 2006). Interestingly, mTOR also targets, Akt resulting in a positive feedback look for Akt activation (Tsang, et al., 2007). The mTOR protein can also affect HIF-1a expression and regulation of pro-angiogenic genes such as VEGF (Guertin and Sabatini, 2007).

NF-κB and IKKα

NF-κB is a ubiquitous, heterodimeric transcription factor that is normally sequestered in the cytosol by the IκB proteins. NF-κB regulates cell survival, chemotaxis, and inflammation (Ueda and Richmond, 2006; Hayden and Ghosh, 2008). The IκB family consists of IκBα, IκBβ, IκBε, IκBγ, and Bcl-3. IκBα is the primary IκB responsible for the cytoplasmic retention of NF-κB. Through phosphorylation of IκB on either Ser32 or Ser36 by the IκB kinase complex, the protein is ubiquitinated and targeted for proteosome-mediated degradation. This allows NF-κB nuclear translocation, and subsequent activation of transcription. IκB is phosphorylated by the IKK complex, which consists of either a dimer of two IKKα (also known as IKK-1), two IKKβ (also known as IKK-2), or one IKKα and one IKKβ. This dimer associates with IKKγ, also known as the NF-κB essential modulator (NEMO). It has been reported that Akt mediates IKKα

phosphorylation at threonine 23, and the subsequent NF-κB activation (Kane et al, 1999; Khwaja, 1999; Ozes, 1999; Romashkova, 1999).

Most studies on NF-κB activation have focused on the role of IKKβ in this process, as IKKβ has been shown to be sufficient for NF-κB activation. IKKα was, until recently, considered a redundant protein since both IKKα and IKKβ are similar in structure and function. However, it has now been shown that the role of IKKα in cell signaling is often separate and distinct from that of IKKβ (Takaesu et al, 2003; Solt, 2007). IKKα can signal through an alternative NF-κB activation pathway whereby IKKα is responsible for the processing of p100 to p52 (Senftleben et al, 2001). In addition, IKKα has been shown to enter the nucleus upon activation, and phosphorylate the H3 protein of the histone complex (Anest et al., 2003; Yamamoto et al., 2003). Together, these data show that IKKα can play a distinct role in NF-κB signaling.

NF-κB and IKKα in tumor growth and angiogenesis

NF-κB activity has long been associated with tumor growth. It plays important roles in tumor-related cell survival, chemotaxis and inflammation (Kiriakidis et al., 2003; Grosjean et al, 2006; Farina, 1999). Accordingly, inhibition of NF-κB activity impairs tumor growth in both *in vitro* and *in vivo* studies (Kim et al., 2006; Basseres et al., 2006). Evidence for the role of NF-κB in almost all aspects of tumor development and progression was put forth recently (Karin et al, 2006). For this reason, there is a worldwide push to develop NF-κB inhibitors for clinical use. NF-κB also plays a role in tumor angiogenesis. Most studies of this process have focused on the regulation of pro-

angiogenic factors, including VEGF, IL-1β, IL-6 and IL-8 (Agarwal et al., 2005).

While NF-κB has yet to be directly linked to endothelial cell motility, indirect evidence does exist. IKKα-null mice die 30 minutes after birth, and show evidence of cardiovascular, and potentially, angiogenic defects (Hu et al, 1999). Several angiogenic factors, such as VEGF and Ang1, have been shown to activate the Akt-IKKα-NF-κB pathway (Marumo et al., 1999; Kim et al., 2001). Also, IKKα has been shown to be important in endothelial cell motility. In support of a role for IKKα in angiogenesis, a recent report shows that over-expression of kinase dead IKKα in tumor cells strongly inhibits both the constitutive NF-κB-dependent promoter and endogenous gene activation. Targeted array-based gene expression analysis reveals that many of the genes downregulated upon inhibition of this pathway are involved in tumor angiogenesis (Agarwal et al., 2005). These data suggest that IKKα could serve as an important anti-angiogenic therapeutic target. Work in Chapters IV and V demonstrate a role for IKKα in tumor angiogenesis, and work in Chapters VI and VII show how two genes regulated by IKKα expression are critical for angiogenesis.

Epithelial to Mesenchymal Transition and Endothelial EMT

Normal epithelia possess cell-to-cell contacts that account for both the proper development of these tissues during embryonic life and for the maintenance of homeostasis and architecture of epithelial structures during adult life. Epithelial to Mesenchymal Transition (EMT) is the process by which epithelial cells lose their epithelial phenotype, and revert to a mesenchymal phenotype. It is characterized by the

adoption of a mesenchymal or spindle cell morphology, the alteration of cell adhesion molecules, and the ability of the cells to migrate (Thiery, 2002). The most studied occurrence of EMT is during tumor cell invasion and metastasis. During this process, tumor cells change from an immobile and orderly sheet of cells, to a motile form. Cell-to-cell contacts are disrupted, as seen by altered expression of both E-cadherin and ZO-1. Cells express mesenchymal markers, such as vimentin. They become motile and migrate into the underlying basement membrane, and metastasize to distant sites (Thiery, 2002). EMT is vital in embryogenesis, and involved in several morphogenetic and organogenetic processes, such as gastrulation movements and the emigration of neural crest cells from the neural tube. Given the physiological and pathological importance of EMT, a considerable effort has been made during the past few years to characterize the mechanisms involved (Arias et al., 2001).

The endothelium is a specialized epithelial tissue lining the blood vessels. Endothelial EMT is an integral step in cardiovascular development during embryogenesis. For example, early cardiac morphogenesis consists of the transformation of a simple tubular structure made up of two concentric cylinders of epithelia separated by a gel-like matrix (the cardiac jelly) into the adult organ. A critical step in this process involves the differentiation of cells from the inner epithelial layer (the endocardium) into mesenchymal cells that invade the cardiac jelly and later form the connective tissue of the valves and septa of the adult heart (Barnett and Desgrosellier, 2003; Eisenberg et al., 1995). Endothelial EMT is initiated by signals originating from outside the endothelial cells. Peptide growth factors, such as those in the epithelial growth factor family (EGF) (Camenisch et al., 2002), the transforming growth factor-β family (TGF-β) (Brown et al.,

1999), and the Notch family (Noseda et al., 2004), have been indicated in the induction of EMT. The work in Chapters IV, VI, and VII show that endothelial EMT is also an essential step during adult angiogenesis in which endothelial cells migrate to form new vessels.

Vav1

Vav1 is a member of the Dbl family of guanine nucleotide exchange factors (GEF) expressed in hematopoietic and hematopoietic-derived cells, and has been shown to play a crucial role in activation, growth, and differentiation of these cells. Since hematopoietic cells and endothelial cells share a common progenitor, Vav1 is also expressed in endothelial cells (Georgiades et al., 2002). Vav1 was first identified as an oncogene when 3T3 cells were successfully transformed with an oncogenic form of Vav1 lacking the first 67 amino acids at the amino terminus of the protein (Katzav et al, 1991). Subsequent studies suggested that Vav1 was a signaling molecule. Genetic inactivation of Vav1 in mouse models impairs the development and function of hematopoietic cells, such as lymphocytes, natural killer cells, and osteoclasts. (Katzav 2004). Conversely, over-expression of Vav1 in lymphocytes enhanced activation of multiple intracellular signaling pathways, including ERK, JNK, and PI3 kinase (Bustelo, 2000).

Vav 1 has also been shown to play a role in the regulation of gene transcription (Houlard et al., 2002), possibly through regulation of transcription factors, such as AP-1, NFAT, and NF-κB (Wu et al., 1995). NFAT activation is impaired in T cells from Vav1-deficient mice (Katzav, 2004). In activated T-cells, Vav1 translocates to the cytoplasmic

membrane and the nucleus (Piccolella et al., 2003; Marinari et al., 2002; Houlard et al., 2002). At the cytoplasmic membrane, Vav proteins act as guanine nucleotide exchange factors for members of the Rac1, Rho1, and Cdc42 proteins. In addition, Vav1 interacts with IKKα and forms a transcription complex in T cells (Houlard et al., 2002; Katzav, 2004). In this study, we found a potential interaction of Vav1 with IKKα in endothelial cells that regulates δ-catenin expression. Chapter VII of this work addresses the role of Vav1 in endothelial cell motility and tumor angiogenesis.

δ-catenin

δ-catenin (Neural Plakophilin-Related Arm-Repeat Protein (NPRAP) /Neurojungin) is a member of the p120 family of proteins containing a region of 10 armadillo repeats (Kosik et al., 2005). The protein was first identified in 1997 in the lab of Werner Franke in a search for plakophilin-related proteins. The sequence was found to be highly homologous to two other members of the p120 family, p120, and p0071 (Hatzfeld, 2005). Expression of the protein was originally thought to be exclusively in neural tissue. This work is the first to recognize δ-catenin expression in the vascular endothelium. It should be noted though, that many proteins have been shown to be expressed in both neural and vascular tissue (Eichmann et al., 2005). δ-catenin is known to play a critical role in neural cell migration. Transfection of fibroblasts with the δ-catenin gene rendered the cells sensitive to HGF scattering (Lu et al., 1999). Chapter VII addresses the role of δ-catenin in tumor angiogenesis.

Summary

Angiogenesis is a complex process that involves multiple steps, including endothelial cell proliferation, migration, survival, and assembly of vascular networks. The combined work in this dissertation identifies a new pathway, Akt-IKKα-Vav1, that regulates endothelial cell survival, cell motility, and angiogenesis (**Figure 1**). We demonstrate that Akt is necessary and sufficient in Ang1/Tie2-mediated endothelial cell survival. Although Akt is sufficient in VEGF-mediated cell survival, it is not required in this process. Interestingly, we show that activation of Akt in endothelial cells also leads to endothelial EMT, increased cell motility, and angiogenesis through the IKKα/Vav1/δ-catenin pathway.

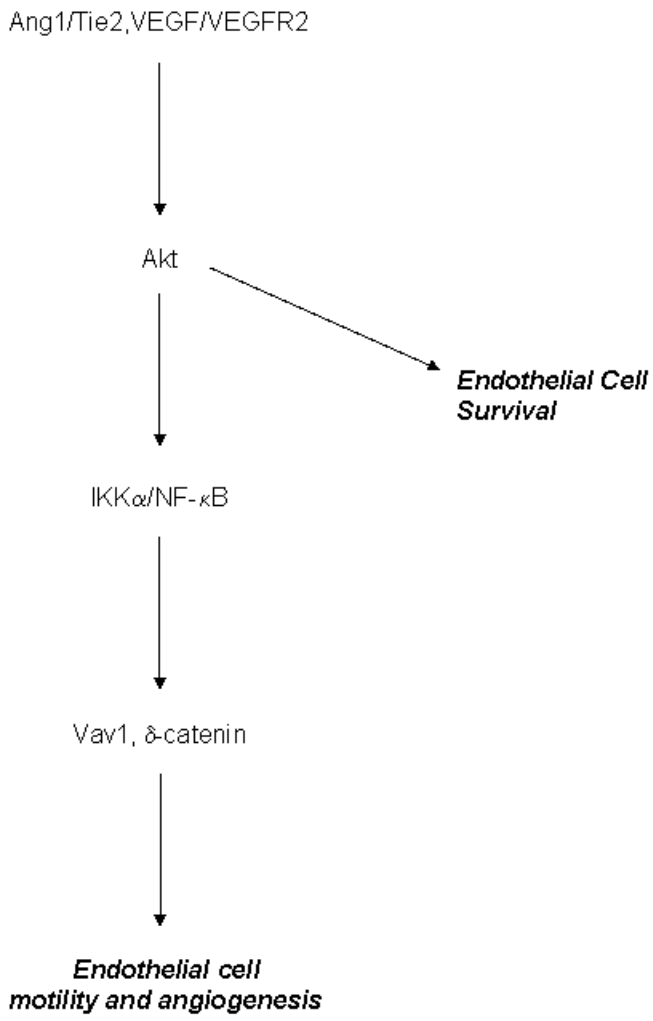

Figure 1. The central signaling pathway for both endothelial cell motility and endothelial cell survival.

CHAPTER II

MATERIAL and METHODS

Reagents

The adenoviral vectors directing the expression of Ang1* (AdAng1) and Ang2 (AdAng2) were provided by Dr. George Yancopoulos from Regeneron Corp. Ang1* is a slightly modified version of Ang1 that is easier to express and purify (Koblizek et al., 1998). The Akt adenoviruses were from the lab of W. Ogawa. The active Akt mutant (AdAkt) has a src myristoylation tag at the amino end and is thus targeted to the cell membrane bringing it into proximity of its activator protein, PDK. The dominant negative form of Akt (Addom-Akt) was made by a K179D mutation.

Adenoviral vectors directing the expression of soluble Tie2 (AdExTek) and soluble VEGF receptor (AdExFlk) were generated as described (Lin et al. 1998 (1); Lin et al. 1998 (2); Geng et al. 2001). The adenovirus directing expression of IKKα, IKKβ and IκBα were kindly provided by Dr. F. Yull (Vanderbilt University) and Dr. Brenner (University of North Carolina at Chapel Hill) (Jobin et al., 1998). The mutant IκB-α adenovirus was from the lab of D. Brenner. It has S32A and S36A mutations preventing phosphorylation and targeting for degradation (Jobin et al. 1998). Adenoviral vectors directing the expression of a green fluorescent protein (AdGFP) and β-galactocidase (β-gal) were used as a vector controls. Viral vectors were propagated in 293 cells, and purified in CsCl columns as described. Viral titers were determined by optical

densitometry, and recombinant viruses were stored in 10% glycerol at -80^0C (Lin et al, 1998 (1)). Antibodies against Akt and phospho-Akt (Ser473) were purchased from Cell Signaling Technology. The VE-cadherin antibody is from Calbiochem. The Cy2 conjugated antibodies and DAPI are from Jackson Labs and Molecular Probes (Eugene, OR) respectively. Methyl cellulose was purchased from Sigma, and Rat Tail Collagen I was purchased from BD Bioscience.

Cell Culture

Human Umbilical Venous Endothelial Cells (HUVECs) and Human Microvascular Endothelial Cells (HMECs) were purchased from Cambrex/Lonza (San Diego). Endothelial cells were grown on 0.1% gelatin-coated tissue culture plates in endothelial growth medium for microvascular cells type two (EGM-2, Cambrex/Lonza, San Diego). Cells between passages 3-7 were used. Lewis lung adenocarcinoma cells (LLCs) were grown on tissue culture plates in DMEM supplemented with 10% FBS and 1% antibiotics. All cells were maintained in a humidified incubator with 5% CO_2 at 37^oC.

Adenoviral infection of endothelial cells

Endothelial cells, either HUVECs or HMVECs, at 75% confluency were infected at a multiplicity of infection (MOI) of 5 with different adenoviral vectors directing expression of the gene of interest. Twenty-four hours after viral infection, the cells were trypsinized, counted, and set up for further experiments. Transfection efficiency was determined by GFP expression in cells infected with AdGFP virus.

RNAi

Pooled siRNAs for Vav1 (Ambion) and δ-catenin (Santa Cruz) or scrambled control siRNA were transfected into endothelial cells using RNAifect system (Qiagen). Protein expression and subsequent experiments were performed 24 hours following transfection.

Cell Survival

Twenty-four hours following adenoviral infection, cells were washed twice with PBS and placed in serum-free media (EBM, Clonetics, Walkersville, MD) for an additional 24 h. Cells were collected and fixed in 80% ethanol overnight at −20°C. The cells were then resuspended in PBS and incubated with propidium iodide (PI) and RNase for 2 h on ice. Flow cytometry analysis was performed by the Veteran's Administration, Nashville FACS core lab.

Cell Sprouting

Either HUVECs or HMVECs grown to 75% confluency were infected with adenovirus directing the expression of the proteins of interest. Twenty-four hours after viral infection, cells were trypsinized and mixed with EGM with 20% FBS and 0.2% methyl cellulose. The cell mixture was then incubated overnight at 37°C in uncoated, sterile Petri culture dishes. The cells formed spheres around the methyl cellulose particles. The next day, the spheres were collected via centrifugation at 800 RPM and resuspended in EGM with 20% FBS and 0.2% methyl cellulose. Equal parts methyl cellulose solution and a collagen I solution of 2 mg/ml rat tail collagen I in PBS plus 1%

NaOH were then combined and plated in 24-well tissue culture plates. The plates were incubated at 37°C for 30 min, overlayed with EGM with 20% FBS, and then incubated at 37°C overnight in a tissue culture incubator. After 24 h, cell sprouts were photographed and counted. A single sprout was counted for every strand of three or more cells that could be traced back to the bead. Two or less cell projections from the beads and cells that were not part of an unbroken strand of cells migrating out of the beads were not counted.

Cell migration

Cells were incubated overnight in serum-free media (EBM, Clonetics, San Diego) plus 1% BSA and then plated at a density of $1x10^5$ in the top chamber of a Transwell modified Boyden chamber with 8 μm pores (Costar) that had been coated overnight with collagen I at a concentration of 10ug/ml. EBM was placed in both the upper and lower chamber. The bottom chamber also contained 25ng/ml VEGF (R&D) as a chemoattractant. Cells were incubated for 5 hours at 37^oC, allowing time for migration through the pores. Membranes were then fixed in 4% paraformaldehyde and stained with crystal violet. Cells that had migrated through the membrane were then counted under microscopy in ten random selected 200X fields as described (Kamiyama et al, 2006). The experiment was performed in duplicate and repeated at least three times.

Endothelial cell tube formation

After viral infection for 36 hours, HUVECs were plated on top of growth factor reduced matrigel (B D Bioscience) and the matrigel was incubated 18 hours at 37^oC.

Vascular branches were counted in 10 randomly selected fields under microscopy. Each experiment was performed in triplicate and repeated three times.

Cell proliferation

An equal number of endothelial cells were plated in multiple tissue culture plate. Cells were harvested and counted 1, 2, and 3 days following plating.

Immunofluorescent Staining of Cells

Cells were cultured on glass tissue culture slides (Nalgene) coated with 0.01% gelatin in PBS. Following a 48 hour incubation, the cells were rinsed with PBS, fixed in 4% paraformaldyhyde, and permeablilized with 0.1% Trition X-100. The cells were then incubated with the primary antibody, rinsed, and incubated with DAPI, for visualization of the cell nuclei, and Cy2 conjugated secondary antibody. Immunofluorescent staining was examined under an Olympus Provis Microscopy connected to a digital camera. The experiment was performed in duplicate and repeated at least three times.

Real-Time PCR

Total RNA was prepared from treated endothelial cells and cDNA was synthesized from 1 μg total RNA with Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative real-time PCR was performed with 5 μL cDNA solution with Universal PCR Master Mix (Qiagen,), SYBR-Green, and 200 nM primer on an ABI PRISM 7000 Sequence Detection System (GE Life Sciences, Fairfield, CT). Relative gene expression levels were normalized

according to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* and was calculated using the comparative Ct method, described in user bulletin 2 (ABI; GE Life Sciences). Expression data represent the average values from 3 different experiments.

Western Blot

For Western blotting of phospho-Akt, after treatment, cells were washed twice with PBS, then lysed by incubation with RIPA buffer plus 1 μg/ml of leupeptin, 1 μg/ml of pepstatin, 1 mM of sodium vanadate, and 1 mM of phenylmethylsulfonyl fluoride for 10 min at 4°C. Cellular lysates were collected, and the protein content was measured using a BCA protein assay kit (BioRad, Hercules, CA). For blots of conditioned media, after treatment, cells were incubated overnight in EBM. Five hundred microliters were collected from each sample, and concentrated down to 100 μl per sample using Microcon-YM-10 filter tubes (Amicon, Bioseparations). Proteins were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Western blotting of cell lysates was performed with primary antibody overnight at 4^{o}C. The membrane was then washed and incubated with horseradish peroxidase-conjugated secondary IgG antibody (Amersham).

Akt Kinase Assay

The kinase assay was performed using the Akt Kinase Assay Kit (Cell Signaling Technology). After treatment, cells were lysed in cell lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM

β-glycerophosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin plus 1 mM PMSF. The lysates were then immunoprecipitated overnight at 4°C with anti-Akt antibody crosslinked to agarose beads. Immunoprecipitated proteins were then incubated with 1 μg GSK-3 protein in a solution containing 25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium vanadate, and 10 mM $MgCl_2$ plus 10 mM ATP for 30 min at 30°C. SDS (3×) sample buffer was then added to the kinase reaction mixtures, and the samples were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Western blotting was performed with a phospho-GSK-3α/β primary antibody and an anti-rabbit HRP-conjugated secondary antibody as described above.

IKK phosphorylation

Cell was immunoprecipitated with anti-IKKα antibody (Santa Cruz) and the immunoprecipitate was run an SDS-PAGE gel and subject to Western Blotting with anti-pIKKα/IKKβ (Cell Signaling).

EMSA

Endothelial cells were infected with adenovirus. Cells were harvested 24 hours later and nuclear proteins were extracted. Nuclear lysate was incubated with ^{32}P-labled DNA containing NF-κB binding sequence. The lysate was then run on a non-denaturing gel and imaged.

Rac, RhoA and Cdc42 activation assay

Rac1, RhoA and Cdc42 activation kits were purchased from Chemicon. The assays were performed following manufacture's instructions.

Atrioventricular (AV) cushion assay

Chick eggs were purchased from Charles River (Wilmington, MA). Fertilized chicken eggs were incubated at 14^{o}C until they reached stage 15 according to the Hamburger and Hamilton staging of chick development (Hamburger and Hamilton, 1951). Endocardial cell EMT was performed as described (Desgrosellier, 2005). Briefly, embryos were harvested and atrioventricular cushions were removed, opened, soaked briefly in a PBS solution containing adenoviruses directing the expression of the proteins of interest. AV cushions were then placed endocardial side down onto a collagen bed containing Rat Tail collagen I (Sigma). Explants were incubated 48 hours at 37^{o}C at 5% CO_2. Endocardial cells that migrated into the collagen matrix were counted as having undergone EMT as described (Brown et al., 1999). The experiments repeated at least three times.

Aortic Ring Assay

Aortas were removed from Vav1 and Wildtype mice. Connective tissue was removed and the aortas were rinsed in PBS, sliced and embedded in a fibrin matrix (Sigma, St. Louis, MO). Endothelial sprouts were counted 7 days later.

Mice

C57/Bl6 and Rag1 mice were purchased from the Jackson Lab (Bar Harbor, ME). Vav1-null mice on a C57/Bl6 background were from the lab of Victor Tybulewicz (NIMR, London) and δ-catenin null mice on a C57/Bl6 background were from the lab of Liu Xin (UCLA, Los Angeles). All mice used in this work were housed in pathogen-free units at Vanderbilt University Medical Center, in compliance with IACUC regulations. Age and sex matched mice were used in the study.

Analysis of tumor growth in vivo

The LLC/endothelial cell co-transplantation experiments were performed as described (Brantley-Sieders et al, 2005). Briefly, HUVECs were infected with adenoviral vector directing the expression of either GFP or IKKα respectively. Twenty-four hours following infection, cells were mixed with LLC tumor cells. Cells were mixed with growth factor-reduced matrigel (BD Bioscience) and injected subcutaneously into the left hind flank of Rag1 mice. Tumor growth was measured every other day with calipers. Tumor volume was calculated as Length X $Width^2/2$.

Apoptotic cells and cell proliferation were determined by TUNEL assay (Chemicon) and PCNA immunostaining (Santa Cruz), respectively, according to manufactures' protocols. The number of positive cells was counted in 10 randomly selected 200x fields under microscopy.

BMT

Vav1-null or wildtype C57Bl/6 mice were irradiated with a lethal dose of 7.5 Gy. Bone marrow cells were isolated from wildtype mice and $5x10^6$ cells were injected in the irradiated mice within 3 hours after irradiation. All bone marrow transplanted mice survived, suggesting incorporation of donor bone marrow cells. Mice were kept in a pathogen free environment and given acidic water with Neosporin antibiotic at a concentration of 2.5ml/L. Tumor cells were injected 6 weeks following irradiation.

Immunohistochemical and immunofluorescent staining of tissue

Tissues were harvested, freshly frozen and embedded in OCT. 7 μm sections were cut and were fixed in pre-chilled acetone for 10 minutes at -20^{o}C, then rinsed in PBS. Sections were then blocked with either 10% normal goat serum or mouse blocking solution from the M.O.M. kit (Vector Labs) for 1 hour at room temperature and incubated with primary antibodies overnight at 4^{o}C. Tissues were stained with antibodies against IKKα (Santa Cruz), pan CD31 for total vascular density against human specific CD31 antibody for human endothelial cells and against murine CD31 for mouse endothelium (Pharmingen). Sections were then rinsed in PBS and incubated with secondary antibodies 1 hour at room temperature.

Statistics

Results are reported as mean $\pm$ SE for each group. Parametric, Student's t-test and nonparametric, Mann-Whitney U test, were used to analyze statistical differences

between control and treated groups. Tests of hypothesis between groups were made with ANOVA with adjusted least square means. Statistical calculations were run on Prism 5 Graph Pad. Differences were considered statistically significant if $p < 0.05$.

CHAPTER III

AKT IS A MAJOR ANGIOGENIC MEDIATOR OF THE ANG1/TIE2 SIGNALING PATHWAY

Introduction

Tie2 and VEGF receptors (VEGFRs) are tyrosine kinases that play essential roles in angiogenesis. Activation of either receptor leads to the activation of (Akt), an important mediator of cell survival and cell motility. However, it is not known if Tie2 and VEGFR mediate a pro-angiogenic phenotype through Akt. In this study, we compared the role of Akt in Tie2-mediated versus VEGF-mediated endothelial cell survival and endothelial cell sprouting.

Our data show that Akt is involved in endothelial cell survival mediated by angiopoietin 1 (Ang 1), a ligand for Tie2. Activating Akt rescues endothelial cells from apoptosis induced by blockade of either Tie 2 or VEGF. However, blocking Akt function abolishes Ang1-mediated endothelial cell survival, but has no effect on VEGF-induced endothelial cell survival, demonstrating that Akt is sufficient but not required for VEGF-mediated endothelial cell survival. In addition, we show that both Ang1 and VEGF induce endothelial cell sprouting in a three-dimensional collagen gel, and this process depends on the activation of Akt. Blocking Akt activity inhibited endothelial cell sprouting induced by either Ang1 or VEGF.

Overall, the data show that Akt is the primary mediator of Ang1-induced endothelial cell survival, while additional pathways for endothelial cell survival are

involved downstream of VEGF. However, Akt is necessary and sufficient to mediate the endothelial cell sprouting induced by both Ang1 and VEGF.

Results

Adenoviral vector is effective in transducing genes into endothelial cells

Transfection efficiency is low in primary endothelial cells using conventional expression vectors; therefore, it is often challenging to analyze signaling pathways in these cells. We observed that adenoviral vectors work very efficiently in infecting endothelial cells. HUVECs were cultured to a75% confluency, and then infected with an adenoviral vector directing the expression of the green fluorescent protein (AdGFP) at a multiplicity of infection of 5 pfu per cell in EGM. GFP expression could be detected 12 h after viral infection. High levels of GFP expression were achieved 24 h after the infection and lasted for several days (**Figure 2A**). Under this condition, an infection efficiency of 100% was achieved without visible cytotoxicity. These conditions were used for subsequent experiments. In addition, AdGFP as a viral vector control was used in every experiment to monitor infection efficiency. Similar efficiencies were achieved in each experiment.

Ang1, VEGF, and a constitutively active Akt construct (act-Akt) activate Akt, and the dominant-negative Akt (dom-Akt) construct inhibits Akt activity in endothelial cells

Both Ang1 and VEGF have previously been shown to activate the PI3K/Akt pathway (Fujikawa et al., 1999; Gerber et al. 1998). To show we could induce Akt

phosphorylation with both Ang1 and VEGF in HUVECs, the cells were infected with adenovirus directing the expression of Ang1* (AdAng1), VEGF (AdVEGF), or control AdGFP. Cell lysates were analyzed by SDS-PAGE, followed by Western Blotting using a phospho-Akt antibody for detection of Akt activation. The Western Blot shows that both AdAng1 and AdVEGF increase Akt phosphorylation in HUVECs as compared to AdGFP (**Figure 2B**). Since over-expression of Akt constructs in HUVECs changes total Akt protein levels and makes it difficult to analyze Akt activation by examining the levels of Akt phosphorylation, we used a kinase assay with a GSK-3 target to determine the activity of Akt in cells infected with different viral vectors as listed in Figure 2C. Our work showed that Ang1, VEGF, and an act-Akt construct significantly increased Akt activity when compared to the viral vector control, with the act-Akt construct achieved a slight stronger Akt activity than either VEGF or Ang1 (**Figure 2C**). In contrast, over-expression of dom-Akt inhibited endogenous Akt activity as well as VEGF- or Ang1-induced Akt activation (**Figure 2C**).

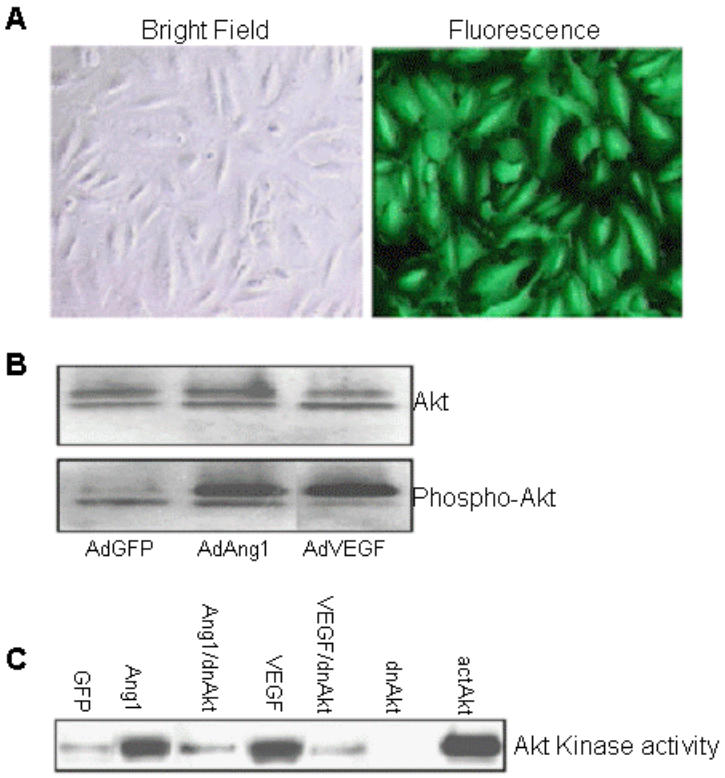

Figure 2. Ang1 and VEGF induce Akt phosphorylation and activation. HUVECs were cultured to 75% confluency, then infected with AdGFP viral vectors. Images were taken 24 hrs after infection. The left panel shows the infected cells under normal light. The right panel shows the same cells under fluorescent light (A) . Ang1 and VEGF induce Akt phosphorylation. HUVECs were cultured to 75% confluency, then infected with AdGFP, AdAng1, and AdVEGF viral vectors. Cell lysate was collected 48 h after infection, and analyzed by SD S-PAGE, followed by Western Blotting using an Akt antibody (top row) or a phospho-Akt antibody (bottom row) for detection of Akt activation (B) . Akt kinase activity was evaluated in HUVECs infected with different viral vectors. Anti-Akt immunoprecipitates from cells infected with different viral vectors expressing the gene of interest were used in kinase assays with the substrate GSK-3 protein. Immunoblotting was performed with a phospho-GSK-3α/β primary antibody to assess Akt activity (C) .

Akt is required and sufficient to mediate Ang1-induced endothelial cell survival

Activation of Tie2 receptor by Ang1 stimulation induces Akt phosphorylation and enhances endothelial cell survival against serum starvation (Fujikawa et al. 1999; Papapetropoulos et al. 2000). However, it is less clear whether the Akt activation is necessary and/or sufficient to mediate Ang1- or Tie2-induced endothelial cell survival. Here, we used more specific inhibitors to examine the role of Akt in the Ang1/Tie2 survival pathway. HUVECs were infected with AdGFP as a control, AdAng1, or AdAng1 plus Addom-Akt, followed by serum starvation. Microscopic photographs were taken 72 h after serum starvation (**Figure 3A**). There were many apoptotic cells in the control-treated plates. In contrast, there were only a few apoptotic cells when over-expressing Ang1 in cells. Interestingly, over-expression of a dominant-negative Akt construct blocked the Ang1-induced cell survival. The cells were collected 72 h later, stained with PI, and analyzed by flow cytometry. Apoptotic cells were counted as sub-G0/G1 cells (**Figure 3B**). The percentage of apoptotic cells was plotted against each treatment group (**Figure 3C**). We showed that serum starvation of HUVECs led to cell death. Over-expression of Ang1 protected the cells from apoptosis. Interestingly, co-expression of a dominant-negative Akt with Ang1 neutralized Ang1-induced cell survival. Over-expression of dom-Akt alone led to cell apoptosis (**Figure 3C**, $p < 0.001$). The cell apoptosis data correlated with Akt activity data (**Figure 2**). In addition, we measured Ang1 levels in cells infected with AdAng1. The culture media were harvested 48 h after the viral infection, concentrated fivefold, and then analyzed by Western blotting probed with an Ang1 antibody (**Figure 3D**). Ang1 was only detected in AdAng1-infected cells. Ang1 concentration was quantified using a densitometer and compared to

the recombinant Ang1 protein. There was approximately 30 ng/ml of Ang1 present in the culture media, which is similar to the concentration commonly used in the *in vitro* assay. Akt expression was verified with Western Blot analysis of cell lysate from AdGFP and Ad-domAkt adenovirus (**Figure 3E**). Collectively, these results confirm Akt is required for Ang1- or Tie2-mediated endothelial cell survival.

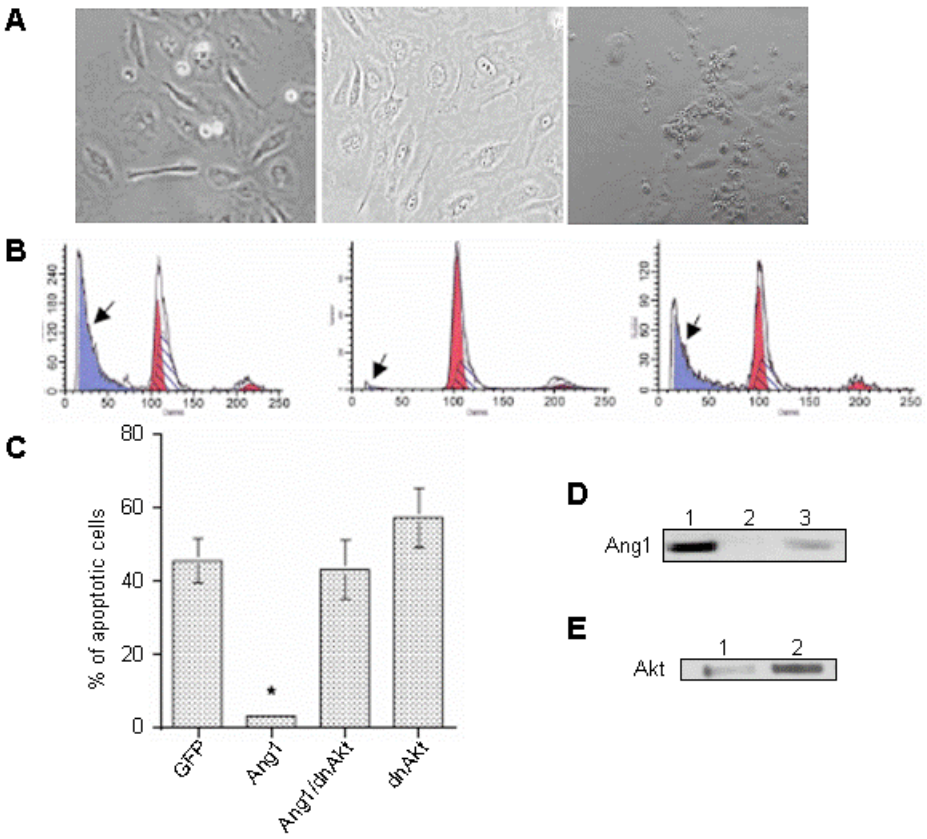

Figure 3. Ang1 enhances endothelial cell survival through Akt. HUVECs were cultured to 75% confluency then infected with adenoviruses directing the expression of the proteins of interest. Control cells were infected with AdGFP virus. Cells were then transferred to serum-free media. Representative microscopic photographs were taken 72 h after serum starvation (A). Cells were then collected and stained with PI solution for flow cytometry. Sub-G1/G0 cells were counted as apoptotic cells as indicated by the arrowhead (B). Percent of apoptotic cells were quantified from three independent experiments. Mean and SE were plotted (n= 3, p< 0.001 by Student's t-test, *$p < 0.001$ by Mann-Whitney U) (C). Cell culture media were collected 48 h after viral infection, concentrated, and then analyzed by Western blotting for Ang1 expression (D). Lane 1 was loaded with 20 ng of recombinant Ang1 protein; lane 2 was HUVECs infected with AdGFP; and lane 3 was cells infected with AdAng1. Cell lysate was collected and analyzed by Western blotting for Akt expression (E). Lane 1 lysate was collected from AdGFP infected HUVECs and lane 2 lysate was collected from Ad-dom Akt infected HUVECs.

To further explore the role of Akt in Ang1-induced endothelial cell survival, we performed the complementary experiment. HUVECs were infected with control AdGFP, AdExTek as a Tie2 inhibitor (Lin et al. 1997), or AdExTek plus Adact-Akt, respectively, followed by serum starvation. Microscopic photographs of cells were taken 72 h after serum starvation (**Figure 4A**). Again, there were many apoptotic cells in the control group. Blocking Tie2 signaling by over-expression of a Tie2 inhibitor (ExTek) increased the number of apoptotic cells, but co-expression of a constitutively active Akt dramatically protected cell apoptosis. Flow cytometry analysis was performed to quantify apoptotic cells in each group. Cells were stained with PI dye followed by flow cytometry analysis (**Figure 4B**). Apoptotic cells were plotted against different treatment (**Figure 4C**). The results showed that serum starvation led to endothelial cell apoptosis. Over-expression of ExTek significantly enhanced cell death ($p < 0.05$). A constitutively active Akt attenuated ExTek-induced cell death ($p < 0.001$). Over-expression of a constitutively active Akt alone also protected HUVECs from serum starvation-induced cell death (**Figure 4C**, $p< 0.001$). Akt expression was verified with Western Blot analysis of cell lysate from AdGFP and Ad-act Akt adenovirus (**Figure 4E**). Collectively, the results showed that Akt is a major endothelial cell survival mediator downstream of the Ang1/Tie2 signaling pathway; Akt activity is necessary and sufficient for Ang1/Tie2-induced endothelial cell survival.

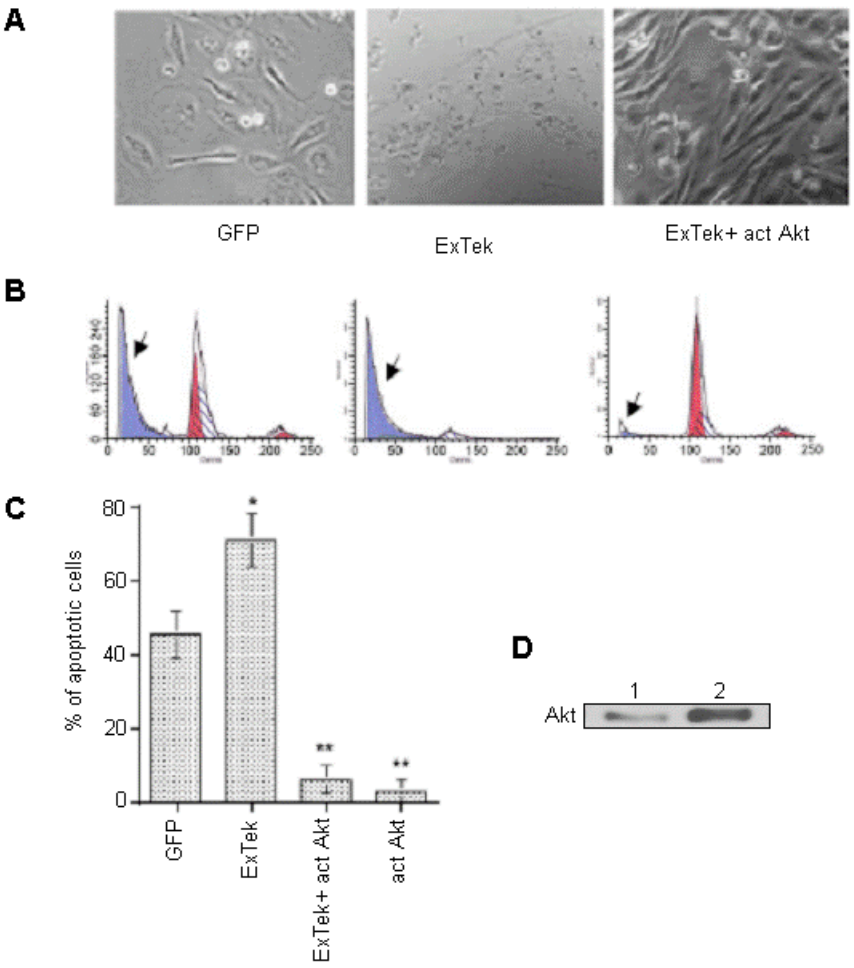

Figure 4. Akt is sufficient to mediate Tie2 activation-induced endothelial cell survival. HUVECs were infected with adenoviruses directing the expression of the proteins of interest followed by serum starvation. Microscopic photographs were taken 72 h after serum starvation (A). Cells were then collected and stained with PI solution for flow cytometry. Sub-G1/G0 cells were counted as apoptotic cells as indicated by the arrowhead (B). Apoptotic cells were quantified from three independent experiments and mean value and SE were plotted (n= 3, F=4.32, $*p < 0.05$, $**p < 0.001$ by ANOVA) (C). when compared to the control GFP group. Cell lysate was collected and analyzed by Western blotting for Akt expression (D). Lane 1 lysate was collected from AdGFP infected HUVECs and lane 2 lysate was collected from Ad-act Akt infected HUVECs.

Akt is sufficient but not required for VEGF-mediated endothelial cell survival

VEGF stimulation of endothelial cells also leads to Akt activation and endothelial cell survival (Gerber et al. 1998; Fujio et al. 1999). We compared the role of Akt in VEGF- and Ang1-induced survival. HUVECs were infected with adenoviruses directing the expression of either the control AdGFP, a soluble VEGF-receptor II (AdExFlk) (Lin et al., 1998; Gerber et al. 1998), or a combination of AdExFlk plus Adact-Akt followed by serum starvation for 72 h. The cells were harvested and stained with PI followed by flow cytometry (**Figure 5A**). After quantification of apoptotic cells in each treatment group, we observed that again, serum starvation caused apoptosis (**Figure 5**). Blocking VEGF signaling by over-expression of ExFlk significantly increased the percentage of apoptotic cells ($p < 0.05$). Furthermore, activation of Akt by co-expression of a constitutively active Akt with ExFlk attenuated cell apoptosis ($p < 0.001$). The results suggest that Akt activation is sufficient to mediate VEGF-induced cell survival signaling.

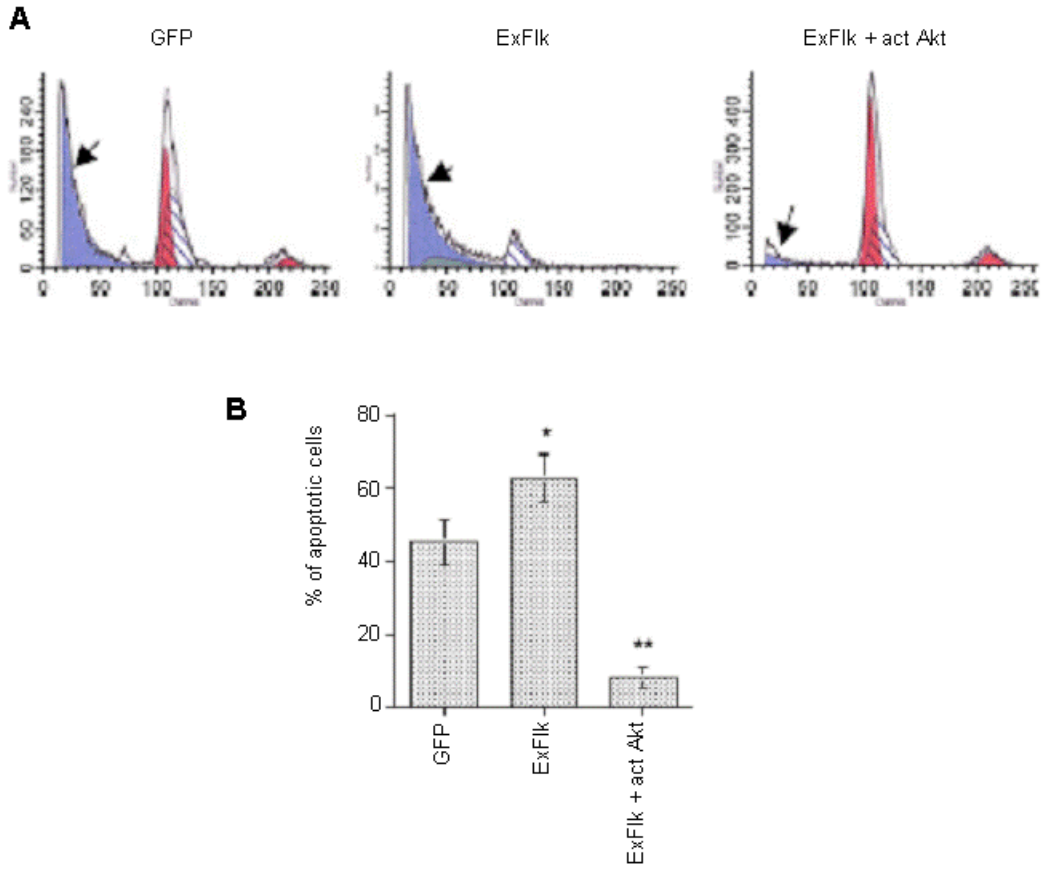

Figure 5. Akt is sufficient to protect endothelial cells from apoptosis induced by VEGF blockade. HUVECs were infected with adenoviruses directing the expression of the proteins of interest followed by serum starvation. For flow cytometry, cells were collected 72 h following serum starvation and stained with the PI (A). Sub-G1/G0 cells were counted as apoptotic cells as indicated by the arrowhead (A). Apoptotic cells were quantified from three independent experiments and the mean value and SE were plotted (n=3, F=5.32, $*p < 0.05$, $**p < 0.001$ by ANOVA)(B).

To determine whether Akt activity was required for VEGF-induced endothelial cell survival, HUVECs were infected with AdGFP, AdVEGF, or AdVEGF plus Addom-Akt, followed by serum starvation. Cells were stained with PI and cell survival was measured by flow cytometry (**Figure 6A**). The number of apoptotic cells were quantified from three independent experiments and plotted against the treatment (**Figure 6B**). The data showed that serum starvation led to cell apoptosis in the control-treated group. Over-expression of VEGF in endothelial cells protected the cells from serum starvation-induced apoptosis ($p< 0.001$). Surprisingly, co-expression of a dominant-negative Akt had little or no effects on VEGF-mediated endothelial cell survival. In addition, VEGF levels were quantified in cells infected with AdVEGF. The media was collected 48 h after the viral infection, concentrated, and then analyzed by Western blotting probed with a VEGF antibody (**Figure 6C**). VEGF protein concentration was quantified using a densitometer and compared to the recombinant VEGF protein. There was approximately 25 ng/ml of VEGF present in the culture media. The concentration of VEGF is in the range of recombinant proteins used in the *in vitro* studies. Collectively, our data showed that Akt was not required for VEGF-induced endothelial cell survival against growth factor depletion, although Akt mediated VEGF-induced endothelial cell survival and Akt could protect cells from VEGF-blockade-induced cell death.

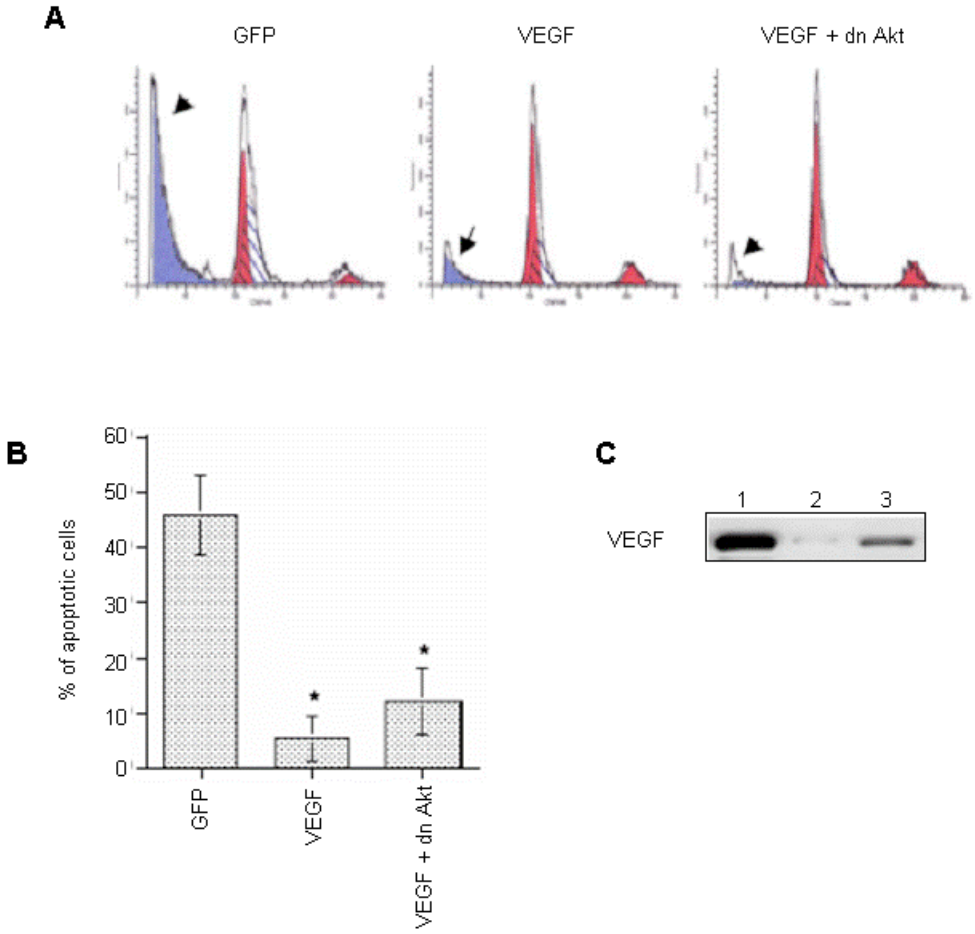

Figure 6. Akt is not required for VEGF-mediated endothelial cell survival. HUVECs were infected with adenoviruses followed by serum starvation. For flow cytometry, cells were collected 72 h following serum starvation and stained with the PI. Sub-G1/G0 cells were counted as apoptotic cells as indicated by the arrowhead (A). Apoptotic cells were quantified from three independent experiments and mean value and SE were plotted (n=3, F=2.41, $^{*}p < 0.01$ by ANOVA) (B). Cell culture media were collected 48 h after the viral infection, concentrated, and then analyzed by Western blotting and probed with a VEGF antibody (C). Lane 1 was loaded with 20 ng of recombinant VEGF protein; lane 2 was HUVECs infected with AdGFP; and lane 3 was cells infected with AdVEGF.

Akt is required to mediate both Ang1 and VEGF-induced endothelial cell sprouting

In addition to being necessary for vascular cell survival, both the Ang1/Tie2 and VEGF/VEGFR pathways play important roles in the process of endothelial cell sprouting in angiogenesis (Koblizek et al. 1998; Kim et al. 2000). Both HUVECs and HMVECs grown to 75% confluency were infected with AdAkt, AdAng1, AdVEGF, or a combination of either AdAng1 and Addom-Akt or AdVEGF and Addom-Akt. AdGFP was used as a control. Cells were collected and mixed with methyl cellulose media. Following an overnight incubation, equal parts cell suspension and a collagen I solution were then combined and plated then overlayed with media. Twenty four hours later, cell sprouts were photographed and counted.

Endothelial cell sprouting was seen with both HUVECs and HMVECs expressing either Akt, Ang1, or VEGF as compared to the control expressing GFP (**Figure 7**). Cells that expressed Ang1, VEGF, or Akt showed a strong sprouting response. HUVECs averaged 1.7 sprouts/bead for AdGFP-infected cells as compared to 3.75 sprouts/bead for AdAng1-infected cells ($p< 0.001$) and 3.6 sprouts/bead for VEGF-infected cells ($p< 0.001$). The sprouting could be attenuated in cells co-expressing Ang1 and dominant-negative Akt, as well as cell co-expressing VEGF and dominant-negative Akt (**Figure 7**). When cells co-expressed both Ang1 and dominant-negative Akt or VEGF and dominant-negative Akt, the cell sprouting response was abolished. The number of sprouts per bead averaged 1.95 for AdAng1 and Addom-Akt coinfected HUVECs, and 1.75 for AdVEGF and Addom-Akt coinfected HUVECs. HMVECs gave a similar sprouting response. There was an average of 4.85 sprouts per bead for AdGFP-infected HMVECs, as compared to 19 sprouts per bead for AdAng1-infected cells and 10.7 sprouts/bead for AdVEGF-

infected cells. Coinfection of HMVECs with AdAng1 and Addom-Akt resulted in an average of 2.7 sprouts per bead. HMVECs coinfected with AdVEGF and Addom-Akt produced an average of 2.3 sprouts per bead. The data demonstrate that Akt is required for the cell sprouting response of both the Ang1/Tie2 pathway as well as the VEGF/VEGFR pathway. The removal of Akt from the downstream signaling of either pathway results in a complete blockade of cell sprouting as demonstrated in a cell sprouting assay.

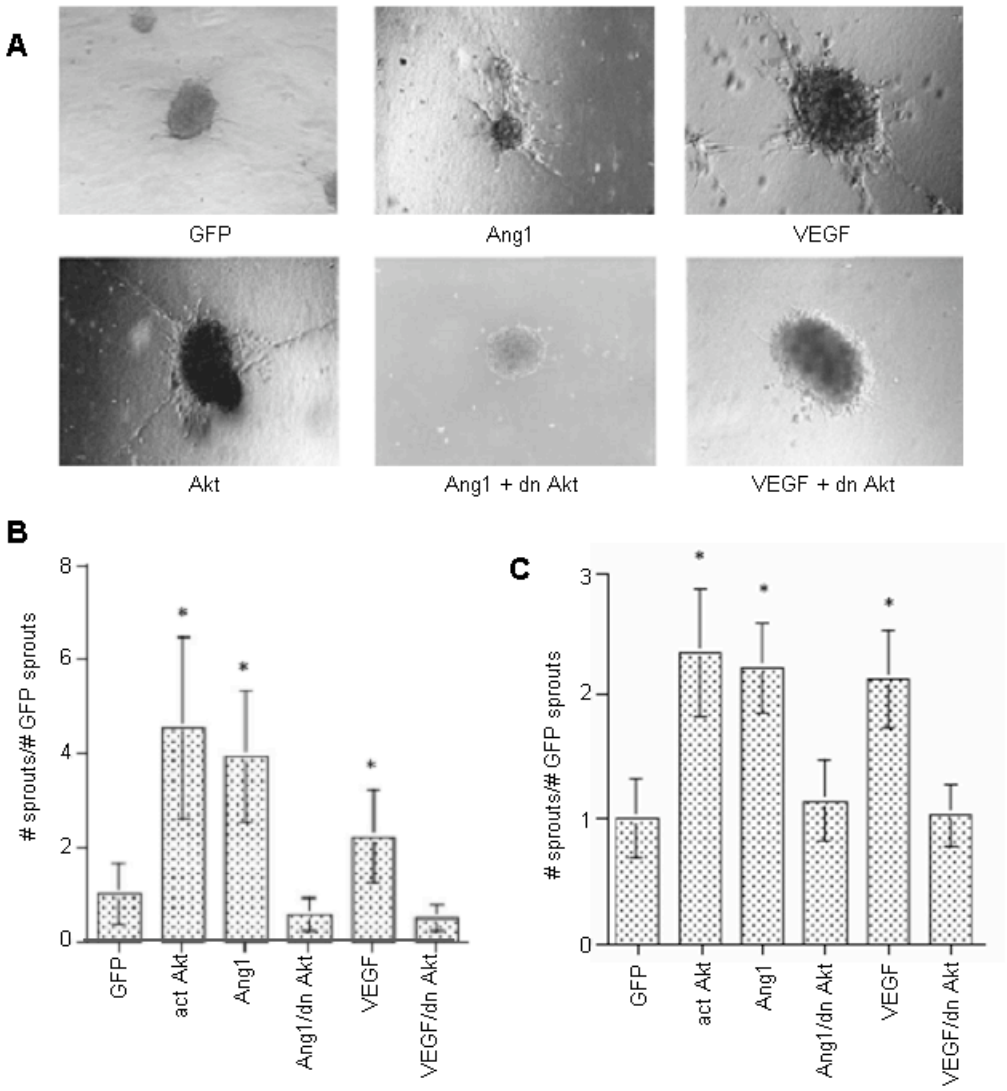

Figure 7. Both Ang1 and VEGF induce cell sprouting in an Akt dependent manner. Endothelial cells were incubated O/N with methyl cellulose. The resulting "beads" were embedded in a collagen I matrix. Cell sprouts were counted and photographed 24 h later. (A) HMVECs beads in collagen I matrix, 24 h after embedding. The ratios of the average number sprouts to the average number of sprouts on GFP beads were calculated for (B) HMVECs and (C) HUVECs. Twenty beads were counted for each sample. Mean and SE are plotted (n=6, F=9.28, $*p < 0.001$ by ANOVA).

Conclusion

In this study, we comparatively examined the role of Akt in Ang1- and VEGF-mediated endothelial cell survival and endothelial cell sprouting. Our data showed that Akt contributes to both Ang1- and VEGF-induced endothelial cells survival and endothelial cell sprouting. Akt activity is necessary and sufficient to mediate Ang1-induced endothelial cell survival. Although Akt activity is sufficient to protect endothelial cells from VEGF-blockade-induced cell death, Akt is not required for VEGF-induced endothelial cell survival, indicative of multiple and redundant survival pathways in VEGF signal transduction. In addition, we showed that Akt activity is required for endothelial cell sprouting in a three-dimensional collagen gel induced by either Ang1 or VEGF.

This work was published as:
DeBusk, L.M., Hallahan, D.E., Lin, P.C. "Akt is a major angiogenic mediator downstream of the Ang1/Tie2 signaling pathway." Exp Cell Res. *298*: 167-177, 2004.

CHAPTER IV

AKT INDUCES ENDOTHELIAL TO MESENCHYMAL TRANSITION THROUGH THE IKKα/NF-κB PATHWAY

Introduction

The process of endothelial EMT is critical for the development of the cardiovascular system, as well for the process of angiogenesis. While examining the role of Akt in endothelial survival (detailed in Chapter III), we surprisingly found that Akt induces EMT in endothelial cells. The purpose of the studies described in this chapter is to clarify the molecular interactions that mediate this effect, specifically involving the NF-κB pathway downstream of Akt.

Our studies show that Akt stimulation of endothelial cells led to a change of cell shape from the typical epithelial like morphology to an elongated shape of a mesenchymal-like phenotype. In agreement with the phenotype of endothelial EMT, immunofluorescent staining showed that VE-cadherin delocalized from the cell membranes of cells that have undergone endothelial EMT. Interestingly, over-expression of a mutant IκBα, an inhibitor of NF-κB, completely blocked Akt-induced endothelial EMT, indicating that EMT is mediated by the NF-κB transcription factor. Further analysis revealed that IκB kinase-α (IKKα), but not IκB kinase-β (IKKβ), induce endothelial EMT. Functional examination showed that over-expression of either active-Akt or IKKα, but not IKKβ, in endothelial cells enhanced cell motility, which could be inhibited by addition of IκBα. Finally, over-expression of either Akt or IKKα induced EMT in endocardial cells in a well-established EMT atrioventricular cushion *ex vivo*

assay, which depended on the activation of NF-κB. Thus, we have identified a novel Akt/IKKα/NF-κB pathway through which EMT is induced in the endothelium.

Results

Akt induces EMT in endothelial cells via NF-κB

Constitutively active Akt (Myr-Akt) was expressed by adenoviral gene transduction in large (HUVEC) (**Figure 8A and 8B**) and microvascular (HMEC) (data not shown) endothelial cells. Thirty six hours after viral infection, there was a shape change from cobblestone like epithelial morphology to spindled, mesenchymal-like morphology, resembling EMT. A significant number of Akt-expressing cells displayed elongated morphology compared to the control, GFP expressing cells (**Figure 8E**).

One hallmark of cells undergoing EMT is the disruption of cell-cell adhesion molecules. VE-cadherin is expressed on endothelium and is the cadherin primarily responsible for endothelial cell-cell adhesion. Therefore, we examined VE-cadherin distribution in HUVECs. In control vector transfected cells that display epithelial morphology, VE-cadherin was located on the cell surface at cell-cell junctions. In contrast, in cells expressing myr-Akt, VE-cadherin localization at the cell membrane was disrupted, consistent with endothelial EMT (**Figure 8F and 8G**). Identical results were observed in microvascular endothelial cells (data not shown).

The next experiments were designed to map the downstream genes responsible for Akt-induced endothelial EMT. Since similar responses were observed in both large and microvascular endothelial cells, HUVECs were chosen for further studies. Although Akt

activates a variety of downstream target genes, we found that NF-κB is responsible for Akt-induced endothelial EMT. Expression of a mutant IκBα known to block NF-κB activity completely abolished Akt-induced EMT in endothelial cells (**Figure 8C and 8E**). Expression of IκBα alone had no effect on endothelial EMT (**Figure 8D and 8E**). Consistent with the morphological changes, membrane localization of VE-cadherin was maintained in cells co-expressing Akt and IκBα (**Figure 8H**), similar to the pattern of cells expressing IκBα alone (**Figure 8I**). Western blot analysis verified that myr-Akt protein and IκBα were expressed in cells infected by Admyr-Akt or AdIκBα, respectively (**Figure 8L**). Collectively, these results demonstrate that NF-κB is responsible for Akt-mediated EMT in endothelial cells.

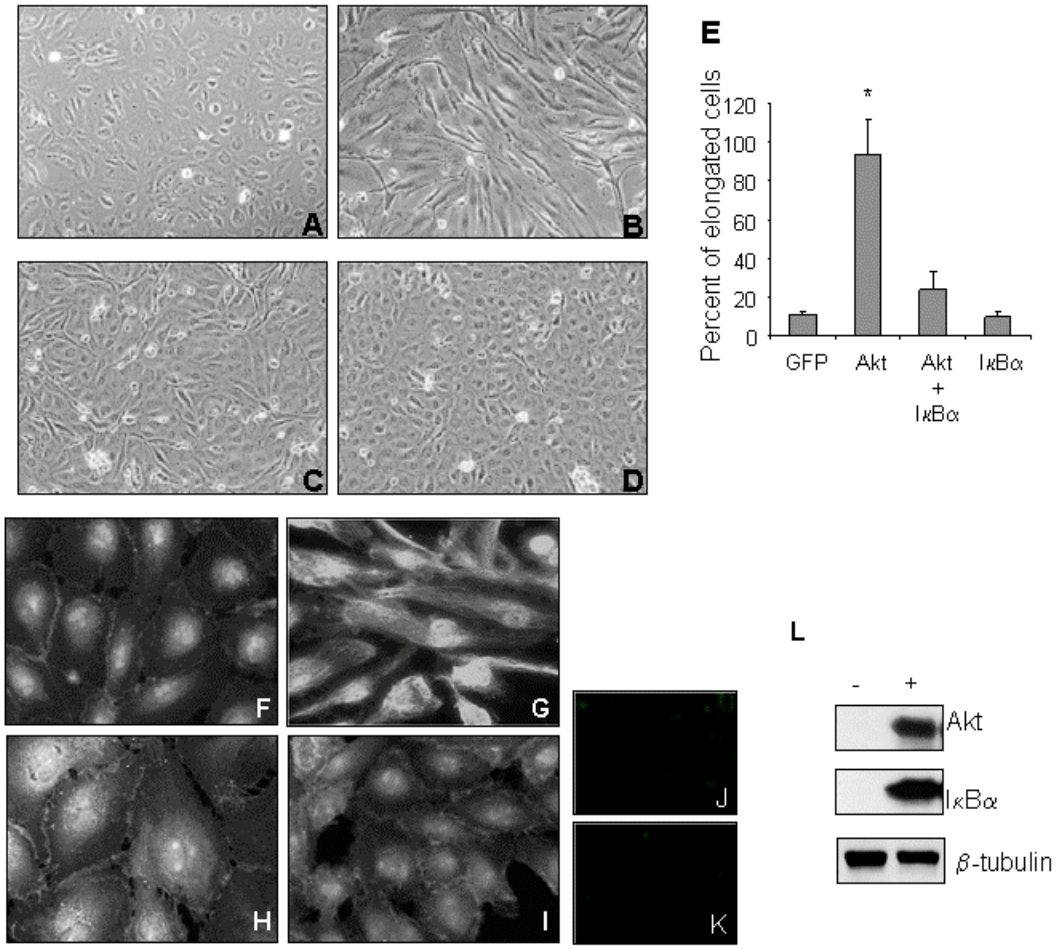

Figure 8. Akt expression induces endothelial to mesenchymal transition. HUVECs were infected with adenoviral vectors directing the expression of GFP (A), myr-Akt (B), myr-Akt and IκBα (C), and IκBα (D). The images were taken 48 hours after viral infection. The percentage of cells that acquired mesenchymal-like spindle morphology over total number of cells was quantified from three independent experiments. Mean and SE were plotted (n=6, $p < 0.001$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (E). Immunofluorescence staining for VE-cadherin was performed in adenoviral vector infected HUVECs 48 hours after infection of β-gal (F), myr-Akt (G), myr-Akt and IκBα (H), IκBα (I), VE-cadherin IgG control (J) and secondary antibody only (K). Western blot analysis was performed on HUVEC lysate infected with Adenoviral vectors for myr-Akt, and IκBα to confirm protein expression (L).

IKKα but not IKKβ induces EMT in endothelial cells

NF-κB is retained in the cytoplasm by interaction with IκBα. Upon stimulation, phosphorylation of IκBα by IKKα or IKKβ leads to degradation of IκBα and nuclear translocation of NF-κB. Therefore, we examined the role of IKKα and IKKβ in endothelial EMT. Over-expression of IKKα induced endothelial EMT (**Figure 9B and 9E**) when compared to vector control infected cells (**Figure 9A**). In contrast, cells expressing IKKβ showed almost no change in EMT (**Figure 9C**). Finally, since Akt-induced endothelial EMT depends on NF-κB activity, which in turn, depends on its phosphorylation by IκBα, we measured the effect of IκBα on IKKα-induced cell elongation. We found that blocking NF-κB with expression of IκBα significantly inhibited IKKα-induced cell elongation (**Figure 9D and 9E**).

Next, VE-cadherin distribution was examined in endothelial cells over-expressing IKKα. Consistent with the EMT phenotype, cells expressing IKKα (**Figure 9B**), but not IKKβ (**Figure 9C**) had VE-cadherin lost from the cell surface, and internalized in these cells (**Figure 9G**), similar to the pattern seen in Akt-expressing cells. Blocking NF-κB activation prevented both the IKKα-induced cell morphological change and VE-Cadherin redistribution (**Figure 9I**). Protein expression was confirmed in each group (**Figure 9L**).

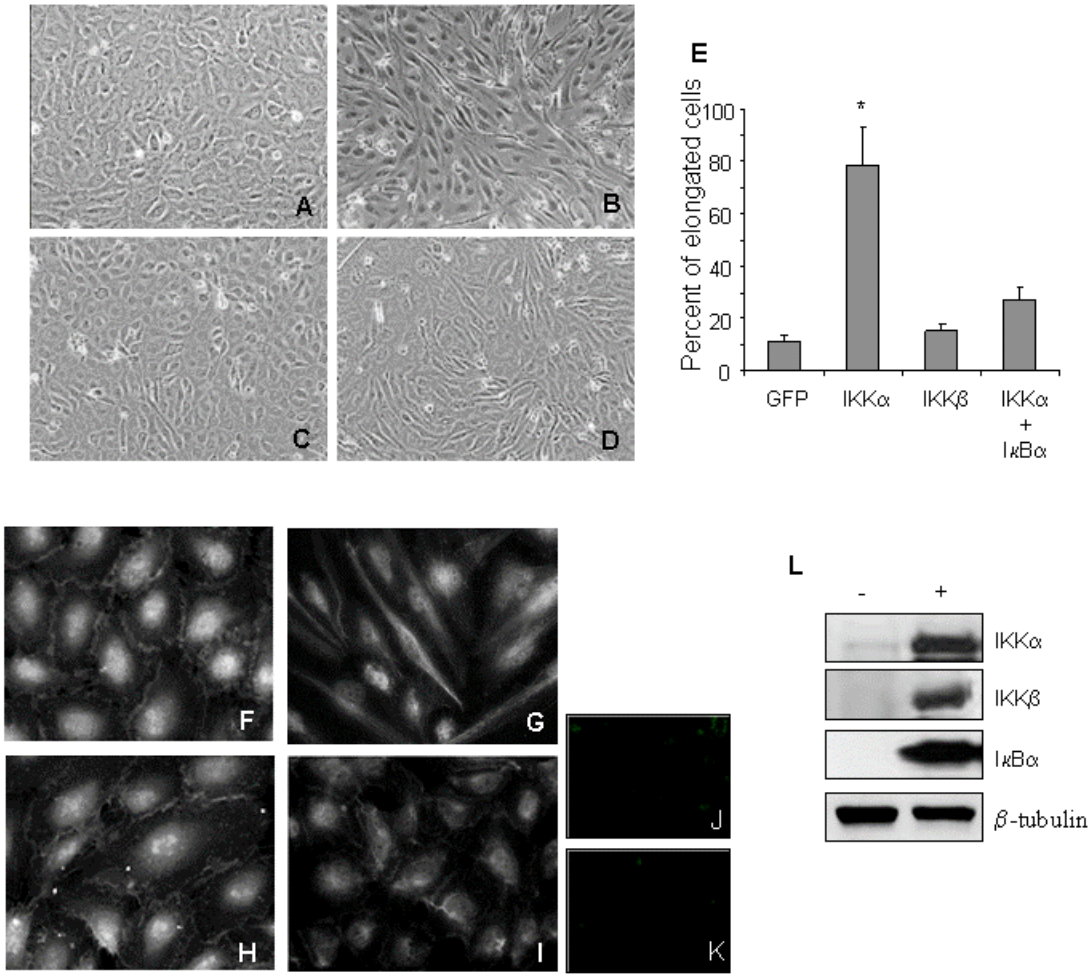

Figure 9. IKKα, but not IKKβ, induces endothelial to mesenchymal transition. HUVECs were infected with adenoviral vectors directing the expression of GFP (A), IKKα (B), IKKβ (C), and IKKα and IκBα (D). The percentage of cells that acquired mesenchymal-like spindle morphology over total number of cell was quantified from three independent experiments. Mean and SE were plotted measured (n=6, $p < 0.001$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (E). Immunofluorescence for VE-cadherin expression was performed in adenoviral vector infected HUVECs 48 hours after infection for the expression of β-gal (F), IKKα (G), IKKβ (H), and IKKα, IκBα (I), VE-Cadherin IgG control (J), and secondary antibody only (K). Western blot analysis was performed on HUVEC lysate infected with AdIKKα, AdIKKβ and AdIκBα to confirm protein expression (L).

To further verify the role of Akt in NF-κB activation in endothelial cells, we examined the effect of Akt on IKKα and κB DNA-binding. Consistent with the morphological results, expression of Akt induced phosphorylation of IKKα (**Figure 10A**) and DNA binding of κB, as demonstrated by Electromobility Shift Assay (EMSA) (**Figure 10B**). Conversely, neutralization of NF-κB using IκBα blocked Akt κB DNA-binding (**Figure 10B**). Together, these findings suggest that Akt phosphorylates IKKα in endothelial cells and leads to endothelial EMT through the NF-κB pathway.

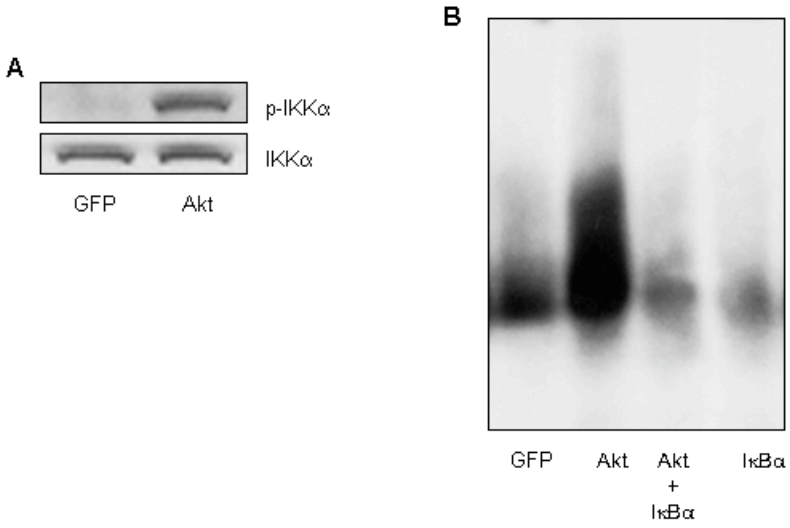

Figure 10. Akt phosphorylates IKKα and activates NF-κB. HUVECs were infected with AdGFP or Admyr-Akt and collected 24 hours later. Total IKKα was immunoprecipitated and then subject to Western blot analysis using an anti-phospho-IKKα antibody and anti-IKKα antibody(A). HUVECs were infected with AdGFP, Admyr-Akt, both Admyr-Akt and AdIκBα or AdIκBα. Cell lysate was collected 24 hours later and nuclear proteins were incubated with ^{32}P-labled DNA containing the NF-κB binding sequence. Protein and DNA were analyzed by SDS-PAGE (B).

NF-κB mediates Akt- or IKKα-induced cell migration in endothelial cells

During EMT, as epithelial/endothelial cells lose their epithelial phenotype and undergo a morphological change, cell migration is initiated. Therefore, it is conceivable that Akt-IKKα signaling, which has been shown to have a prominent role in endothelial EMT, may also be involved in mediating endothelial cell motility. To study this hypothesis, endothelial cells were infected with adenoviral vectors expressing Akt, either with or without IκBα, or control GFP for 36 hours, at which time the Akt-expressing cells displayed elongated EMT morphology. The cells were harvested and plated in a modified Boyden chamber coated with collagen I, and allowed to migrate for 6 hours. Significantly more cells migrated through the Transwell filter in Akt-expressing cells than in GFP-expressing cells (**Figure 11A**). Consistent with morphological data, blocking NF-κB activation with IκBα significantly inhibited Akt-induced endothelial cell migration (**Figure 11A**).

Given that IKKα, but not IKKβ, induced endothelial EMT, the effects of IKKα on endothelial cell migration was next examined. HUVECs were infected with adenoviral vectors directing expression of IKKα, either with or without IκBα, or control GFP. Thirty six hours later, IKKα had induced endothelial EMT. Cell migration was tested as above and showed that cells over-expressing IKKα displayed enhanced cell migration when compared to control cells. Blocking NF-κB activity using IκBα significantly attenuated IKKα induced cell migration (**Figure 11B**). In contrast, over-expression of IKKβ had no effect on endothelial cell motility, consistent with morphological observation (data not shown).

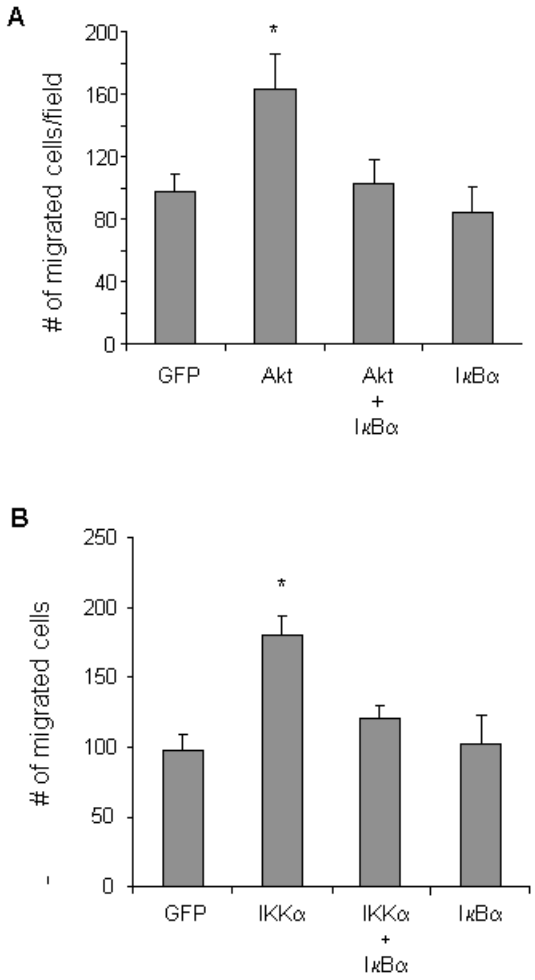

Figure 11. Akt and IKKα induce endothelial cell motility *in vitro*. HUVECs were infected with adenoviral vectors expressing GFP, myr-Akt, myr-Akt plus IκBα or only IκBα, for 48 hours. Cells were plated on a modified Boyden chamber for 6 hours. Migrating cells were fixed, stained with crystal violet, and counted in randomly selected high power (400X) fields under microscopy Mean and SE were plotted (n= 6, $p< 0.01$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (A). Endothelial cells were infected with adenoviral vectors expressing GFP, IKKα, IKKα plus IκBα or IκBα, for 48 hours. Cells were then plated on a modified Boyden chamber for 6 hours. Migrated endothelial cells from 10 randomly selected high power fields under microscopy were quantified from three independent experiments. Mean and SE were plotted (n= 6, $p< 0.001$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (B).

Akt and IKKα induce endothelial EMT in AV chick cushion explants via NF-κB

During heart development, the endocardium undergoes EMT and invades the cardiac jelly, and later forms the valves and septa of adult heart (Barnett and Desgrosellier, 2003; Eisenberg et al, 1995). Thus, the atrioventricular *ex vivo* explant assay is commonly used for examining EMT in the endocardium (Brown et al, 1999). We used this explant model to confirm the functional role of Akt and IKKα in endothelial EMT. We harvested the atrioventricular regions of the cardiac tube of a stage 15 chick and infected it with adenoviral vectors expressing the genes of interest. Explants were placed onto a collagen matrix and incubated for 24 hours. Cells that underwent EMT and migrated into the collagen matrix were scored (Desgrosellier et al., 2005). The results show efficient transgene expression in endocardial cells undergoing EMT when infected with a control vector expressing GFP (**Figure 12A**). In addition, there was no significant difference in the number of transformed cells between GFP viral vector-infected group and PBS group (data not shown). Using this *ex vivo* assay, it was found that expression of either myr-Akt or IKKα significantly increased endocardial cell EMT when compared to control GFP expressing cells (**Figure 12B**). In agreement with previous findings, co-expression of IκBα significantly attenuated Akt- and IKKα–induced endocardial EMT (**Figure 12B**). Over-expression of IKKβ had no effect in this regard (data not shown). These results in a well-described model of endocardial cell EMT verify our previous findings in HUVECs, and support the hypothesis that Akt mediates endothelial EMT through IKKα and NF-κB.

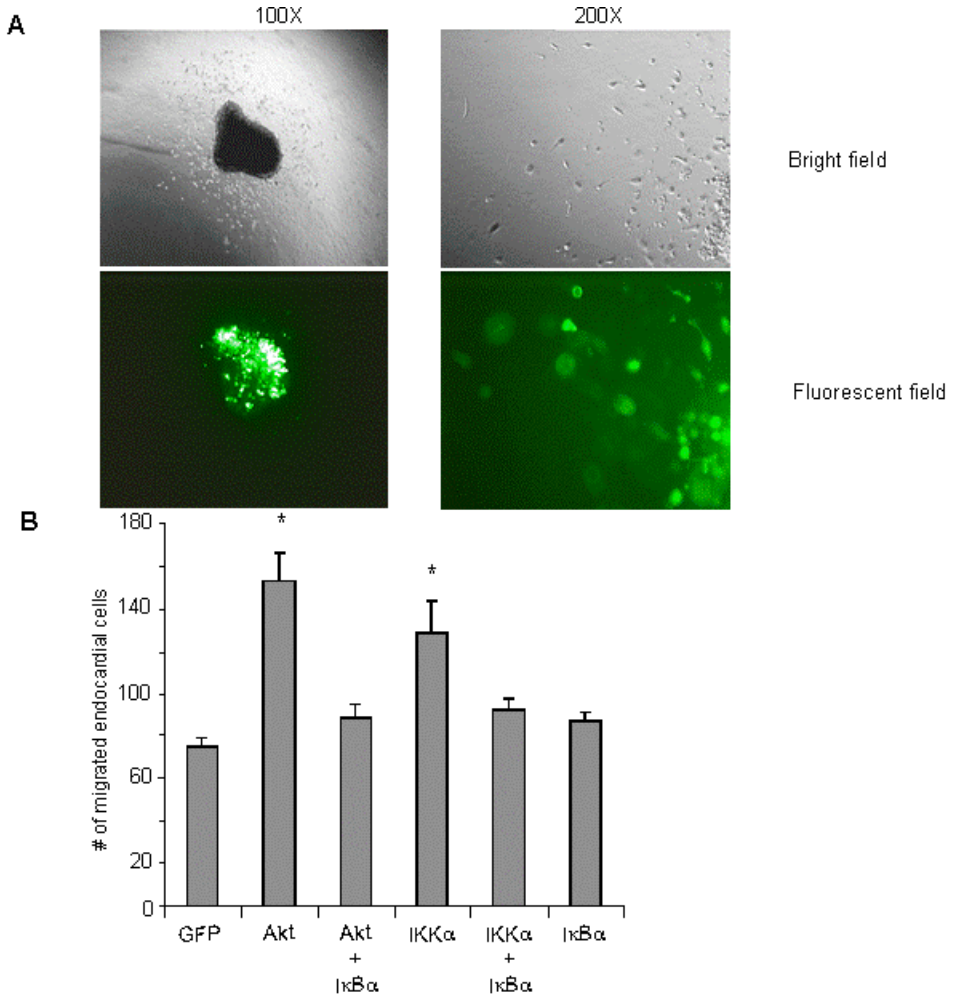

Figure 12. Akt-IKKα/NF-κB signaling mediates endothelial EMT in an *ex vivo* AV cushion assay. Chick embryos at stage 15 were harvested and AV cushions were removed, opened and then infected with a control vector expressing GFP. AV cushions were then placed endocardial side down onto a collagen I matrix. Explants were incubated 48 hours, and endocardial cells that migrated into the collagen matrix were image at bright field (top panel), and fluorescent field (bottom panel) (A). AV cushions were infected with adenoviral vectors to direct the expression of genes of interest as shown in the graph, followed by placing onto a collagen I matrix for 48 hours. Cells that migrated into the collagen matrix were scored as having undergone EMT from 10 randomly selected high power fields under microscopy were quantified from three independent experiments. Mean and SE were plotted (n=9, F=4.65, $p< 0.01$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U).

Conclusion

In summary, we showed that endothelial EMT can be induced through the action of Akt and IKKα upstream of NF-κB. Over-expression of a constitutively active Akt gene altered cell morphology, induced delocalization of VE-cadherin from cell membrane, and increased endothelial cell motility. Further, Akt induced endocardial EMT in a well-established endothelial EMT *ex vivo* assay (Brown et al, 1999). Inhibition of NF-κB activation completely abolished Akt-induced endothelial EMT. In endothelial cells, Akt phosphorylates IKKα, and over-expression of IKKα, but not IKKβ, led to endothelial EMT. With this data, we have identified a novel pathway that regulates endothelial EMT, involving NF-κB effect mediated by Akt activation of IKKα.

This work is in revision as:
DeBusk, L.M., Kamiyama, M., Barnett, J.V., Lin, P.C. "Akt induces endothelial to mesenchymal transition through the IKKα/NF-κB pathway" Dev Dynamics *in revision*.

CHAPTER V

IKKα REGULATES ENDOTHELIAL CELL MOTILITY *IN VITRO* AND TUMOR ANGIOGENESIS *IN VIVO*

Introduction

The transcription factor NF-κB is constitutively activated in many types of cancers and has been implicated in gene expression important for angiogenesis, tumor growth, progression and metastasis. Here we demonstrate that the NF-κB activator, IκB kinase-α (IκB-α) promotes tumor angiogenesis. IKKα is elevated in tumor vascular endothelium compared to normal, quiescent, endothelium. Over-expression of IKKα in endothelial cells enhanced cell motility and vascular tube formation in a 2-D culture assay compared to control cells. Co-injection of IKKα-over-expressing endothelial cells with tumor cells in mice resulted in a significant increase in tumor vascular formation compared to controls, which contributed to increased tumor growth, tumor cell proliferation and a decrease in tumor cell apoptosis. Collectively, these findings provide direct evidence that IKKα in endothelium promotes tumor angiogenesis and tumor progression.

Results

IKKα expression is increased in tumor endothelium

NF-κB is normally sequestered in cytosol by the IκB proteins. Phosphorylation of IκB by the IκB kinase complex (IKK) leads to proteosome-mediated degradation and activation of NF-κB mediated transcription. IKK contains two catalytic subunits, IKKα and IKKβ, which share a similar structure. While it is clear that the NF-κB pathway plays a critical role in tumor development, most of the focus has been placed on the role of IKKβ. To investigate the role of IKKα in tumor development, IKKα expression in tumor tissues was examined. LLC tumor cells were subcutaneously implanted in the hindlimb of C57/Bl6 mice for 2 weeks. Tumor tissues were harvested and processed for immunofluorescent staining with antibodies against IKKα (**Figure 13A and C**) and a vascular marker, CD31 (**Figure 13B and C**). Surrounding normal tissues were used as a control (**Figure 13 D-F**). The data show a significant increase of IKKα expression in tumor tissues compared to controls. Interestingly, double staining with the CD31 antibody indicates that IKKα is highly expressed in the tumor vasculature (**Figure 13A**). In addition, IKKα levels in human lung cancer samples were evaluated. A significant increase of IKKα was observed in lung cancer biopsies compared to adjacent normal tissues (**Figure 13K**). Together, these results indicate a role for IKKα in tumor angiogenesis.

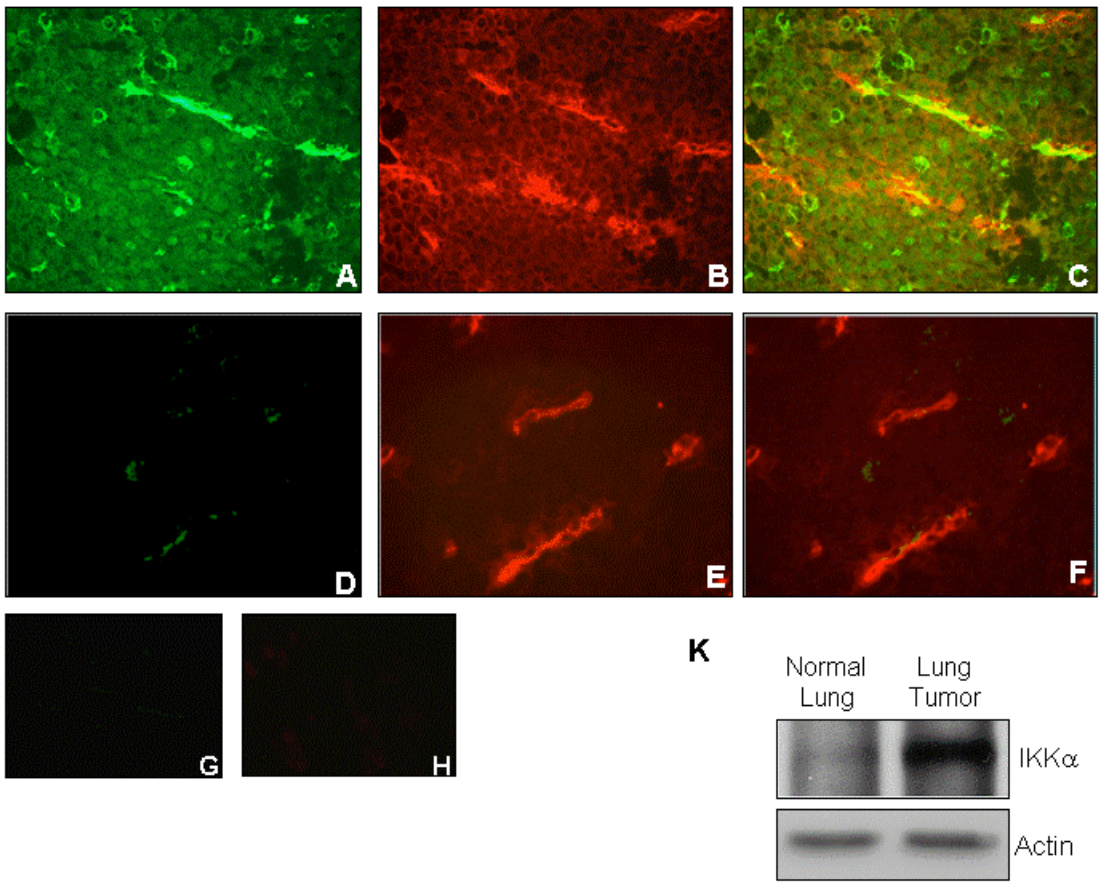

Figure 13. IKKα is expressed in tumor vasculature. Frozen tissue sections from LLC tumor tissues (top panels) and normal skin tissues (bottom panels) were immunostained with antibodies against IKKα in green (A and D), and CD31 in red (B and E). Image overlays show co-localization of IKKα and CD31 expression (C and F). Staining controls are IgG control for IKKα (G) and secondary antibody for CD31 (H). Human lung cancer biopsy and surrounding normal lung tissues were analyzed by Western blotting (K).

IKKα induces a proangiogenic phenotype in vitro

Based on the findings that IKKα expression is elevated in tumor vasculature, we reasoned that it might have a role in tumor angiogenesis. Angiogenesis is a multi-step process that includes endothelial cell migration, proliferation, and tube formation. Therefore, the function of IKKα in each of these areas was examined. Adenoviral vector was used for efficient gene delivery in endothelial cells as described (Kobayashi et al, 2006). HUVECs were infected with adenoviral vector directing the expression of IKKα, IκBα, IKKβ, or IKKα plus IκBα for 36 hours. The viral vector expressing GFP was used as a control, and it was also used to evaluate transgene transduction efficiency. Over 85% transfection efficiency, by examination of GFP expression, was achieved in cells (data not shown). Western blot analysis confirmed sufficient transgene expression in endothelial cells (**Figure 14D**).

For evaluation of cell migration, viral vector-infected HUVECs were seeded in transwells. We found that over-expression of IKKα significantly increased endothelial cell migration compared to GFP vector transfected cells (**Figure 14A**). Interestingly, the induction of migration could be completely abolished with the co-expression of IκBα, an inhibitor of NF-κB. These results suggest that IKKα promotes endothelial cell migration through activation of NF-κB signaling. In contrary, IKKβ has no significant effect on endothelial cell migration (**Figure 14A**). Consistent with the cell migration results, expression of IKKα, but not IKKβ, significantly increased vascular tube formation in 2-D Matrigel compared to control cells, and blocking NF-κB activity by co-expression of IκB completely inhibited IKKα-induced vascular network formation (**Figure 14B and 14C**). Furthermore, there is less vascular network formation in the IκB group than the

vector control (**Figure 14C**), which supports a role of NF-κB in angiogenesis. In contrast, no difference in endothelial cell proliferation was observed between these two groups by measuring the amount of BrDU incorporation in cells (data not shown). Collectively, these data suggest that IKKα mediates angiogenesis via an effect on cell motility.

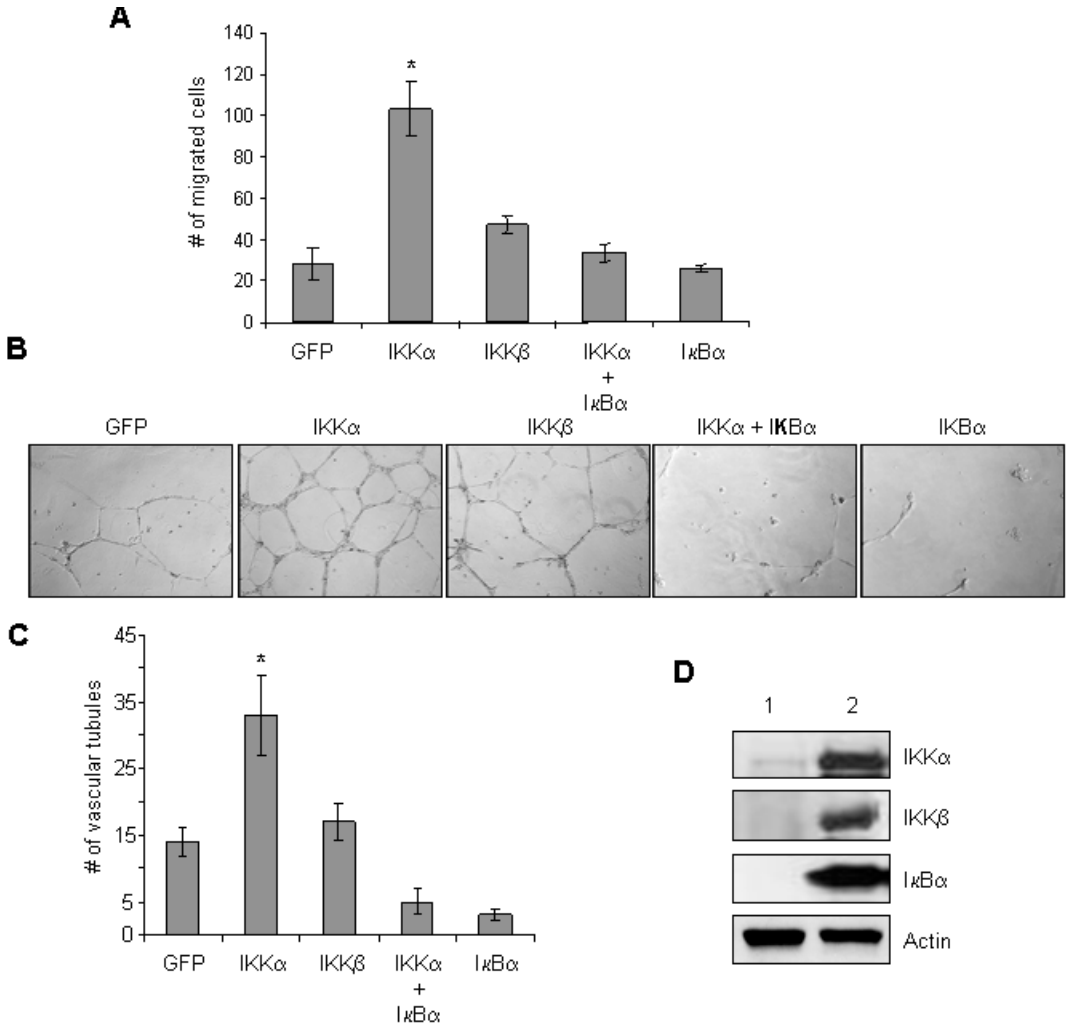

Figure 14 Expression of IKKα in endothelial cells promotes angiogenesis *in vitro*. HUVECs were. infected with adenoviral vectors directing the expression of genes as listed for 36 hours. The cells were seeded on top of Transwells, and cell migration was evaluated 5 hours later. Migrated endothelial cells from 10 randomly selected high power fields under microscopy were quantified from three independent experiments. Mean and SE were plotted (n= 6, $p< 0.001$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (A). Adenoviral vector transfected HUVECs were seeded on top of Matrigel, followed by incubation for 24 hours (B). Vascular branch points were counted in 10 randomly selected fields under microscopy. Mean and SE were plotted (n= 6, $p< 0.01$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (C). Infected HUVECs expressing IKKα, IKKβ, and IκBα were analyzed by Western blotting for transgene expression (D).

IKKα promotes tumor angiogenesis and tumor growth in vivo

To further investigate the role of IKKα in tumor angiogenesis, a tumor cell and endothelial cell co-transplantation assay was used as described (Brantley-Sieders et al, 2005). LLC-tumor cells were mixed with adenoviral vector infected-HUVECs expressing either IKKα or GFP in a Matrigel matrix, and then injected subcutaneously in immune deficient Rag-1 mice. Controls also include mice injected with tumor cells alone in Matrigel. Tumor volume was measured over time. The data show that tumors mixed with IKKα-expressing endothelial cells grew more quickly, and the tumors were much larger, than tumors containing GFP-expressing endothelial cells and control tumors (**Figure 15**).

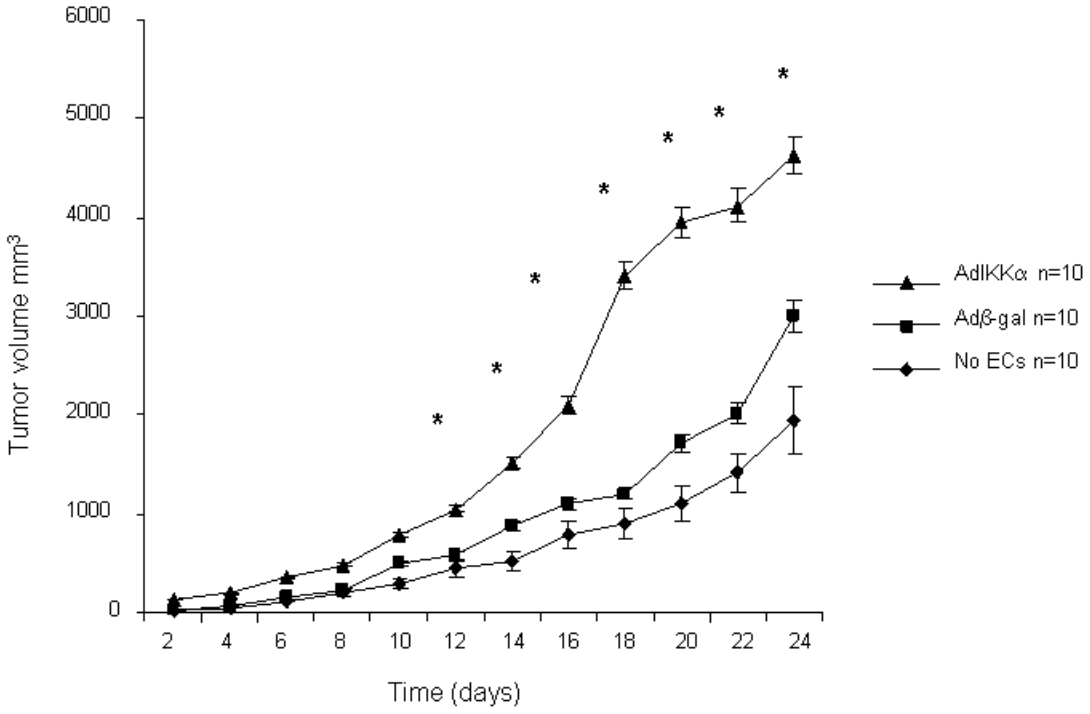

Figure 15. IKKα over-expression endothelial cells promotes tumor growth. LLC-tumor cells ($1x10^5$) were mixed in a Matrigel matrix with HUVECs ($5x10^5$) expressing either IKKα or β-gal, then co-injected sub-cutaneously in Rag-1 mice. Control mice were injected with tumor cells alone in Matrigel. Tumor volume was measured every other day by calipers. Tumor volume was calculated and graphed. Mean and SE were plotted (n= 10, F= 3.54, $*p < 0.01$ by ANOVA)

To investigate the molecular mechanism, tumors were harvested and processed for histological analysis. Tumor sections were immunostained with an antibody against pan CD31, which recognizes both murine and human endothelium. Vascular density was calculated by counting CD31 positive vessels in tumor sections. The data show that tumor cells mixed with IKKα-expressing endothelial cells had a significantly higher vascular density than tumors mixed with GFP-expressing endothelial cells. Control tumors, containing no endothelial cells, had the lowest vascular density (**Figure 16A-E**).

Based on *in vitro* data that IKKα promotes endothelial cell migration and vascular network formation, we postulated that IKKα-expressing endothelial cells increased tumor angiogenesis through direct incorporation into tumor vasculature. To test this hypothesis, a CD31 antibody specific for human endothelial cells, for detecting the infected HUVECs, and a CD31 antibody specific for murine endothelium for detecting the host-derived vasculature, were used. The co-staining confirmed incorporation of exogenous human endothelial cells into the tumor vasculature in both groups (**Figure 16F-G**). Most importantly, there are significantly more IKKα-expressing HUVECs incorporated into the tumor vasculature than β-gal expressing HUVECs. (**Figure 16J**). This is consistent with the *in vitro* data demonstrating that IKKα over-expression mediates a more angiogenic phenotype in endothelial cells. Together, these data demonstrate that activation of IKKα in endothelial cells promotes tumor angiogenesis.

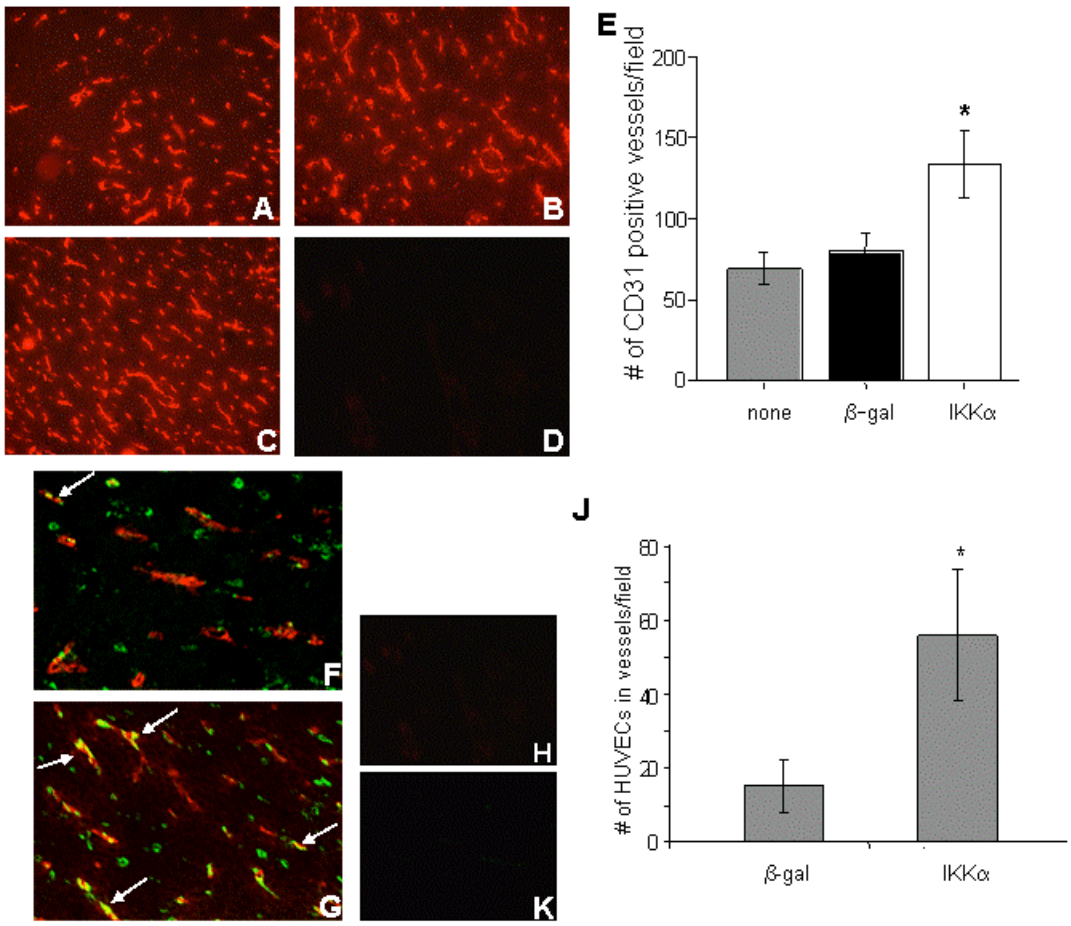

Figure 16. IKKα over-expression in endothelial cells promotes tumor vascular formation through direct incorporation into tumor vasculature. Tumors grown with no HUVECs (A), β-gal expressing HUVECs (B) or IKKα expressing HUVECs (C), were harvested 8 days after implantation. Tumor sections were immunostained with antibody against pan CD31, which recognizes both human and murine endothelium or secondary antibody only (D). The number of CD31 positive blood vessels were counted in 10 randomly selected 200X field. Mean and SE were plotted (n=8, *p<0.05 by ANOVA)(E). Sections from tumors grown with b-gal expressing HUVECs (F) or IKKα expressing HUVECs were double-stained with antibodies against human specific CD31 (green), murine specific CD31 (red), or with secondary antibody only (H) or an IgG control for IKKα (K). The total number of HUVECs incorporated into the vasculature was counted in both the AdIKKα and Adβ-gal tissues (n=6, p< 0.001 by Student's t-test, *p < 0.05 by Mann-Whitney U) (J).

Next, apoptosis and cell proliferation were examined in these tumor samples. Tumors tissue sections were subjected to TUNEL staining to quantify the number of cells undergoing apoptosis. We found there was less apoptosis in the tumor cells mixed with IKKα-expressing endothelial cells than both control groups (**Figure 17A-D**). Similarly, an analysis of cell proliferation using PCNA immunostaining demonstrated an increase in cell proliferation in tumor cells mixed with IKKα-expressing endothelial cells compared to either control (**Figure 17E-H**). These findings are consistent with the observation that IKKα promotes tumor angiogenesis that improves tissue perfusion. As a result, IKKα expression increases cell proliferation, reduces apoptosis, and increases tumor growth and progression.

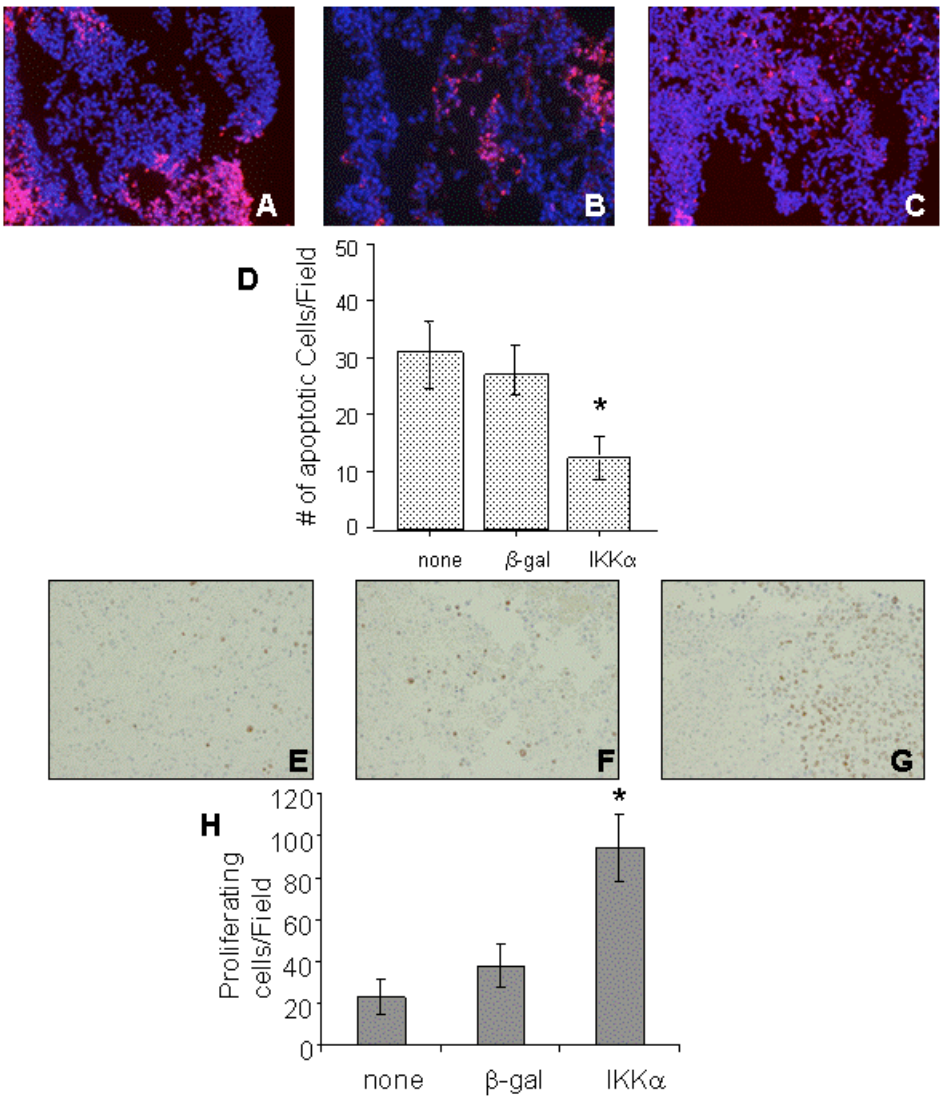

Figure 17. IKKα expression in the endothelium decreased tumor apoptosis and increased cell proliferation. Tumors grown with no HUVECs (A and E), β-gal expressing HUVECs (B and F) or IKKα expressing HUVECs (C and G)), were harvested 8 days after implantation. Tumors tissue sections from each group were subject to TUNEL staining (A through C). TUNEL positive apoptotic cells (pink) were quantified in 10 randomly selected high power fields under microscopy Mean and SE were plotted (n=8, $p< 0.01$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (D). *p<0.01 . Tumosections were also immunstained with PCNA (E through G). Proliferating cells were counted in 10 randomly selected high power fields under microscopy Mean and SE were plotted (n=8, $p< 0.001$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U)(H).

Conclusion

In summary, this study reveals a new role of IKKα in angiogenesis that is dependent on NF-κB activity. We observed an elevation of IKKα levels in tumor endothelium compared to quiescent endothelium. IKKα induces a proangiogenic phenotype both *in vitro* and *in vivo*. Tumor cells mixed with IKKα-over-expressing endothelial cells grew larger and were more vascularized, resulting in decreased tumor cell apoptosis and increased tumor cell proliferation. A more complete understanding of the molecular mechanism of tumor angiogenesis offers the promise for the development of therapeutic strategies for cancer treatment. This study identifies IKKα signaling in the endothelium as a pro-angiogenic pathway.

This work is in preparation as:
DeBusk LM and Lin, PC. "IKK-alpha, but not IKK-beta, enhances endothelial cell motility in vitro and tumor angiogenesis in vivo."

CHAPTER VI

THE IKKα REGULATED PROTEIN, VAV1, IS REQUIRED FOR IKKα MEDIATED ENDOTHELIAL EMT AND IS PRO-ANGIOGENIC

Introduction

The previous chapters describe the roles of Akt in endothelial cell survival and endothelial cell sprouting (Chapter III) and both Akt and IKKα in the induction of endothelial EMT and endothelial cell motility (Chapter IV). In addition, the effect of endothelial cell IKKα expression on tumor angiogenesis was determined (Chapter V). IKKα has been shown to activate the NF-κB signaling pathway, as well as promote transcription by phosphorylating histones. Since IKKα plays such an important role in gene regulation, we identified gene targets upregulated by over-expression of IKKα. A gene array analysis was performed and genes upregulated in IKKα-over-expressing endothelial cells were identified. Two of these upregulated genes, Vav1 and δ-catenin (discussed in chapter VII), were shown to be important in the induction of endothelial EMT, endothelial cell motility and tumor angiogenesis. In this chapter, we show that Vav1 is required for IKKα-induced endothelial EMT. We also demonstrate that Vav1 induces a pro-angiogenic phenotype *in vitro* and promotes endothelial sprouting in an *ex vivo* assay. Finally, we show that Vav1 expression in the vascular endothelium promotes tumor angiogenesis and subsequent tumor growth.

Results

IKKα regulates Vav1 expression in endothelial cells

Since IKKα was shown to induce endothelial EMT and induce a proangiogenic phenotype *in vitro* and *in vivo* in earlier chapters in this work and since IKKα is known to be involved in gene regulation, a gene array analysis was conducted to identify genes that are regulated by IKKα. Candidate genes were identified with gene array analysis where gene upregulation was compared in endothelial cells over-expressing IKKα and endothelial cells expressing the β-gal gene (Data not shown). The analysis revealed a dramatic, 12-fold increase in the expression of the Vav1 gene as well as a dramatic increase of δ-catenin expression (to be described in Chapter VII). Real-time PCR and Western blot analysis showed significantly greater expression of this gene and gene product, respectively, in IKKα- transfected endothelial cells compared to control cells (**Figure 18A-C**).

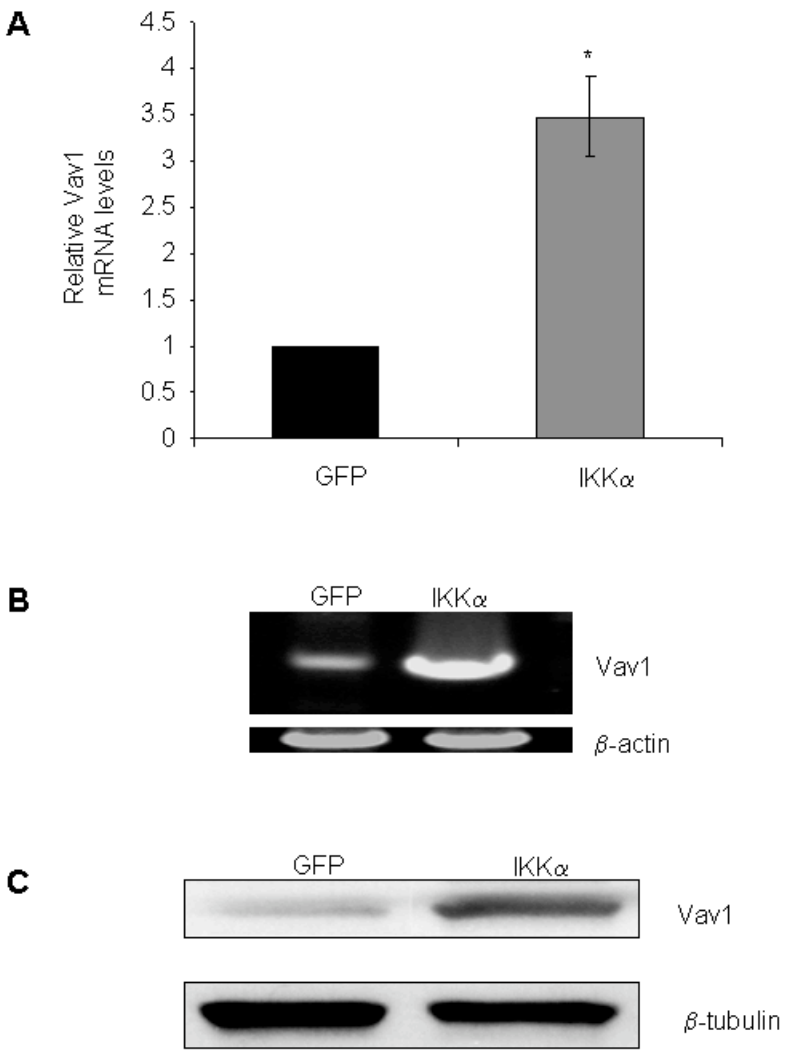

Figure 18. IKKα increases Vav1 expression. IKKα upregulates Vav1 expression in endothelial cells. HUVECs were infected with adenovirus directing the expression of GFP or IKKα. RNA was collected and subject to reverse transcription and the resulting cDNA was subject to Real-time PCR for Vav1. Relative levels of mRNA were quantified Mean and SE were plotted (n=4) (n=8, F=4.58, $p < 0.001$ by Student's t-test, $*p < 0.001$ by Mann-Whitney U) (A). The Real-time PCR product was run on an agarose gel (B). Cell lysate was collected and Western Blot analysis was performed for expression of Vav1 protein (C).

Vav1 is expressed in endothelial cells and vascular endothelium, but not in epithelial cells

With Vav1 identified as a candidate gene and as a potential target of IKKα in EMT and angiogenesis, the specificity of Vav1 expression was next determined. Vav1 has previously been shown to be expressed in hematopoietic cells and endothelial cells, but not in other type of cells. To verify this finding, we checked primary lung endothelial cells isolated from wildtype mice and Vav1-null mice, an immortalized mouse embryonic endothelial cell line, and the mouse epithelial cell lines, 4T1, LLC, and UG251, for Vav1 expression. Western blot analysis demonstrated clear Vav1 expression in endothelial cells from both freshly isolated primary lung endothelial cells and immortalized cell line, while all the epithelial cells tested and negative control, Vav1-null endothelial cells, did not express Vav1 protein(**Figure 19A**)..

To further examine the expression of Vav1 in vivo, immunofluorescence staining was conducted on tissue samples (**Figure 19B**). Liver tumor tissue removed for biopsy from patients was double stained with antibodies for both Vav1 and the vascular marker, von Willebrand factor. Overlay of the images show that Vav1 was expressed in many tumor blood vessels. Therefore, these data show that Vav1 is expressed in endothelial cells *in vitro*, and more importantly, it is expressed in the tumor vascular endothelium *in vivo*.

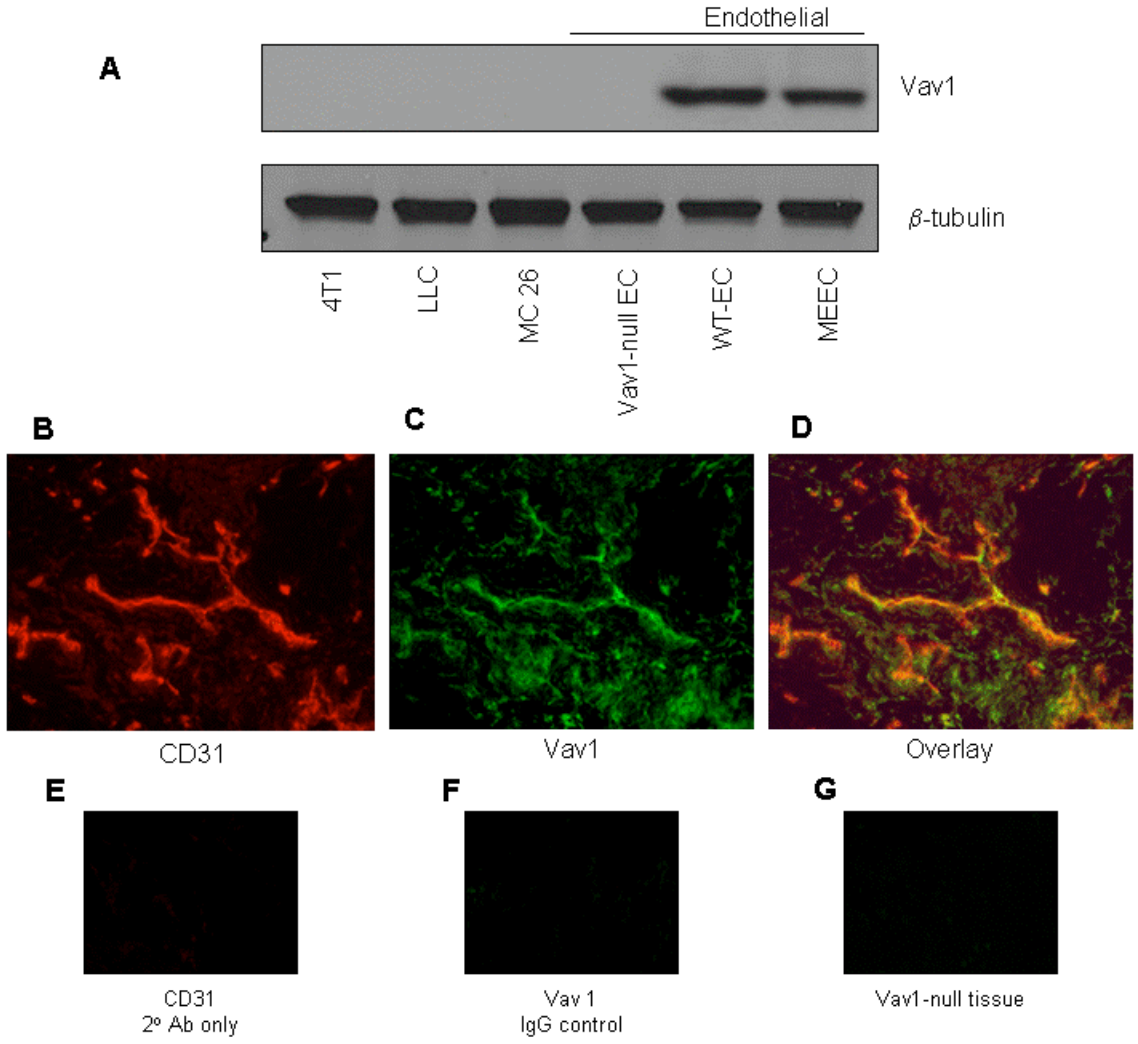

Figure 19. Vav1 is expressed in endothelial cells and vascular endothelium but not in epithelial cells. Mouse epithelial and endothelial cell lines and primary cells were analyzed by Western blotting for Vav1 and β-tubulin expression (A). Frozen tissue sections from human liver tumor tissues and normal skin tissues were immunostained with antibodies against CD31 (red) and Vav1 (green). Image overlay show co-localization of Vav1 and CD31 expression. Staining controls are secondary antibody for CD31 and IgG control for Vav1 (B).

Vav1 expression is required for IKKα-induced endothelial EMT

We have established that Vav1 is expressed in endothelial cells in response to IKKα stimulation. To determine if Vav1 plays a functional role in the induction of EMT in endothelial cells, primary lung endothelial cells isolated from either Vav1-null or wildtype mice were infected with adenovirus directing the expression of IKKα or GFP. EMT was induced with IKKα overexpression in the wild-type endothelial cells, but not in Vav1-null endothelial cells, as determined by cell shape change and VE-cadherin localization (**Figure 20A**). These results were verified by knocking down Vav1 expression using siRNA (data not shown). Endothelial cells transfected with Vav1 shRNA vector, and then infected with AdIKKα, failed to undergo EMT, while the cells transfected with control shRNA and then infected with AdIKKα clearly demonstrated EMT. These data suggest Vav1 mediates IKKα induced endothelial EMT and cell motility.

Since Vav1 is absent in epithelial cells (**Figure 19**), we therefore determined whether these cells would respond to IKKα stimulation. Consistent with the data in endothelial cells, expression of IKKα in epithelial cells failed to induce EMT, as determined by cell shape change and E-cadherin localization (**Figure 20B**). Most excitingly, transfection of the epithelial cells with the Vav1 gene, restored sensitivity to IKKα stimulation, resulting in induction of EMT compared to controls. (**Figure 20B**). Collectively, our findings strongly support that Vav1 mediates EMT in response to IKKα stimulation.

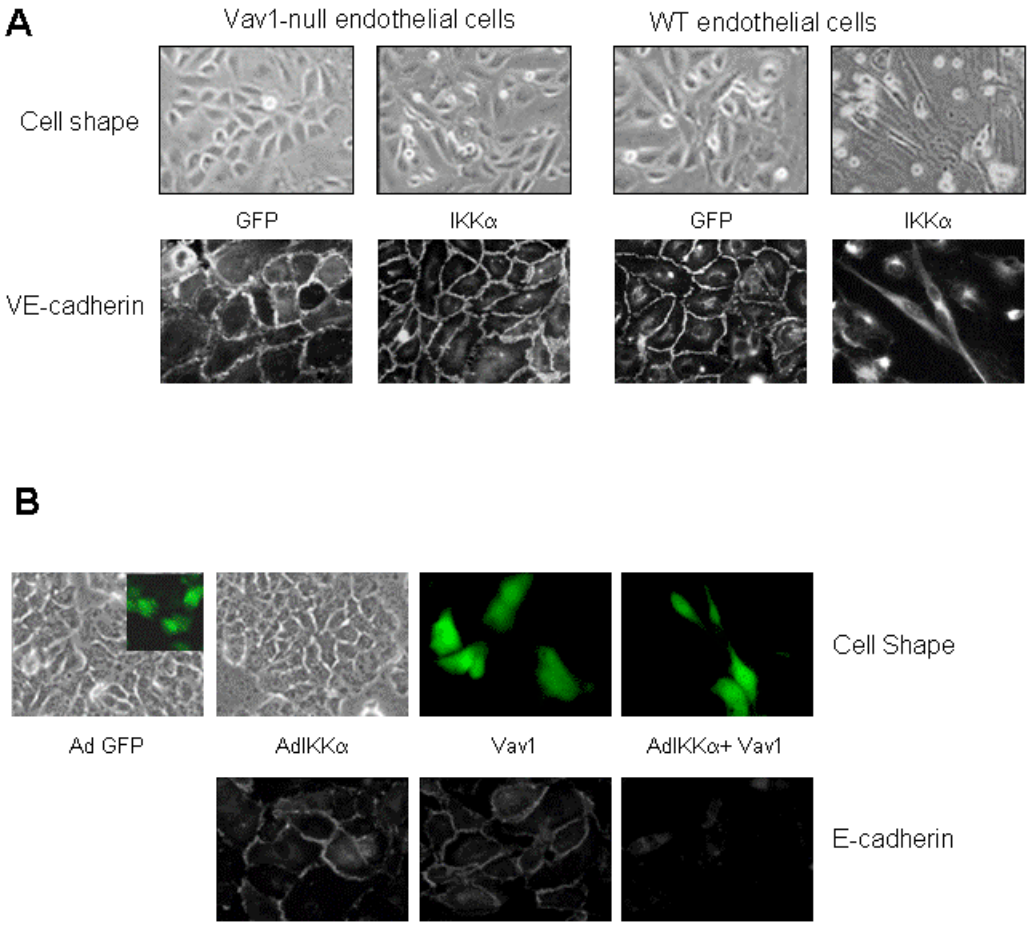

Figure 20. Deletion of Vav1 impairs IKKα mediated EMT and, reconstitution of Vav1 in epithelial cells restores IKKα induced EMT. Endothelial cells were harvested from Vav1-null and wildtype control mice. Both Vav1-null and control endothelial cells were infected with adenovirus directing the expression of GFP or IKKα and were either imaged 48 hours later or subject to immunocytochemistry in order to visualize VE-cadherin localization (A). A431 tumor cells were either infected with AdIKKα, transfected with plasmid pEF-Vav1 plasmid, or transduced with both IKKα and Vav1. Cells were imaged 48 hours later or subject to immunocytochemistry in order to visualize E-cadherin localization (B).

Vav1 mediates the angiogenic phenotype in vitro

Based on the findings that Vav1 mediates IKKα induced endothelial EMT, we next examined the effect of Vav1 on the IKKα-induced pro-angiogenic phenotype, specifically in cell migration, proliferation and assembly of vascular structures. Using a modified Boyden chamber assay, we found a significant impairment of cell migration in endothelial cells isolated from Vav1-null mice compared to the ones from wild type mice. Interestingly, reexpression of Vav1 in Vav1 null endothelial cells totally restored the migratory ability (**Figure 21A**). Consistent with the defective phenotype in cell motility, Vav1 null endothelial cells display impaired vascular tubule formation capability in a Matrigel assay compared to wild type cells. Reciprocally, when Vav1 expression was restored to these cells, tubule formation was also restored (**Figure 21B**).

Next, we investigated the role of Vav1 in endothelial cell proliferation by counting live cells in culture over time. There was no significant difference in cell counts between Vav1-null and Vav1-expressing endothelial cells. (**Figure 21C**). Thus, the collective data show that Vav1 regulates angiogenesis through effects on cell migration and tubule formation, but not cell proliferation.

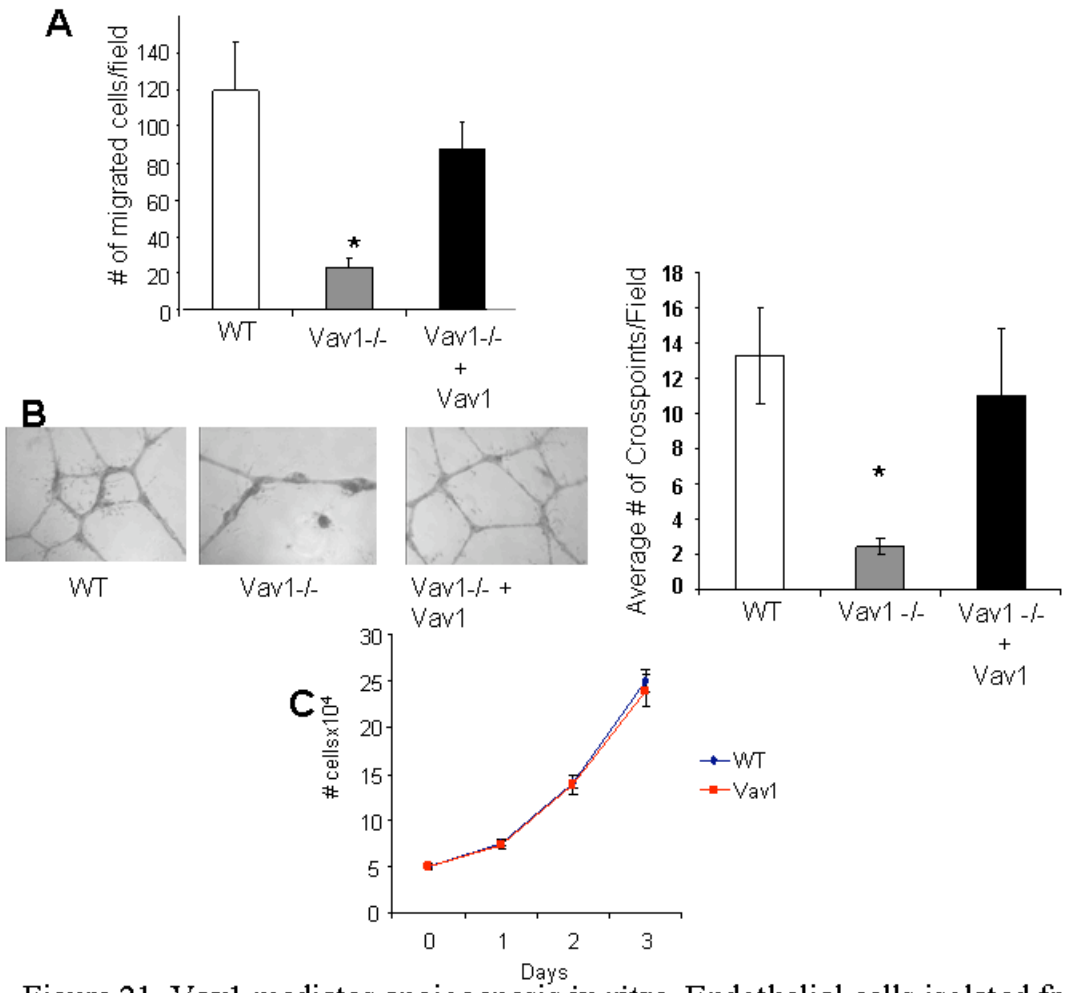

Figure 21. Vav1 mediates angiogenesis *in vitro*. Endothelial cells isolated from wild-type and Vav1 null mice and Vav1 null cells transfected with a Vav1 expression vector were analyzed for migration in a modified Boyden chamber *using VEGF (25 ng/ml) as a chemoattractant. Migrated endothelial cells from 10 randomly selected high power fields under microscopy were quantified from three independent experiments. Mean and SE were plotted (n=4, $p < 0.001$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (A). Primary endothelial cells were subject to a vascular tube formation assay in Matrigel. The images were acquired 24 hours after incubation. Tubules were counted in 10 randomly selected fields and quantified from three independent experiments. Mean and SE were plotted (n=4, $p < 0.001$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (B). Primary endothelial cells were cultured in growth media. Live cells were counted every 24 hours (n=3, $p < 0.001$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (C).

Endothelial cell expression of Vav1 promotes tumor growth and angiogenesis in vivo

To continue investigating the role of Vav1 in angiogenesis, we used an *ex vivo* aortic ring assay. Small pieces of aortas from Vav1-null or wild-type mice were embedded in a fibrin matrix, and endothelial sprouts were counted 7 days later. There were significantly more endothelial sprouts from the aortic rings of wild-type mice compared to those of Vav1-null mice (**Figure 22A**), consistent with the findings from the in vitro studies.

Finally, we verified the angiogenic function of Vav1 in vivo. Since Vav1 is also expressed in hematopoietic cells in addition to endothelial cells, that could contribute to vascular formation. To distinguish the contribution of Vav1 in endothelium (angiogenesis) from hematopoietic cells, we performed bone marrow transplantation. Recipient Vav1-null and wild-type mice were lethally irradiated to eliminate endogenous hematopoietic stem and progenitor cells, followed by injection of bone marrow cells harvested from donor wild type mice. Six weeks after the bone marrow transplantation when the majority of the hematopoieitc cells of the Vav1-null mice were replaced with wild-type cells, limiting the contribution from hematopoietic cells, LLC tumor cells were injected subcutaneously in the hind-limb of these mice. We found a significant inhibition of tumor growth in the Vav1-null mice that had received wild type bone marrow compared to the control wild-type mice that had received wild type bone marrow transplantation (**Figure 22B**). Similarly, tissue analysis for vascular density confirmed a significant impairment in the Vav1 null group compared to controls, suggesting deletion of Vav1 in endothelium impaired tumor angiogenesis that contributed to inhibited tumor

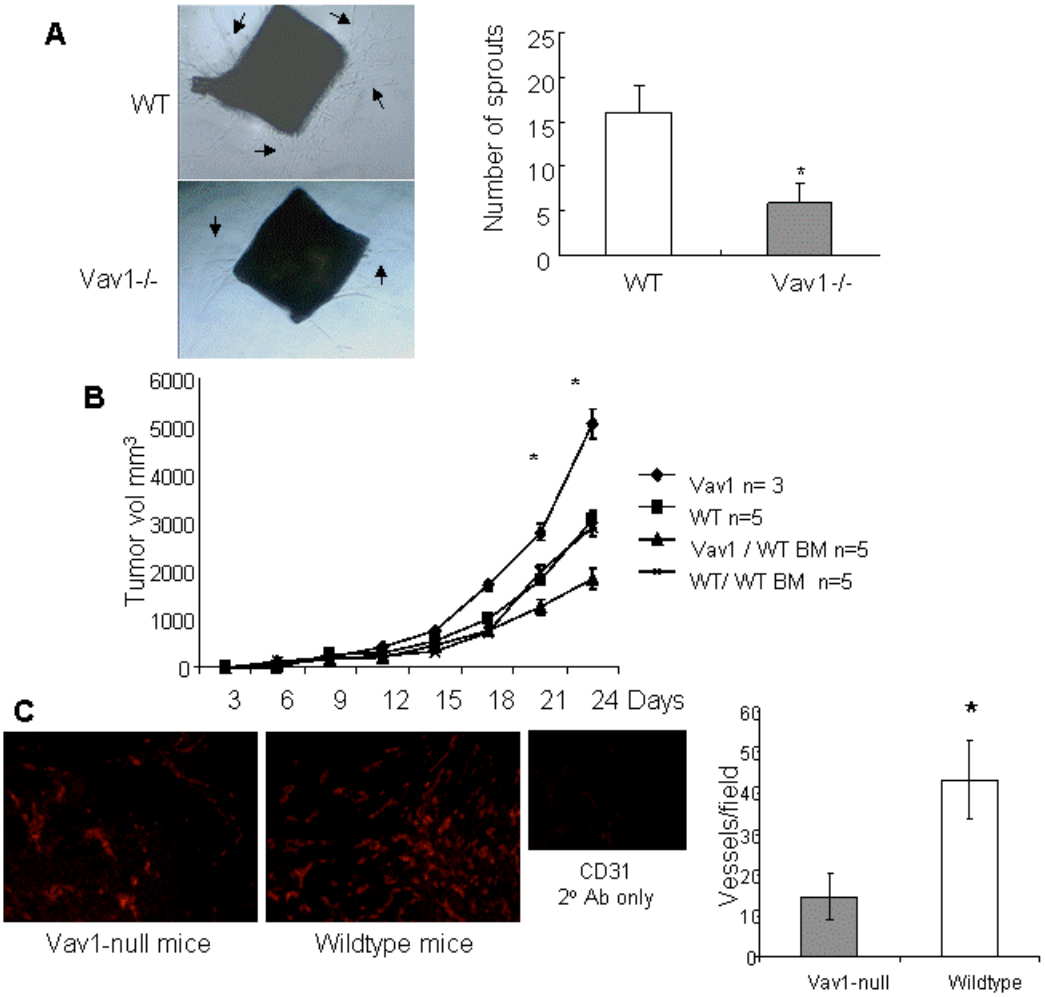

Figure 22. Vav1 is angiogenic *ex vivo* and *in vivo*. Aortas from Vav1-null and wildtype mice were removed, sliced and embedded in fibrinogen. Endothelial sprouts were counted 7days later. Mean and SE were plotted (n=5, $p < 0.001$ by Student's t-test, $*p < 0.05$ by Mann-Whitney U) (A). Vav1-null and wildtype mice were subject to bone marrow transplantation with wildtype bone marrow. LLC tumor cells were injected subcutaneously 4 weeks later. Tumor volume was measured for the next 24 days(n values on graph, $F=3.22$, $*p < 0.01$ by ANOVA) (B). Frozen tissue sections from the LLC tumor tissues were immunostained with antibodies against CD31. The number of CD31 positive blood vessels were counted in 10 randomly selected 200X field and were quantified from three independent experiments. Mean and SE were plotted (n= 6, $p < 0.001$ by Student's t-test, $*p < 0.05$ by Mann-Whitney U)(C).

growth in vivo (**Figure 22C**).

Conclusions

In conclusion, the data reveal that Vav1 is specifically expressed in endothelial cells and IKKα regulates its expression. Vav1 mediates IKKα induced endothelial EMT. Accordingly, overexpression of Vav1 in endothelia cells increases endothelial cell motility and vascular network formation, and deletion or knockdown of Vav1 in endothelial cells impairs angiogenic function. We further confirmed the angiogenic function of Vav1 in tumor angiogenesis. Together, this study identifies a novel function of Vav1 in angiogenesis. It may identify a potential therapeutic target for anti angiogenic therapy.

This work is in preparation as:

Huang, J., **DeBusk, L.M.,** Vaughan, D.E., Min, Y. and Lin, P.C. "Vav1 regulates angiogenesis in heart and protects from ischemia-induced heart failure." (submitted)

Min, Y.*, **DeBusk, L.M. *** , Ren, X., Huang, J. and Lin, P.C. "Opposing functions of Vav1 in tumor angiogenesis and vasculogenesis." *** equal contribution**

CHAPTER VII

THE IKKα REGULATED PROTEIN, δ-CATENIN, IS REQUIRED FOR IKKα MEDIATED ENDOTHELIAL EMT AND IS PRO-ANGIOGENIC

Introduction

In the search for IKKα target genes in endothelial EMT and angiogenesis, we identified δ-catenin, a gene that was previously reported only present in neuronal cells and regulates neuronal cell migration. Interestingly, we detected δ-catenin expression in primary endothelial cells, as well as vasculature in vivo. Overexpression of δ-catenin induced a pro-angiogenic phenotype *in vitro*, as demonstrated by increased endothelial cell migration and tubule formation, while knockdown of δ-catenin expression blocked this phenotype. Since Rho family GTPases are critical for cell motility, the effects of δ-catenin expression on the activation of these proteins was examined. Cdc42 activation was increased, while RhoA activation was decreased, in endothelial cells overexpressing δ-catenin. Knockdown of δ-catenin reversed this phenotype. On the other hand, Rac1 activation remained unchanged. Furthermore, δ-catenin-null mice were used to examine the role of δ-catenin on tumor angiogenesis *in vivo*. 3LL cells, a highly metastatic form of Lewis Lung Carcinoma (LLC) cells, were injected subcutaneously in δ-catenin homozygous null, δ-catenin heterozygous, and wildtype mice. Tumor growth and vascular density were measured. Tumors grew much slower in δ-catenin homozygous null mice compared to wildtype controls. Interestingly, tumors grown in δ-catenin heterozygous mice had a growth rate very similar to that of tumors grown in null mice. Likewise, the vascular densities of tumors grown in both δ-catenin heterozygous and

homozygous null mice were significantly less than in tumors grown in wildtype mice. These data show that δ-catenin plays a role in endothelial cell motility and in tumor angiogenesis.

Results*IKKα and Vav1 regulates δ-catenin expression in endothelial cells*

As described above, a gene array analysis was performed to identify genes regulated by IKKα expression. Both δ-catenin and Vav1 were identified in this array, and Vav1 was characterized in Chapter VI. Since Vav1 has also been shown to play a role in gene regulation in T-cells (Houlard et al., 2002), we performed a second gene array to determine the effect of Vav1 on IKKα-induced gene expression. IKKα was over-expressed in both wildtype endothelial cells and Vav1-null endothelial cells, followed by microarray analysis. We found increased δ-catenin expression levels in wildtype endothelial cells, while Vav1-null cells showed no such increase suggesting IKKα induced δ-catenin expression depends on Vav1. The gene array results were verified with Real-Time PCR (**Figure 23A and B**). Both IKKα and Vav1 over-expression could increase δ-catenin expression. This finding was verified with Western blot analysis (**Figure 23C**). Interestingly, we also found that IKKα-induced expression of δ-catenin increased only in the presence of Vav1 when compared to β-actin expression levels in the same cell population (**Figure 23D**). Endothelial cells isolated from Vav1-null mice were infected with either AdGFP, AdIKKα or AdIKKα plus transfection of Vav1. Over-expression of IKKα in the Vav1-null cells did not increase δ-catenin expression. Co-expression of both IKKα and Vav1, however did increase levels of δ-catenin protein in

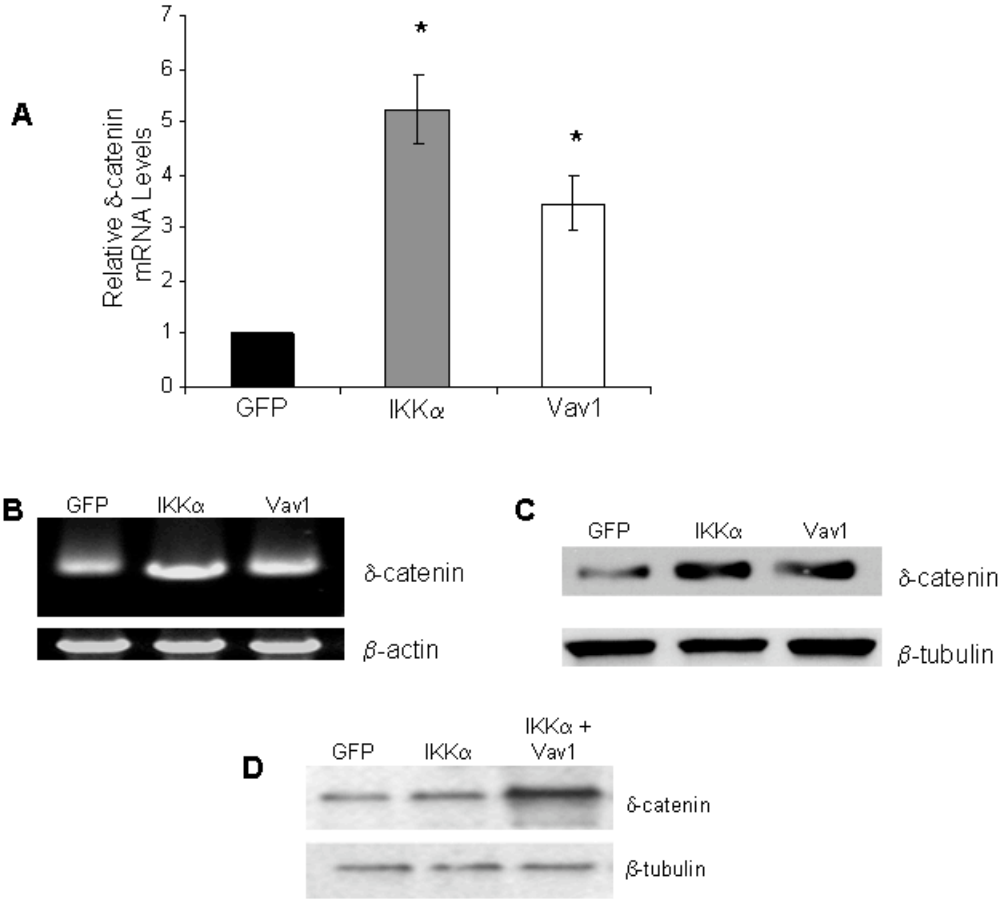

Figure 23. IKKα and Vav1 increase δ-catenin expression and Vav1 is necessary for IKKα regulation of δ-catenin expression. HUVECs were infected with adenovirus directing the expression of GFP or IKKα or transfected with the a Vav1 expression vector. RNA was collected and subject to reverse transcription, and the resulting cDNA was used for Real-time PCR. Relative levels of mRNA were quantified from three independent experiments. Mean and SE were plotted (n=4, $p < 0.001$ by Student's t-test, $*p < 0.001$ by Mann-Whitney U) A). The Real-time PCR product was run on an agarose gel (B). Cell lysates were collected and Western Blot analysis was performed for expression of δ-catenin protein (C). Vav1-null endothelial cells were infected with AdGFP, AdIKKα or AdIKKα + Vav1 transfection. Cell lysates were collected and Western Blot analysis was performed for expression of δ-catenin protein.

the cells. These results show that expression of δ-catenin in endothelial cells is controlled by both IKKα and Vav1.

δ-catenin expression in endothelial cells is verified

δ-catenin was first characterized in neuronal tissues. We were able to demonstrate its expression in endothelial cells for the first time. To examine the cell type specificity, we analyzed δ-catenin expression in endothelial cells, neuronal cells (HT-22), epithelial cells (U251 MG and 293), and hematopoietic cells (U937 and THP-1) by Western Blotting using a δ-catenin antibody. We found that δ-catenin was expressed in large and small endothelial cells in addition to neuronal cells, but not in any of the other cells (**Figure 24A**). Immunostaining with δ-catenin antibody showed that δ-catenin expression in endothelial cells was localized intracellularly and at cell-cell junctions (**Figure 24B**).

To investigate δ-catenin expression *in vivo*, we performed double-staining immunofluorescence for both δ-catenin and a vascular marker (CD31 or von Willebrand factor) on mouse skin tissue and human tumor tissue (**Figure 24C**). δ-catenin expression was found in vascular endothelium in both mouse and human tissue.

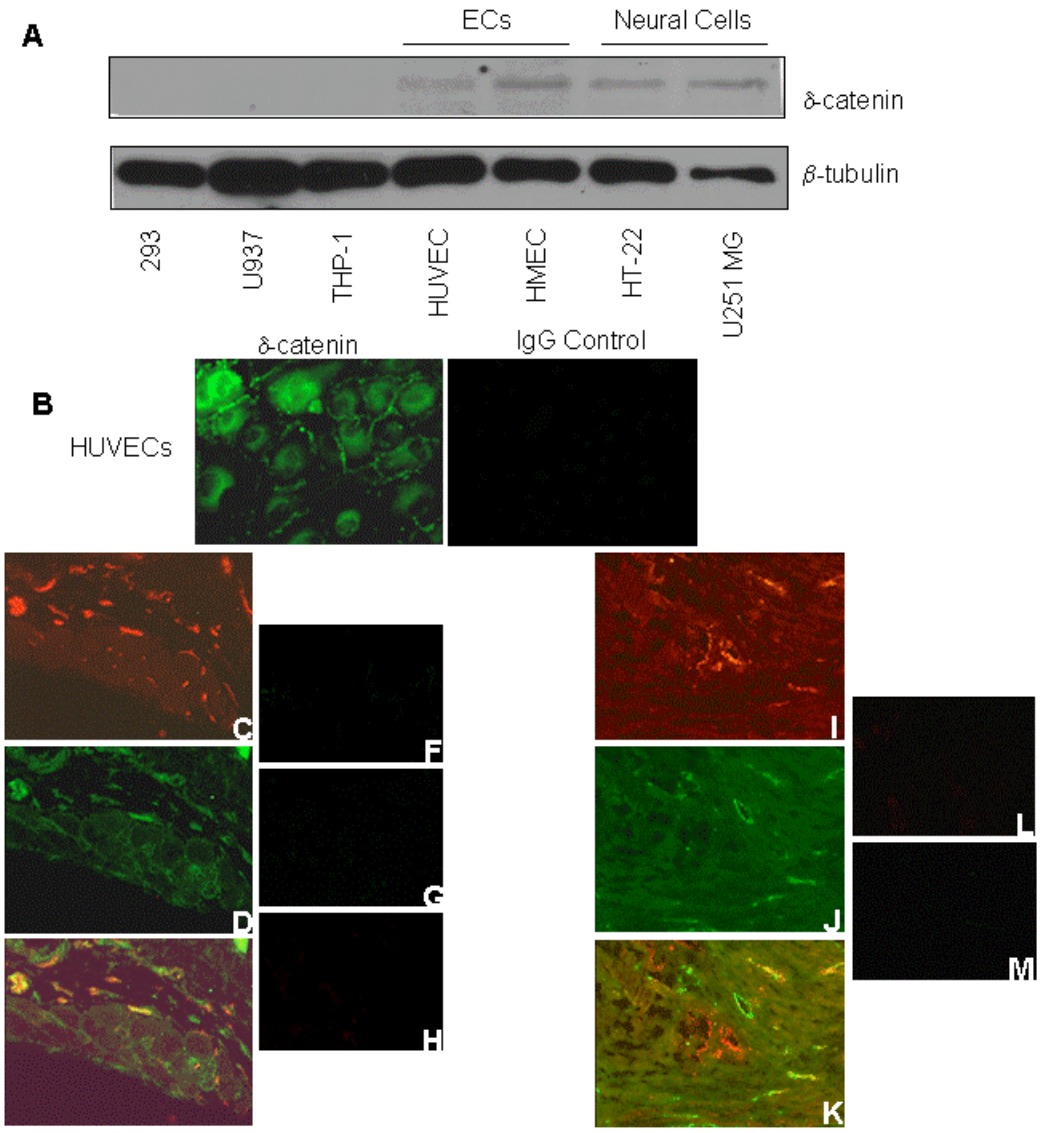

Figure 24. δ-catenin expression is verified in endothelial cells. Protein lysate of human cell lines were subject to western blot analysis (A). Immunofluorescence staining for δ-catenir. in HUVECs (B). Mouse skin tissue was subject to immunofluorescence double-staining for the vascular marker, CD31(C) and δ-catenin (D), with overlay (E). Staining controls were δ-catenin null mice (F), IgG control for δ-catenin (G) and secondary antibody for CD31 (H). Human colon cancer tissue was subject to immunofluorescence double-staining for δ-catenin (I) and CD31 (J) and image overlay (K). Staining controls were IgG control for δ-catenin (L) and secondary antibody for CD31 (M).

Over-expression of δ-catenin induces an angiogenic phenotype in vitro

Next, we investigated the role of δ-catenin in angiogenesis. δ-catenin was over-expressed in endothelial cells, and δ-catenin expression was verified with Western Blot analysis (**Figure 25B**). Using a Transwell assay, we found that cells transfected with the δ-catenin expression vector showed significantly increased migration as compared to either the vector control or the untransfected cells (**Figure 25A**). Similarly, expression of δ-catenin in endothelial cells enhanced vascular network formation in a 3-D Matrigel assay compared to controls (**Figure 25C**). In contrast, δ-catenin did not have an effect on endothelial cell proliferation (**Figure 25D**). These data are consistent with our findings in IKKα and Vav1.

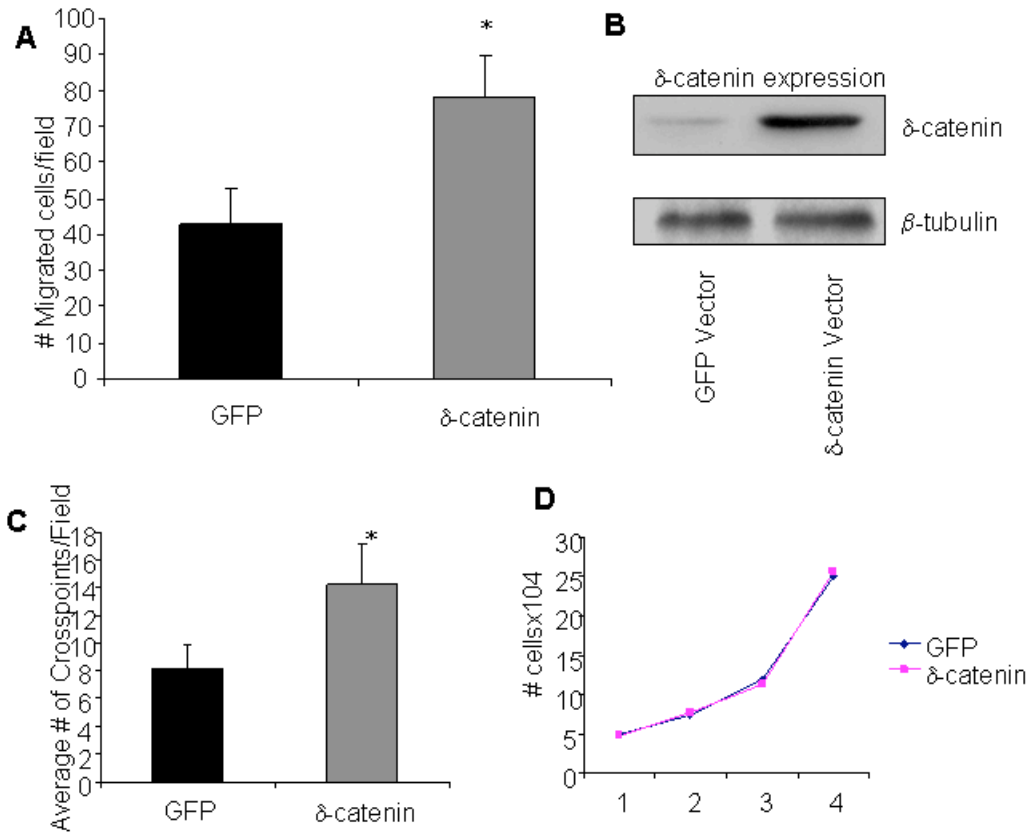

Figure 25. δ-catenin is pro-angiogenic *in vitro*. HUVECs were transfected with plasmid expressing either GFP or δ-catenin. Twenty-four hours after transfection, cell migration was measured in a modified Boyden chamber Migrated endothelial cells from 10 randomly selected high power fields under microscopy were quantified from three independent experiments. Mean and SE were plotted (n=6, $p < 0.001$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (A). Cell lysate was collected to verify δ-catenin expression levels (B). HUVECs were transfected with plasmid expressing either GFP or δ-catenin and then plated in growth factor reduced Matrigel 24 hrs later. Tube formation was then measured 12 hrs after cell plating from 10 randomly selected high power fields under microscopy and were quantified from three independent experiments. Mean and SE were plotted (n=6, $p < 0.01$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (C). Transfected endothelial cells were cultured in growth media. Live cells were counted every 24 hours and quantified from three independent experiments. Mean and SE were plotted (D).

Knockdown of δ-catenin with siRNA inhibits the angiogenic phenotype in vitro

To confirm the role of δ-catenin in angiogenesis, we used siRNA to knockdown δ-catenin in endothelial cells. Scrambled control siRNA, as well as untreated cells, were used as controls for all experiments. Cells were treated with the siRNA for 24 hours, and expression levels of δ-catenin were verified using Western blot analysis (**Figure 26B**). As expected, the knockdown of expression of δ-catenin profoundly decreased both tubule formation and endothelial cell migration, but has no effects on cell proliferation (**Figure 26A and C-D**). These data demonstrate a pro-angiogenic role of δ-catenin via modulating endothelial cell motility.

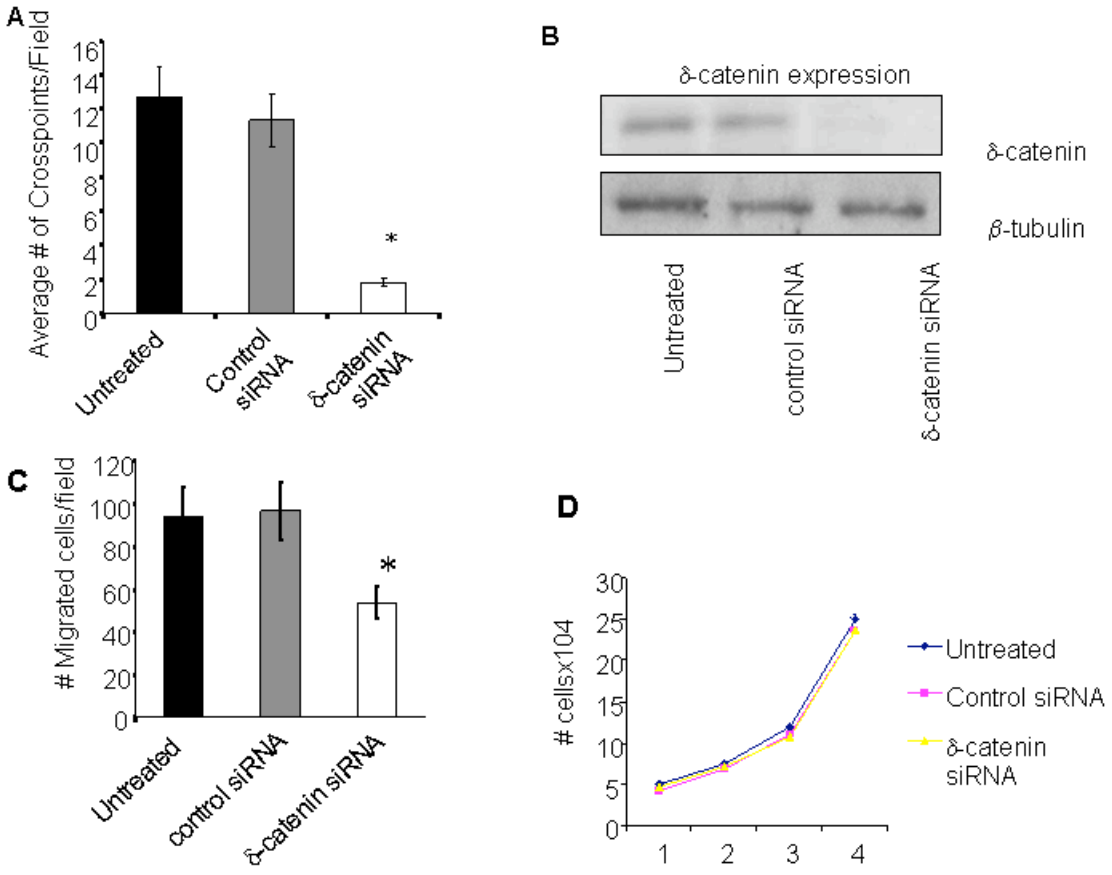

Figure 26. Knockdown of δ-catenin inhibits angiogenic phenotype *in vitro*. HUVECs were treated with siRNA in order to knock down δ-catenin expression. Cell migration was measured in a modified Boyden chamber 24 hrs later Migrated endothelial cells from 10 randomly selected high power fields under microscopy were quantified from three independent experiments. Mean and SE were plotted (n=4, $p < 0.001$ by Student's t-test, $*p < 0.001$ by Mann-Whitney U) (A). Cell lysate was also collected to verify δ-catenin expression, knock down (B). HUVECs were treated with siRNA in order to knock down δ-catenin expression and then plated in growth factor reduced matrigel 24 hrs later. Tube formation was then measured 12 hrs after cell plating from 10 randomly selected high power fields under microscopy and were quantified from three independent experiments. Mean and SE were plotted (n=4, $p < 0.001$ by Student's t-test, $*p < 0.01$ by Mann-Whitney U) (C). Transfected endothelial cells were cultured in growth media. Live cells were counted every 24 hours and quantified from three independent experiments. Mean and SE were plotted (D).

δ-catenin inhibits RhoA activity and induces Cdc42 activity

We have shown that δ-catenin plays a role in tube formation and endothelial cell migration but not endothelial cell proliferation. This observation raises the question of defining the mechanism by which δ-catenin induces the angiogenic phenotype. Other proteins of the p120 family of proteins that includes δ-catenin have been shown to affect RhoA, Rac1 and Cdc42 activation (Wildenberg et al., 2006). δ-catenin-induced activation of these proteins has been identified in neuronal cells. Therefore, we next determined if such activation occurs in endothelial cells as well. The experiments showed that over-expression of δ-catenin inhibited RhoA activity (**Figure 27A and B**). Knock down of δ-catenin increased RhoA activation (**Figure 27A and B**). Over-expression of δ-catenin activated led to increased Cdc42 activation, but knock down of δ-catenin did not significantly alter the activation of Cdc42 (**Figure 27C and D**). Both over-expression and knock down of δ-catenin did not significantly affect Rac1 activity in endothelial cells (data not shown). Thus δ-catenin, which is regulated by IKKα and Vav1 in endothelial cells activates Cdc42 activity and inhibits RhoA activity, but does not affect Rac1 activity in these cells.

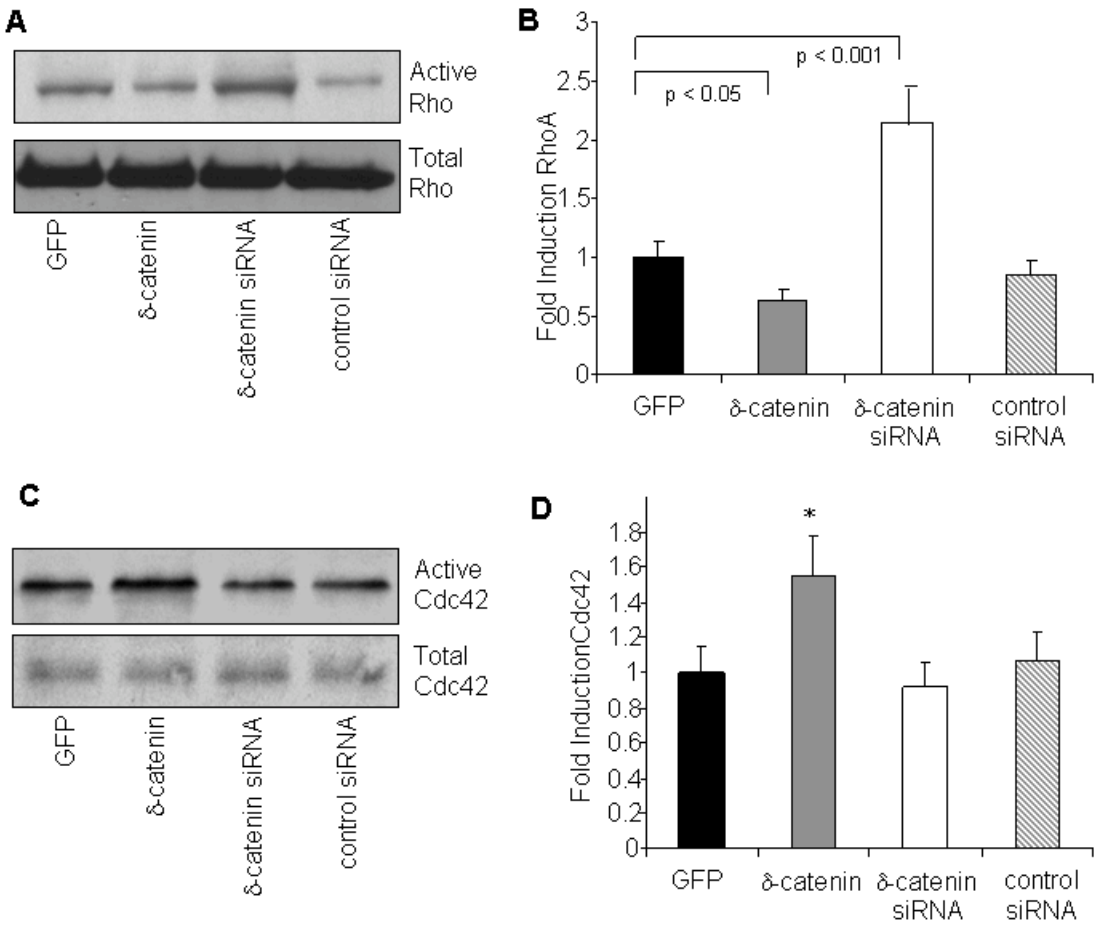

Figure 27. δ-catenin inhibits RhoA activity and induces Cdc42 activity in endothelial cells. HUVECs were transfected with plasmid or treated with siRNA. Cell lysates were collected 24hrs later and active RhoA was pulled down with Rhotekin-Agarose beads. Precipitated RhoA was subject to western blot analysis (A) and levels were compared to total levels of RhoA (n=3, F=1.32, *$p < 0.001$ by ANOVA) (B). HUVECs were transfected with plasmid or treated with siRNA. Cell lysates were collected 24hrs later and active Cdc42 was pulled down with PAK1-Agarose beads. Precipitated Cdc42 was subject to western blot analysis (C) and levels were compared to total levels of Cdc42 (n=3, $p< 0.001$ by Student's t-test, *$p < 0.01$ by Mann-Whitney U) (D).

δ-catenin promotes tumor growth and angiogenesis

Our *in vitro* data demonstrate a pro angiogenic function of δ-catenin, which point to a potential role for δ-catenin in tumor angiogenesis in vivo. To test this hypothesis, we implanted 3LL tumor cells in δ-catenin homozygous-null, heterozygous-null, and control wild type mice. Tumor growth was measured over time. Interestingly, tumor growth was significantly inhibited in both homozygous-null and heterozygous-null δ-catenin mice compared to wild-type mice (**Figure 28A**), demonstrating that a single copy of δ-catenin is not sufficient for normal tumor progression. Tumor angiogenesis corresponds to tumor growth, and both heterozygous and homozygous-null mice had significantly decreased vascular density compared to wild-type mice (**Figure 28B and C**). These data suggest that deletion of δ-catenin impairs tumor angiogenesis and thus contributes to impaired tumor growth in vivo.

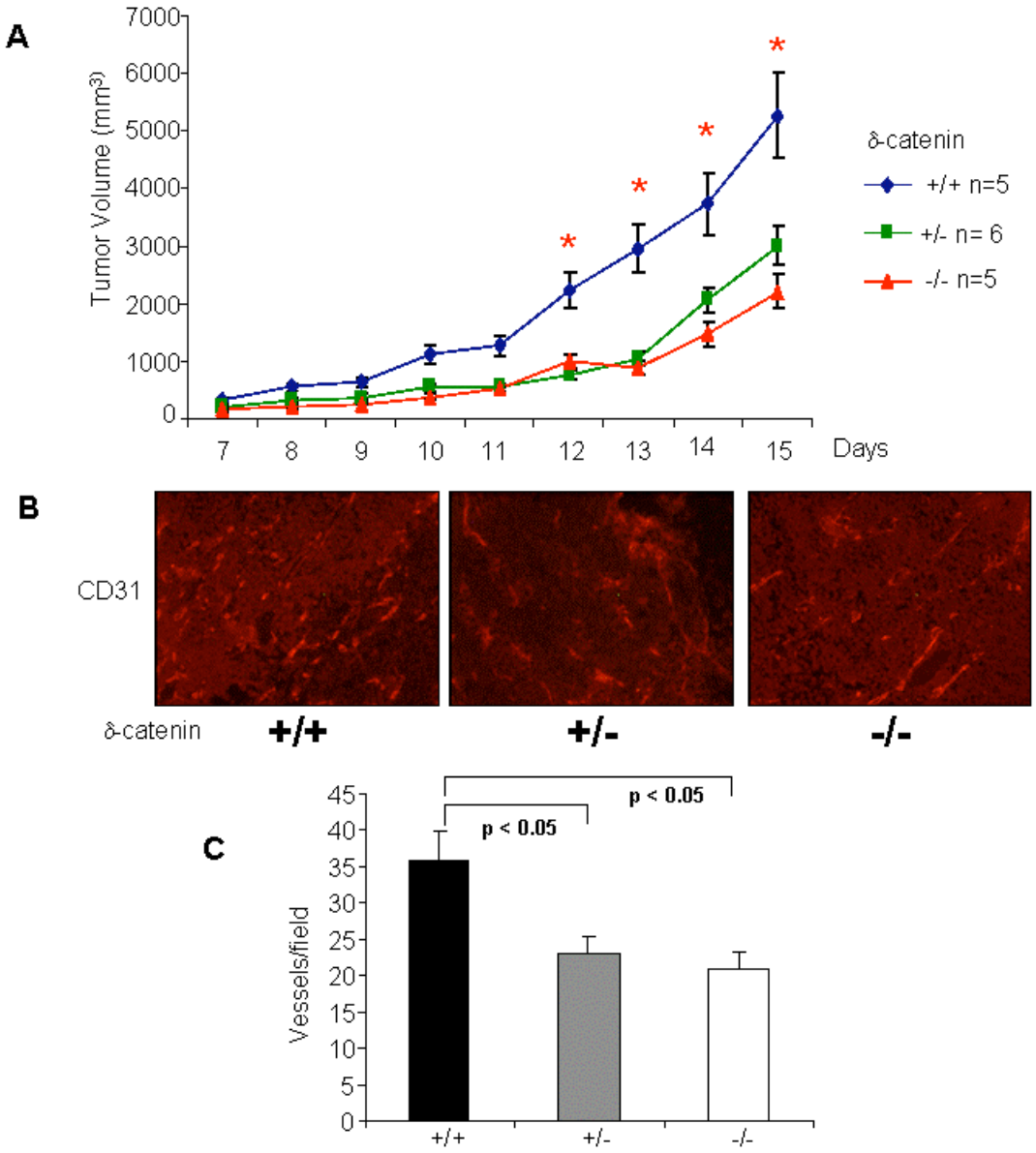

Figure 28. δ-catenin promotes tumor growth and angiogenesis. 3LL cells were injected subcutaneously into δ-catenin homozygous null, heterozygous and WT mice. Tumor growth was monitored for the next 15 days (n values on graph , F=8. 64 *$p < 0.001$ by ANOVA) (A). Tumor tissue sections were subject to immunofluorescence staining for the vascular marker, CD31 (B). Vascular density was then measured from 10 randomly selected high power fields under microscopy and was quantified from three independent experiments. Mean and SE were plotted (n=3, F=4.53, *$p < 0.05$ by ANOVA) (C).

Conclusion

In summary the data in this chapter showed that δ-catenin is expressed in endothelial cells and is regulated by IKKα. These studies also show that over-expression of δ-catenin induces an angiogenic phenotype while knockdown of δ-catenin with siRNA inhibited this phenotype *in vitro*. In addition δ-catenin was shown to inhibit RhoA activity and induce Cdc42 activity, suggesting a role in tumor angiogenesis. We found further evidence for the role this protein in tumor angiogenesis in the promotion of tumor growth and increased vascularity induced by δ-catenin expression *in vivo*. Therefore this study has identified δ-catenin as a novel protein involved in angiogenesis.

This work is in preparation as:
DeBusk L.M., Min, Y. and Lin, P.C. "δ-catenin regulates endothelial motility and tumor angiogenesis."

CHAPTER VIII

DISCUSSION

There are many steps involved in the process of angiogenesis. These steps include endothelial cell survival, which is critical for vascular maintenance, and endothelial cell motility, which is vital for vascular formation. The work in this dissertation describes aspects of both of these processes. We have identified a novel pathway, Akt/IKKα/Vav1/δ-Catenin, that regulates endothelial survival and cell motility.

Akt is well known for its anti-apoptotic activity (Datta et al. 1999). However, whether Akt is necessary or even sufficient to mediate Ang1- or VEGF-induced endothelial cell survival was not clear. In addition, Ang1 stimulation induces endothelial cell sprouting, but the molecular mechanism is unknown. In Chapter III, the roles of Akt in Ang1- and VEGF-mediated endothelial cell survival and endothelial cell sprouting were comparatively examined. Our data showed that Akt activity is necessary and sufficient in Ang1/Tie2-mediated endothelial cell survival. Akt protected endothelial cells from Tie2 blockade-induced cell apoptosis. Reciprocally, blockade of Akt activity inhibited Ang1-induced cell survival. Likewise, VEGF stimulation led to Akt activation and endothelial cell survival. Blocking VEGF activity enhanced endothelial cell apoptosis, but cells could be rescued by over-expression of a constitutively active Akt. Interestingly, a blockade of Akt had no effect on VEGF-induced cell survival, suggesting Akt is sufficient but not required for VEGF-induced endothelial cell survival. In contrast, Akt activity is required for both Ang1- and VEGF-induced vascular sprouting. These

findings highlight the fact that different growth factors function through complicated signal transduction mechanisms that utilize various mediators in regulating similar biological functions (DeBusk, et al. 2004).

Growth factors mediate cell survival through multiple signaling pathways, such as MAPK, NF-κB, PKA, and PKC, in addition to Akt (Franklin et al. 2000). Stimulation of Tie2 with Ang1 leads to Akt activation as well as MAPK/ERK activation (Fujikawa et al., 1999). Blocking PI3 kinase activity, a major activator of Akt, or Akt activity inhibited Ang1-induced endothelial cell survival (Papapetropoulos et al. 2000). However, blocking MAPK/ERK activity using a specific inhibitor PD98059 did not affect endothelial cell survival. The published data support the observation that Akt is the main, if not exclusive, mediator of Ang1-induced cell survival. While Akt activity mediated endothelial cell survival through the VEGF signaling pathway, a blockade of Akt function failed to inhibit VEGF-induced endothelial cell survival, indicating the existence of redundant survival pathways downstream of the VEGF signaling pathway. Compared to Ang1 signaling, VEGF stimulation induces ERK phosphorylation in addition to Akt activation. Blocking MAPK/ERK activity using the specific inhibitor PD98059 inhibited VEGF-induced ERK activation and endothelial cell survival (Gupta et al. 1999; Baek et al. 2000), which is distinct from Ang1-mediated endothelial cell survival (Fujikawa et al. 1999). In addition, studies have shown that VEGF enhances cell survival through the induction of cell survival proteins, including survivin and Bcl-2, in endothelial cells (Tran et al. 1999). These data are in agreement with the findings that Akt regulates, but is not required for, VEGF-induced cell survival.

Addition of a soluble Tie2 receptor (ExTek) or VEGF receptor (ExFlk) enhanced

apoptosis in cultured endothelial cells, which suggests that the survival of endothelial cells in culture depends on an autocrine function of endogenously expressed VEGF and angiopoietins. It has been reported that endothelial cells express VEGF, and stress conditions increase its production potentially as a protection mechanism (Inoue et al., 2001; Pai et al., 2002; Wang et al., 1999; Robbins et al., 1997). A study showed that serum starvation increased VEGF production in adipocytes (Wang et al., 2003). The same scenario may also occur in endothelial cells. Although no detectable Ang1 was reported in endothelial cells, endothelial cells express Ang2 and Ang4, and certain stress conditions are known to regulate the production of Ang2 and Ang4 (Pichiule et al., 2003; Cerimele et al., 2003; Yamakawa et al., 2003). Ang2 has been thought of as an antagonist ligand for Tie2 (Davis et al., 1999). However, biochemical studies on Ang2 yield controversial results. Ang2 blocks Ang1-induced Tie2 activation in endothelial cells, but induces Tie2 phosphorylation when Tie2 is genetically introduced into NIH3T3 fibroblast cells (Maisonpierre et al., 1997). One report shows that high levels of Ang2 activate Tie2 in endothelial cells (Kim et al., 2000), and Ang2 induces vascular tubule formation (Mochizuki et al., 2002), revealing the complexity of Ang2 function in angiogenesis. It was also observed that long-term stimulation of endothelial cells with Ang2 induced a delayed activation of Akt and protected endothelial cells from TNFα-induced cell death (Chen et al., 2004). These findings suggest that the role of Ang2 is context dependent, and it may enhance endothelial cell survival under certain conditions. The biological role of Ang4 has not been identified; however, it has been shown that Ang4 functions as an agonist ligand like Ang1 (Valenzuela et al. 1999), indicating that Ang4 may regulate endothelial cell survival. Soluble Tie2 receptor can bind all types of angiopoietins,

leading to a cessation in function and affecting cell survival, regardless of which angiopoietin is the mediator.

The process of endothelial cell sprouting is a critical step in angiogenesis. Similar to cell survival, both Ang1 and VEGF are known to play a role in cell sprouting (Koblizek et al., 1998; Kim et al., 2000), but the role of Akt in this process has yet to be fully explored. Previous studies have shown that PI3K phosphorylation of focal adhesion kinases plays a role in Ang1-induced endothelial cell sprouting (Kim et al. 2000). We demonstrate that Akt has a vital role in both Ang1- and VEGF-induced endothelial cell sprouting. In this study, HMVECs sprouted more robustly than HUVECs for all samples. This difference likely reflects the origins of the two lines, as HMVECs are a microvascular endothelial cell line, while HUVECs are large vessel endothelial cells. Since angiogenesis occurs in the microvasculature rather than in large vessels, the HMVEC cell line may more accurately represent *in vivo* events.

AKT-IKKα signaling and endothelial cell motility and EMT

We showed that Akt phosphorylates IKKα in endothelial cells, and induces cell motility. However, Akt has other target proteins, including GSK3b and PAK-1, that can also mediate this process. GSK3b, which was our target protein in the Akt kinase assay described in chapter III of this work, has been shown to inhibit cell motility. Akt phosphorylation of GSK3b inhibits the effect of the protein thus enhancing cell migration (Grimes et al., 2001). Akt activation of PAK-1 promotes microtubule elongation and increased cellular motility. In addition, cell motility can be regulated by other signaling pathways. For example, Src has been shown to play a role in FAK turnover as well as

induction of actin polymerization, resulting in increased migration (Kim and Chung, 2002). This redundancy in motility-inducing signaling also explains why there was not a total obliteration of endothelial cell motility in cells with a knockdown of either IKKα expression (data not shown) or δ-catenin expression or in Vav1-null cells.

EMT is the process by which epithelial cells lose their epithelial phenotype and revert to a mesenchymal phenotype to facilitate migration. Endothelial EMT is an integral step in cardiovascular development. In Chapter IV of this study, it was demonstrated that Akt induces EMT in endothelial cells via NF-κB signaling. Over-expression of a constitutively active Akt altered cell morphology, induced delocalization of VE-Cadherin from the cell membrane, and increased endothelial cell motility. Further, Akt induced endocardial EMT in a well-established endothelial EMT *ex vivo* assay (Brown et al., 1999). Inhibition of NF-κB activation completely abolished Akt-induced endothelial EMT. In endothelial cells, Akt phosphorylates IKKα, and over-expression of IKKα, but not IKKβ, led to endothelial EMT. Together, these data reveal that Akt activates IKKα, which induces EMT through NF-κB, and identify a novel pathway that regulates endothelial EMT.

Several peptide growth factors have been implicated in endothelial EMT. Cardiac endothelial cells that undergo epithelial to mesenchymal transformation express transforming growth factor-β (TGF-β) receptor II, TGF-β receptor III, or Alk2, and neutralization of their functions inhibit mesenchyme formation and migration in atrioventricular cushion explants (Brown et al., 1999). Others have shown that ErbB2 and ErbB3 regulate endothelial EMT, and are required for early cardiac valve formation (Camenisch et al., 2002). VEGF and Notch signaling were also implicated in endothelial

EMT and atrioventricular cushion morphogenesis (Enciso et al., 2003; Noseda et al., 2004). Akt is a serine/theronine protein kinase B that can be activated by tyrosine kinase receptors such as ErbB and VEGF receptors, as well as TGF-β and Notch receptors (Remy et al., 2004; Sade et al., 2004). Akt-null mice show profound cardiovascular defects (Chen et al., 2005; Ackah et al., 2005). In this study, evidence demonstrating that Akt is a mediator of endothelial EMT is provided, linking receptor signaling and EMT in endothelial cells.

Akt plays important roles in endothelial cell migration and survival (Scheid et al., 2001). Multiple Akt downstream targets have been identified, including NF-κB. Akt activates NF-κB through direct association with the IKK complex, and subsequent phosphorylation and degradation of IκB (Kane et al., 1999; Khwaja et al., 1999; Romashkova et al., 1999). Interestingly, Ozes and colleagues show that Akt associates with and phosphorylates IKKα. When a non-phosphorylatable IKKα mutant was introduced into cells, it blocked TNFα-induced degradation of IκB, thereby inhibiting translocation of NF-κB to the nucleus. In addition, Akt has been implicated in EMT in tumor cells through NF-κB (Irie et al., 2005; Julien et al., 2007; Larue and Bellacosa, 2005). Consistent with these data, we show that Akt phosphorylates IKKα in endothelial cells, and most importantly, we identify a novel function of this signaling pathway in endothelial EMT.

The IKK complex contains two catalytic subunits, IKKα and IKKβ, which share a similar structure. IKKα and IKKβ differ, however, in their physiological functions. IKKβ mediates NF-κB activation in response to pro-inflammatory cytokines and microbial products. IKKα is dispensable for these functions, but is essential for

developing the epidermis and its derivatives through an alternative pathway (Bonizzi et al., 2004; Hu et al., 1999; Hu et al., 2001; Senftleben et al., 2001). Our data reveal an IKKα-specific role in endothelial cell EMT. IKKα-induced EMT can be attenuated by co-expression of IκBα; thus, IKKα is acting though the canonical pathway, rather than the IKKα-specific non-canonical pathway. Surprisingly, IKKα, but not IKKβ, the primary IKK subunit in canonical NF-κB signaling, induced EMT through this mechanism. These results suggest additional levels of complexity in NF-κB signaling, and show an added importance for IKKα.

NF-κB regulates many physiological processes in a variety of cells, including EMT. Studies have shown that NF-κB mediates EMT through inhibition of GSK-3 in epithelial cells via Snail expression (Bachelder et al., 2005). The IKKβ/NF-κB pathway is required for the induction and maintenance of EMT in breast cancer epithelial cells (Huber et al., 2004). Constitutive *de novo* RelB synthesis is selectively active in invasive estrogen receptor alpha-negative breast cancer cells, and induction of Bcl-2 by RelB promoted the more invasive phenotype of cancer cells. These findings suggest that increased NF-κB levels induce expression of Bcl-2 to both suppress apoptosis and induce EMT in tumor cells (Wang et al, 2007). Although the role of NF-κB in endothelial EMT has not previously been shown, a chemical blockade of NF-κB in chick embryos induced cardiac abnormalities, primarily outflow tract disruptions (Hernandez-Gutierrez et al, 2006). IKKα-null mice display vascular defects, and the null mice die within 30 minutes after birth, indicating a potential cardiac abnormality (Hu et al, 1999). In this study, we demonstrate a role for Akt-IKKα-NF-κB signaling in endothelial EMT. The data point to a new function of Akt and IKKα-NF-κB in cardiovascular development.

There are various markers used to identify the induction of EMT in general and endothelial EMT in particular. General EMT markers include internalization of cadherin, upregulation of Snail and SLUG, and increased vimentin expression (Theiry, 2002). In our studies, we used cadherin localization, specifically VE-cadherin internalization, as our marker for induction of EMT, since it is has been widely used in other studies of EMT. In addition we found that the SLUG gene was upregulated in IKKα over-expressing cells. We could not use vimentin expression in our studies, since vimentin is normally expressed in endothelial cells. Internalization of VE-cadherin and upregulation of SLUG give us confidence that IKKα-treated cells underwent EMT.

Fibroblast Specific Protein (FSP-1) has recently been identified as a potential marker for endothelial EMT (Zeisberg et al., 2007). We looked at expression of FSP-1 protein endothelial cells in IKKα-treated cells and found that the protein is expressed in endothelial cells that have undergone endothelial EMT, induced by Akt or IKKα (data not shown). The upregulation of FSP-1 gives further strength to our conclusion that cells over-expressing IKKα have indeed undergone endothelial EMT.

In summary, the data presented in Chapter IV provides insight into the cellular and molecular mechanisms involved in the process of endothelial EMT. With a greater understanding of this process, progress can be made in the development of new therapies targeting EMT in cardiac defects and endothelial cell motility in angiogenesis.

IKKα and Tumor angiogenesis

Although NF-κB has been shown to be critical for tumor development and angiogenesis, most of the studies are focused on IKKβ. Here, we show that IKKα is

elevated in tumor endothelium compared to normal tissues. Over-expression of IKKα in endothelial cells increased cell motility and vascular network formation *in vitro*, and tumor angiogenesis *in vivo*. As a result, it reduced apoptosis, increased cell proliferation, as well as promoted tumor growth. In contrast, over-expression of IKKβ did not affect angiogenic properties in endothelial cells. Interestingly, IKKα-mediated angiogenic responses are dependent on NF-κB activation. Together, these findings reveal a new function of IKKα through the NF-κB canonical pathway in tumor angiogenesis.

The IKK complex contains two catalytic subunits, IKKα and IKKβ both of which share a similar structure. IKKα and IKKβ differ, however, in their physiological functions (Ghosh et al., 2002; Baldwin et al., 1996). Published data indicate that IKKβ is sufficient to mediate NF-κB activation in response to pro-inflammatory cytokines and microbial products ((Ghosh et al., 2002; Baldwin et al., 1996) . IKKα seems to be dispensable for these functions. Studies have identified essential functions of IKKα for developing the epidermis and its derivatives, and tooth, however that is mediated through an alternative pathway (Senftlenben et al., 2001; Hu et al., 1999; Hu et al., 2001; Ohazama et al., 2004). In this study, we identify an IKKα-specific role in angiogenesis though canonical NF-κB signaling. IKKα-induced angiogenic functions could be completely attenuated with co-expression of IκBα thus IKKα was acting though the canonical, rather than the IKKα-specific non-canonical pathway. Most surprisingly, IKKα, but not IKKβ, the primary IKK in canonical NF-κB signaling, could induce endothelial cell motility and vascular network formation. This finding adds another level of complexity to NF-κB signaling in general and shows an added importance for IKKα in this pathway.

IKKα has been shown to play a role in epithelial cell motility by activating of b-catenin signaling (Kraft et al. 2001). IKKα also regulates angiogenesis via up-regulation of pro-angiogenic genes such as VEGF, IL-6, and IL-8 in tumor cells (Agawal, 2005) and VEGF in tumor endothelial cells (Meng, 2007). This study provides another mechanism, namely regulation of Vav1 and δ-catenin, by which IKKα can induce endothelial cell motility and angiogenesis.

NF-κB is implicated in tumor angiogenesis (Karin et al., 2006). Most studies of this process have focused on the regulation of pro-angiogenic factors, including VEGF, IL-1β, IL-6 and IL-8. In supporting a role of IKKα in angiogenesis, a recent report shows over-expression of kinase dead IKKα in tumor cells strongly inhibits both the constitutive NF-κB-dependent promoter and endogenous gene activation. Targeted array-based gene expression analysis reveals that many of the genes downregulated upon inhibition of this pathway are involved in tumor angiogenesis (Agarwal et al., 2005). There are also reports showing that IKKα can act separately from IKKβ to regulate gene transcription (Takaesu, et al., 2003; Solt et al., 2007) . In this study, we also found that over-expression of IKKα in endothelial cells increased VEGF production (data not shown), which could contribute to increased tumor vascular formation. IKKα-null mice die 30 minutes after birth and show evidence of cardiovascular and potential angiogenic defects (Hu et al., 1999). In addition, angiogenic factors such as VEGF and angiopoietin 1 have been shown to activate the Akt-IKKα-NF-κB pathway (Marumo et al., 1999; Kim et al., 2001; DeBusk et al., 2004), supporting a role of IKKα in vascular motility and angiogenesis.

TGF-β induction of tumor cell migration has been shown to be mediated through

the canonical IKKβ/NF-κB pathway (Wei et al., 2007). However, IKKβ had no effects on endothelial cell motility and tubule formation in our study, which suggests that IKKβ may not play a role in tumor angiogenesis.

According to a recent review, there have been over 750 NF-κB inhibitors developed (Gilmore et al., 2006). However, none of the developed inhibitors are currently being used clinically. One reason for this may be the potentially severe side effects of inhibiting the NF-κB pathway in its entirety. Drugs targeting IKKα rather than IKKβ or the entire NF-κB signaling pathway would prove more beneficial in a potential clinical setting. Potential side effects with IKKβ inhibitors and general NF-κB inhibitors might be avoided with the targeting of IKKα alone.

In summary, the studies presented in Chapter V reveal a new role for IKKα in angiogenesis that is dependent on NF-κB activity. An understanding of the molecular mechanism of tumor angiogenesis offers the promise for development of therapeutic strategies for cancer treatment.

Vav1

Vav1, a guanine nucleotide exchange factor (GEF), is specifically expressed in hematopoietic and hematopoietic-derived cells (Tybulewicz et al., 2003) and regulates hematopoietic cell activation, cell growth and differentiation (Katzav et al., 2004; Bustelo et al., 2000; Houlard et al., 2002). Since hematopoietic cells and endothelial cells share a common progenitor, it is no surprise that Vav1 is also detected in vascular endothelium (Georgiades et al., 2002). However, its function in the vascular system is unclear. In this study, we demonstrate that Vav1 is required for IKKα-induced endothelial EMT and cell

motility. IKKα failed to induce EMT in epithelial cells, and Vav1 is absent in epithelial cells. Interestingly, expression of Vav1 in epithelial cells makes the cells responsive to IKKα-induced EMT and cell motility. Due to the fact that Vav1 is a GEF protein, which activates Rho-family GTPases and regulates cell motility in lymphocytes, we analyzed the function of Vav1 with relation Rho GTPases in endothelial cells. We found that deletion of Vav1 dramatically inhibited activation of Cdc42 when compared to wild-type controls, which could explain the defective motility in Vav1 null cells. Collectively, these results reveal an endothelial specific function of IKKα in EMT and angiogenesis that is mediated through Vav1.

The Vav family includes three mammalian members (Vav1, Vav2 and Vav3), which have a common structure as well as similar regulatory and catalytic properties. Despite the structural similarities, the Vav genes differ in their expression patterns. Vav1 is restricted to hematopoietic cells, with expression found from the pluripotent stem cells to the most mature stages of the lymphoid and myeloid-erythoid lineages, as well as endothelial cells (Georgiades et al., 2002). On the other hand, Vav3 shows a much broader expression profile, and Vav2 displays an almost ubiquitous distribution (Bustelo et al., 2000). Genetic experiments using knockout mice have shown that Vav proteins are important for the development and function of hematopoietic–derived cells, such as lymphocytes, natural killer cells and osteoclasts (Turner et al., 2002; Faccio et al., 2005; Tybulewicz, 2003). In the vascular system, Vav3 has been identified as the GEF that links the EphA2 receptor to Rho family GTPase activation and angiogenesis (Hunter et al., 2006). Despite the presence of Vav2 and Vav3 in endothelial cells, genetic knockout or biochemical knockdown of Vav1 is sufficient to impair IKKα-mediated endothelial

EMT and angiogenesis, suggesting Vav proteins have distinctive and non-redundant functions despite the fact that they share very similar structure motifs. Similarly, epithelial cells express Vav2 and Vav3, but the cells do not respond to IKKα stimulation with regard to EMT and cell motility. Expression of Vav1 in epithelial cells restores cellular response to IKKα, consistent with our assessment that Vav1 specifically mediates IKKα-induced cell motility and angiogenesis.

Vav1 was initially identified as an oncogene (Katzav et al. 1989; Katzav et al. 1991). Later, it was found to be an important signal transducer with a pivotal role in hematopoietic cell activation (Bustelo et al., 2000; Katzav, 2004). Numerous studies have provided insight into the role of Vav1 as an intracellular signaling molecule during cell growth and differentiation in hematopoietic cells (Vicente-Manzanares et al., 2005; Whetton et al., 2003; Houlard et al, 2002).

Vav1 has been shown to play a role in cell motility in hematopoeitic cells (Katzav, 1997) in part due to interaction with members of the RhoGTPase family (Bustelio 2004). However, studies of a Vav1-GEF mutant identified other, non-GEF mechanisms for Vav1. In fact, cell spreading and movement were still possible in T-cells expressing a Vav1-GEF mutant (del Pozo et al, 2003).

In addition, a study indicates a role of Vav1 in gene regulation in T-cells (Houlard et al., 2002). Interestingly, we found that Vav1 translocated to the nucleus of endothelial cells that had undergone IKKα induced EMT. Transfection of Vav1-null cells with wildtype Vav1, but not with a cytoplasmically-trapped Vav1 mutant, could restore IKKα induced EMT in these cells (data not shown). Most importantly, Vav1 is required for IKKα-induced δ-catenin expression in these cells (Figure 23). We have preliminary data

that suggests that IKKα and Vav1 can form a complex, and wish to address this further in future studies. These results support a role of Vav1 in gene regulation.

δ-catenin

In the previous studies, we have identified a novel Akt/IKKα/Vav1 pathway that regulates endothelial EMT, cell motility and angiogenesis. To continue this line of research and identify down stream mediators, we performed microarray analysis and found δ-catenin as a critical mediator in endothelial EMT and angiogenesis. We found that IKKα upregulates the expression of δ-catenin in endothelial cells, and that this depends on Vav1 function. Over-expression of δ-catenin in endothelial cells induces an angiogenic phenotype, while knockdown of δ-catenin with siRNA inhibited this phenotype *in vitro*. These findings were confirmed by using primary endothelial cells isolated from lungs of δ-catenin null mice and wild type controls. Furthermore, we observed a significant reduction in tumor angiogenesis and a growth delay of tumors implanted in δ-catenin null mice compared to wildtype mice, consistent with our findings suggesting a role of δ-catenin in endothelial motility and angiogenesis. Most strikingly, the reduction of tumor angiogenesis and tumor growth was similar in mice that were heterozygous and homozygous-null for δ-catenin expression, indicative of possible haploinsufficiency for this gene.

Expression of δ-catenin was originally thought to be exclusively in neural tissue. This work is the first to characterize δ-catenin expression in the vascular endothelium. However, the expression pattern is not surprising, as there have been numerous proteins that are expressed in both neural and endothelial tissues. These molecules include

members of the Ephrin-Eph family, Slit/Robo families, Netrins and Semaphorins (Eichmann et al., 2005). δ-catenin is yet another protein that plays a role in both tissue types. The role that δ-catenin plays in neural cell motility is well characterized (Paffenholz et al., 1997; Paffenholz et al., 1999; Fujita et al., 2004). We show that expression of δ-catenin can induce motility and other pro-angiogenic processes in endothelial cells.

We believe that δ-catenin plays a role in cell motility by affecting RhoA and Cdc42 activity. We did not see translocation of δ-catenin to the cell nucleus in the endothelial cells used in our studies. There is no evidence that δ-catenin can act as a GEF protein. Rather, there are modulating proteins mediating the effect of δ-catenin and RhoA and Cdc42. It has been shown that the highly-homologous p120 protein induces a similar phenotype in epithelial cells (Wildenberg, 2006). The p120 protein inhibited RhoA activation and increased Rac1 activation in epithelial cells. Moreover, this effect was not due to direct interaction between p120 and these proteins, but rather, is mediated through p190. (Wildenberg 2006). We believe the effect that δ-catenin plays on RhoA and Cdc42 may be mediated through the same protein or by other modulators. We speculate that expressing dominant negative Cdc42 or constitutively active RhoA would alter the cellular phenotype and render the cells less motile. We did not see an effect on Rac1 activity and thus speculate that Rac1 mutants would not affect δ-catenin-induced cell motility.

Finally, patients with the Cri-du-chat syndrome lack one copy of the δ-catenin gene due to a deletion of regions of the short arm of chromosome 5. They show a profound delay in development, as well as congenital heart defects, including ventricular

septal defects in 15-20% patients (Hills et al. 2006). In this study, we have identified δ-catenin as a critical mediator of endothelial EMT that functions downstream of the Akt/IKKα/Vav1 pathway. Based on the facts that endothelial EMT is an important process in cardiac valve and ventricular septal development, and that our data point to haploinsufficiency of δ-catenin in angiogenesis, we postulate that deletion of one copy of the δ-catenin gene in Cri-du-chat syndrome patients leads to impaired endothelial EMT and cardiac vascular development. Clearly, further examination of the role of this protein during cardiac development would be informative and clinically important.

Summary

Akt/IKKα/Vav1 signaling forms the core signaling pathway of our model for endothelial cell survival and motility (Figure 29). Ang1/Tie2 and VEGF/VEGFR signaling activates Akt and induces cell survival. We will study potential downstream mediators of endothelial cell survival in further studies.

Akt and IKKα both induce endothelial EMT and endothelial cell motility. This can be blocked with co-expression of IκB-α, suggesting that endothelial EMT is mediated through the NF-κB canonical pathway. However, non-canonical signaling can affect the canonical pathway (Lessard et al., 2007). IKKα is found in both canonical and non-canonical signaling. In future work, we want to fully elucidate the potential interplay between these two NF-κB signaling pathways. IKKα can also translocate to the nucleus and act as a gene regulator (Anest et al., 2003; Yamamoto et al., 2003).

Vav1 and δ-catenin are both upregulated by IKKα. Vav1 is required for induction of endothelial EMT, and induces endothelial motility and tumor angiogenesis. We have preliminary data that suggests that IKKα and Vav1 can form a complex. We plan to examine the potential role Vav1 and the Vav1/IKKα complex plays in gene transcription in future studies. Vav1 may also regulate members of the Rho-GTPase family, thus regulating cytoskeletal changes and cell motility. δ-catenin also induces endothelial cell motility and tumor angiogenesis, through regulation of RhoA and Cdc42 activity. We have preliminary data that δ-catenin interacts with VE-cadherin and want to examine this interaction further.

The proposed model advances the study of angiogenesis. We demonstrate a novel mechanism for endothelial cell survival and endothelial cell motility. More importantly, we show that the Akt/IKKα/Vav1 signaling pathway can induce endothelial EMT and that this process plays a role in tumor angiogenesis.

Angiogenesis has long been identified as a target for anti-tumor therapy. While there have been some clinical successes in this field, many potential treatments have failed to live up to initial expectations. The work in this dissertation identifies novel targets for anti-angiogenic therapies, and furthers our knowledge of the endothelial cell survival, sprouting, and motility involved in the process of angiogenesis. The work on cell motility is especially interesting, as the role of EMT in tumor vascular development has not been explored. This work will be very beneficial due to the identification and characterization of this process in endothelial cells.

The role of IKKα in tumor angiogenesis is noteworthy because of the great interest surrounding NF-κB in tumor development and growth. Due to this interest, there

has been active research focused on potentially targeting NF-κB in patients. Therefore, this work could result in the use of currently developed NF-κB inhibitors in novel and previously unimagined ways in the treatment of cancer. Targeting tumor vessels as therapy is an underdeveloped field.

This work identified both Vav1 and δ-catenin as potential anti-angiogenic targets. Neither of these proteins had previously been discussed in the context of angiogenesis,

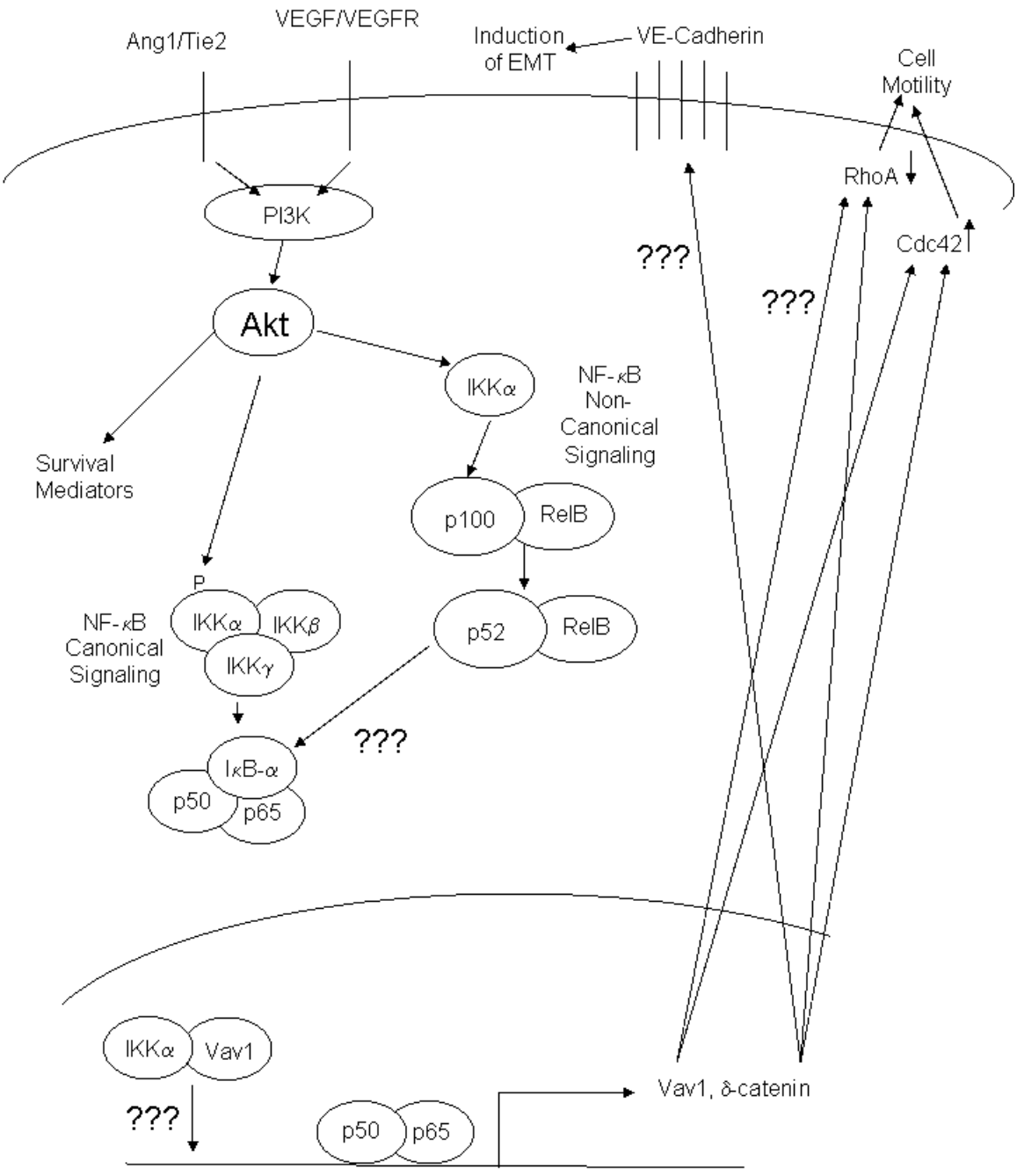

Figure 29: The central signaling pathway for both endothelial cell motility and endothelial cell survival

and our work has helped to bring both of these molecules into the discussion of angiogenesis and potential anti-angiogenic targets.

In total this work has contributed to the study of cancer biology and anti-angiogenic therapies. It has shown the signaling link between different processes involved in angiogenesis, and helped to further characterize known angiogenic factors. It has also demonstrated that endothelial EMT is involved in endothelial cell motility, and has identified previously unknown targets for anti-angiogenic therapies.

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