DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

The vasculature is an integral part of all tissues and its patterning is specific to each tissue. The development of the vasculature has been extensively studied, and many genes that regulate the formation, proliferation, and morphogenesis of endothelial cells have been identified. However, it is not known how blood vessels develop within the context of an organ. Vascular patterning may be controlled by the local tissue environment. Tissue cells may elaborate secreted or cell surface associated molecules that direct vascular formation and patterning. Conversely, the function of the vasculature may be more than just to supply the metabolic requirement of the tissues. It is becoming increasingly evident that endothelial cells may indeed provide specific cues to direct tissue morphogenesis. My hypothesis is that there are reciprocal inductive interactions between a tissue and its vasculature during organogenesis. This is strongly suggested in the lungs by the intimate relationship between airways and blood vessels. In the mature lungs, blood vessels accompany the airways and form capillary networks surrounding the terminal gas-exchange air-sacs or alveoli. Despite the importance of the vasculature as a critical functional component of the mature lungs, the molecular mechanisms that regulate lung vessel development, and in particular how it is coordinated with airway development, are unknown. We propose that the families of Vascular Endothelial Growth Factor (VEGF) and their receptors play important roles in the coordinated development of lung airways and blood vessels, due to their known activity on endothelial cell development and their temporal and spatial expression in the mouse embryonic lungs. We have initiated a study to elucidate the function of these molecules in lung vascular development. However, to directly test the hypothesis of epithelial-endothelial interactions, a new aim is proposed to ablate in vivo either lung epithelial or endothelial cells at specific stages of lung development and determine the consequences of the absence of one cell type on the development of the other cell type. Studying the development of the lung vasculature not only addresses questions on fundamental developmental processes, but may also provide insights into pathogenesis of, and therapeutic targets for, lung diseases in which normal vascular development is a component. These may include developmental diseases such as vascular malformations, pulmonary capillary atresia, pulmonary hypoplasia, and bronchopulmonary dysplasia, as well as acquired diseases such as cancer, fibrosis and abnormal lung repair following injury.

PERFORMANCE SITE(S) *(organization, city, state)*

Department of _____

University of _____

_____, _____

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
_____	_____	Principal Investigator

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ **Yes** ☐ **No**

RCA TOC Substitute Page

Candidate *(Last, first, middle):* _____

Use this substitute page for the Table of Contents of Research Career Awards. The name of the candidate must be provided at the top of each printed page and each continuation page.

RESEARCH CAREER AWARD
TABLE OF CONTENTS
(Substitute Page)

Page Numbers

Note: Type density and size must conform to limits provided in the Specific Instructions.

*Include these items only when applicable.

CITIZENSHIIP

☒ U.S. citizen or noncitizen national

☐ Permanent resident of U.S. (If a permanent resident of the U.S., a notarized statement must be provided by the time of award.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory: The _____, in which Dr._____'s laboratory is situated, occupies 7200 sf. Dr. _____'s own laboratory occupies 1000 sf (square feet). Shared facilities in the Center include two core cell culture facilities (600 sf), a darkroom (100 sf), equipment room (220 sf), walk-in cold room (100 sf), fluorescence microscopy room (80 sf), 200 sf dedicated to flow cytometry, tissue sectioning and microscopy, and a 300 sf small animal surgery, physiology and necropsy laboratory.

Clinical: N/A

Animal: Mice will be housed and bred either in the barrier facility or in conventional animal facility in _____ on the _____ campus. The barrier facility is a modern facility for housing transgenic animals in a closed pathogen-free colony. All surgical procedures will be performed in the 300 sf small animal lab within the _____.

Computer: Dr. _____'s laboratory has 3 Macintosh computers connected through ethernet for data analyses and word processing. In addition, the _____ has available for shared use several Macintosh computers and PCs connected through ethernet, a scanner and five laser printers.

Office: Dr. _____ has a 125 sf office adjacent to the laboratory. The _____ administrative office includes 4 administrative analyst and 2 administrative assistants who will provide grants management and limited secretarial support for this proposal.

Other: Dr. _____ has access to a DNA sequencing facility, transgenic mouse core facility for the generation of genetically modified mice, and histology service on campus. Other groups in the _____ provide expertise in a variety of areas, including transgenic mouse models and antibody generation. In addition, _____ has a number of groups active in animal models, transgenic mouse studies, and vascular biology. The graduate program in Biomedical Sciences and Developmental Biology provide additional intellectual resources.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Within Dr. _____'s laboratory: major equipment needed for molecular biology, animal, and histology studies. These include –80^{o}C and –20^{o}C freezers, refrigerator, high speed centrifuge, gel photodocumentation system, thermocycler, hybridization oven, dissecting microscope, and rotary microtome.

Within _____: a Beckman LS5000 scintillation counter, a Beckman J2 21 M high speed preparative centrifuge, a Beckman LS-80M ultra-centrifuge, 2 Beckman bench-top refrigerated centrifuges, 5 Baker Sterile Gard laminar flow hoods, 12 Forma water jacketed CO_2 incubators, a Beckman DU 65 spectrophotometer, a Forma shaking incubator a Leica Cryocut 1800 Cryostat, a Shandon tissue processor and Leica tissue embedding equipment, a Consolidated autoclave, a Zeiss fluorescence microscope, 3 Leitz Labovert FS with microinjection apparatus and videomicroscopy, a Biorad 583 gel dryer, X-ray film processor, ELISA microplate reader, fluorometer, liquid nitrogen freezers, tissue embedding equipment, and dishwashing facility.

SECTION II: Specialized Information

1. Introduction to Revised Application

2. Letters of Reference

3. The Candidate

A. Candidate's Background

B. Career Goals and Objectives: Scientific Biography

My goal is to become an independent physician-scientist who engages in basic research that has clinical relevance. My past and continuing efforts have been directed at the accomplishment of this goal. I acquired training in the Medical Scientist Training Program leading to both an M.D. and a Ph. D. degree because I believe that a combination of the depth of clinical knowledge acquired with clinical training and the rigor of a basic scientist's training will be most productive for investigators in medical research. After finishing my graduate studies, I continued to obtain further clinical and research training by finishing an Internal Medicine residency program and a fellowship program in Pulmonary and Critical Care Medicine. The fellowship program includes both training in clinical Pulmonary Medicine and opportunity for postdoctoral basic science research.

I have always been interested in developmental processes, especially morphogenetic interactions between tissue components, either through soluble paracrine factors or through extracellular matrix (ECM) components. I did my doctoral thesis under the tutelage of Dr. _____, an internationally recognized expert in the field of peptide growth factors. I examined the role of _____ and its receptor during mouse embryonic development. I discovered a novel, truncated form of the _____ receptor expressed in early embryonic cells (_____). The characterization of this receptor was a technically challenging task, due to the limited amount of embryonic materials. To achieve my objectives, I became proficient in a number of molecular biological approaches, including Northern and Southern blotting, DNA cloning, DNA sequencing, immunoprecipitations and immunoblottings, and the then newly developed polymerase chain reaction (PCR). I also acquired knowledge in the biology of peptide growth factors. After a period devoted to clinical training, I returned to research activity since January 1996. I elected to obtain further research training in the laboratory of Dr. _____, a pioneer in the field of extracellular matrix (ECM). I wanted to learn more about the biology of the ECM, especially in the context of tissue morphogenesis. I chose to study the skeletal growth plates initially because it is a very useful system to study interactions between different tissue components during morphogenesis. The skeletal growth plate is the ultimate paradigm in tissue remodeling because one tissue (cartilage) is completely converted into another (bone). In this system, there are clear temporally and spatially defined processes that can be studied with perturbation experiments. I studied the function of _____ in endochondral ossification at the growth plates. _____. I found that in the growth plates of mice deficient in _____, the remodeling of hypertrophic cartilage (HC) into bone is impaired, resulting in its accumulation. Because _____ is an _____, the most straightforward explanation for the phenotype would have been that _____ deficiency causes a defect in HC resorption. However, I proposed that the phenotype might be due to impedance in the vascularization of hypertrophic cartilage, and _____ may function by regulating the net angiogenic activity in HC. This novel idea was supported by in vitro assays comparing angiogenic activities of wild type and _____-null HC (_____). The study led to an entirely new view of the function of the _____ family, giving evidence that these enzymes may do more than just _____. One of their functions may indeed be to regulate the levels and/or bioavailability of signaling molecules. The paradigm of _____ regulating net angiogenic activity subsequently motivated a study by our collaborators showing that _____ is responsible for triggering the angiogenic switch in a model of _____ (_____). I also carried out other studies that provided significant insights into the different aspects of endochondral ossification and identify the function of other genes in this process (_____). I have learned many invaluable techniques and approaches in my postdoctoral research work, and continued doing so during the period of mentored research development supported by the NIH Mentored Physician Scientist Development (K08) award. I have become proficient in genetic approaches to the study of developmental and pathological processes and have learned additional

skills in animal handling, tissue processing, immunohistological techniques, in situ hybridizations, organ cultures, microdissection, organ transplantation, and genetic manipulations.

Since July of 2000 I have started a faculty position at _____ that provided me with the space and research support that allowed me to initiate an independent research program to study processes in lung development, especially development of the lung vasculature and how it is coordinated with development of the airways. This is the area of my particular interest. My research program addresses a very important yet poorly investigated area of developmental biology, that of the regulation of organ-specific vascular patterning and of the role of the vasculature in tissue morphogenesis. Since the vasculature develops concomitantly with the tissue, and has a specific pattern in relation to the tissue, it seems self-evident that there must be inductive interactions between the vasculature and the tissue during their development. My research program aims to elucidate the molecular and cellular mechanisms underlying this cross talk. One of my approaches is to study candidate genes that have been shown to function in vascular development and whose pattern of expression in the lungs suggests that they are the intermediaries of airways-vasculature interactions. Thus I have initiated a study on the role of the families of vascular endothelial growth factor (VEGF) their receptors in lung vascular and epithelial development. This project is currently supported by a NIH R01 grant and for which protected time is requested. However, to test the fundamental hypothesis of epithelial-endothelial interactions, it is crucial to be able to ablate either lung epithelial or endothelial cells and determine the consequences of the absence of one cell type on the development of the other cell type. This is the additional research proposed for the Independent Scientist (K02) award.

My long-term goal is to continue to develop my research program and to enhance my knowledge and skills in the areas of Lung Development and Vascular Biology to become an expert in these areas. This will enable me to contribute significantly to our knowledge of pulmonary biology and diseases and to training of future researchers in these areas.

C. Career Development Activities During Award Period

The support of the Independent Scientist (K02) award will be essential for me to be able to carry out my proposed studies in an expedite manner since it will allow me to devote at least _____ to research activities. Because I have been supported by a K08 award which provided a substantial amount of my salary, I have not been required to participate in much clinical activity except for attending in the _____ one half day a week and on the inpatient service one month in a year. This amounts to less than _____. The protected time afforded by the K08 award has been instrumental in allowing me to obtain mentored research training, to initiate my independent research program and to compete successfully for a NIH R01 grant support. However, the K08 support will end on July 2003, and without further assured salary support, I will need to increase my clinical activity in order to have salary support from clinical revenue. This may have a detrimental impact on my research career development. Support of a K02 award will allow me to have continued protected time for research activities. During the time of the K02 award, I will continue to have the same amount of clinical activity, attending in the _____ one half day a week (_____) and on the inpatient services one month out of the year (_____). I may choose to be involved in teaching medical students to develop and improve my teaching skills, but will not dedicate more than 2 hours per week (_____) for this activity. The rest of my time (_____) will be devoted to research.

The support of the K02 award will allow me to continue to develop my research program and to acquire further knowledge and skills to become a highly productive scientist. Carrying out the proposed experiments will allow me to get more in depth knowledge of the generation of genetically modified mice and interpreting the results of the experiments will give me the opportunities to learn more about lung development and vascular biology. I will continue to read the literature and to consult with experts in the field, both at the campus and elsewhere in order to deepen and expand my knowledge and skills. I will thus become an expert in my own right in the field of lung development and vascular biology. I will recruit postdoctoral fellows and graduate students to study in my laboratory and I will learn the mentoring skills needed to train future researchers from other senior scientists on campus. The protected time devoted to research activities will give me the luxury of having time to take didactic courses available at _____ such as courses in Developmental Biology, Genetics, Cell Biology, and Mechanisms of Diseases. There are also mini-symposia and workshops on many interesting and relevant topics such as Branching Morphogenesis, Stem Cell Biology, Angiogenesis, Genomics, Proteonomics, and Transgenic Mouse

Models offered at _____or other sites such as at _____. I will also be able to attend scientific meetings such as the American Thoracic Society (ATS) annual meeting, the Keystone symposia, and the Gordon conferences, during which I can meet other investigators, present my work, and have scientific exchange.

A course on The Responsible Conduct of Science is offered at _____each Fall. This course is required of most research trainees and open to all faculty across the campus. It is an 8-week seminar that focuses on a critical review of problems of responsible scientific conduct, including the analysis of fictitious cases and the group discussion of current ongoing issues in the scientific lives of the seminar participants. The emphasis is on bringing community standards to the forefront and helping participants to have the tools to solve the dilemmas they face. Topics included in the course are: why is this an issue?, problems of authorship, reviewing papers and grant proposals, publication practices, conflicts of interest, data management, scientific misconduct, use of humans and animals in research, ownership of data, intellectual property issues, sharing of research materials, mentorship issues, harassment and discrimination. Recently a yearly seminar series on "Professional and Academic Success Skills" covering many of the same topics has been initiated at_____. I have taken the course on The Responsible Conduct of Science in the Fall of 1998 and will require the trainees in my laboratory to attend these courses as well.

4. Statements by Sponsor(s), Consultant(s), and Collaborator(s)

Please see letters from Dr. _____and Dr. _____ who provide the transgenic mouse lines to be used in the proposed studies. Drs. _____ will be available to provide advice on the generation of mice with lung epithelium-specific gene expression and inactivation and on analyses of the lung phenotypes. Dr. _____ has expertise in angiogenesis and Dr_____ has expertise in lung development.

5. Environment and Institutional Commitment to Candidate

A. Description of Institutional Environment

The University _____ is a premier medical and research center that offers an outstanding, scientifically stimulating and at the same time supportive and collaborative research environment. The senior investigators show great interest in helping young and newly established investigators to advance their research career. There is a diverse range of researchers with expertise in a wide range of research areas. In the areas of my research interest, there are nationally recognized experts in lung biology, developmental biology, vascular biology, and transgenic mouse models such as _____, to name a few. There are core facilities that offer technical and methodological expertise and services. The _____ animal care facility has a modern facility for housing transgenic animals in a closed pathogen-free colony that will be a great support for the proposed animal studies. There are also excellent transgenic mouse core facilities that will help with the generation of genetically modified mice. The Department of Medicine is completely supportive of young investigators who wish to engage in basic research into the molecular and cellular mechanisms of physiological and pathological processes. The _____, to which I belong, consists of a group of investigators interested in different aspects of pulmonary biology and diseases. The _____ has a variety of equipment and the investigators in the Center have expertise in a variety of research questions and experimental approaches related to the lungs. The interactions among different members of the _____ either through informal daily contacts or formal weekly seminars have been very productive in catalyzing ideas and collaborations. _____ truly offers all the necessary elements to help newly established investigators to develop their research career.

B. Institutional Commitment to Candidate's Research Career Development

Please see letter from Dr. _____, Vice Chairman, Department of Medicine.

6. Research Plan

A. Statement of Hypothesis and Specific Aims

The vasculature is an integral part of all tissues, yet little is known about either the mechanisms that regulate blood vessel development during tissue formation, or whether tissue vascularization is critical for normal organogenesis. In the lungs, the intimate relationship between airways and blood vessels suggests that there may be reciprocal inductive interactions that coordinate their development. The goal of our research proposal is to identify cellular and molecular mechanisms that regulate lung vessel

development and to determine the contribution of vessel development to epithelial development and vice versa. One of our hypotheses is that factors that regulate angiogenesis may be part of the epithelial-mesenchymal interactions that coordinate airway and vessel formation during lung morphogenesis. A family of endothelial cell-specific growth factors, the Vascular Endothelial Growth Factor (VEGF) family, has been shown to be important for endothelial cell growth, differentiation, and morphogenesis. Many VEGF family members and their receptors are expressed in the embryonic lungs, suggesting a role in lung vascular development. In addition, VEGF-A is expressed in lung epithelium, and its two receptors, VEGFR-1 (flt-1), and VEGFR-2 (flk-1), are expressed in the mesenchyme, suggesting paracrine interactions. Our ongoing peer-reviewed research project (specific aims 1-3) focuses on determining the function of VEGF and VEGF receptor family members in lung development. However, to determine the role of epithelial cells on the development of the lung vasculature and the role of endothelial cells on the development of the airways, we propose a new study in specific aim 4.

Ongoing Specific Aim 1: We will determine the function of Vascular Endothelial Growth Factor (VEGF, VEGF-A) in lung development. VEGF-A is one of a few angiogenic factors shown to play critical roles in blood vessel formation during embryogenesis. The role of VEGF-A in lung development is not known, as VEGF-A deficiency results in early embryonic lethality. We will generate and analyze lung vascular and epithelial development in mice with targeted inactivation of the VEGF-A gene in lung epithelium.

Ongoing Specific Aim 2: We will determine the function of other members of the VEGF family in lung development. The expression of two other VEGF family members, VEGF-C and VEGF-D, in embryonic lungs suggests that they may also play important roles in lung vascular development. We will determine the function of these two molecules in lung development by inhibiting their function using a soluble receptor in a novel model of lung development developed in our laboratory that follows closely lung development in utero, the grafting of embryonic lung rudiments under the kidney capsule.

Ongoing Specific Aim 3: We will determine the function of VEGF receptors in lung development. Mice lacking any of the three VEGF receptor family members die in early embyrogenesis with vascular defects. This precludes analysis of their deficiency on lung vascular development. Since each receptor can bind multiple members of the VEGF family, the function of individual receptor is not clear. We will determine the function of individual receptor in lung development by selectively activating each receptor individually or in combination in the renal capsule graft model using newly characterized receptor-specific ligands.

New Specific Aim 4: We will determine the interdependence of epithelial and endothelial cells in their development. We will generate transgenic mouse lines in which cells can be ablated in a spatially and temporally regulated manner. Using such mice we will specifically ablate either lung epithelial cells or endothelial cells during specific stages of lung development and determine the consequences of the absence of one cell type on the development of the other cell type.

Peer-Reviewed Project for Which Protected Time Is Requested: This project aims to study the role of the Vascular Endothelial Growth Factor (VEGF) and VEGF receptor family members in lung development (ongoing specific aims 1-3).

B. Background, Significance, and Rationale

1) Development of the vascular system

Blood vessels develop through two fundamental mechanisms, vasculogenesis and angiogenesis (Risau, 1991). Vasculogenesis is the de novo formation of blood vessels from the differentiation of endothelial cells from mesenchymal angioblast precursors, and their organization into a network of primitive tubules termed the primary plexus. Angiogenesis is the remodeling of these primitive vascular networks into mature vessels, as well as the branching and sprouting of new blood vessels from existing ones. In the embryo, both vasculogenesis and angiogenesis occur. A primary vascular plexus forms via vasculogenesis, which remodels, grows, and establishes connections by angiogenesis. In adults, angiogenesis accounts for all normal and abnormal blood vessel development.

a. Blood vessel development is regulated by the VEGF family

The genetic and molecular regulation of blood vessel morphogenesis has only been recently elucidated, with the identification of paracrine factors that regulate the development and function of endothelial cells (Risau, 1997; Gale and Yancopoulos, 1999; Yancopoulos et al., 2000). Endothelial cell precursors are termed angioblasts. These cells differentiate from mesodermal precursor cells and coalesce to form a network of homogeneously sized primitive tubules termed the primary plexus. Members of the fibroblast growth factor (FGF) family are important for the generation of angioblasts from mesodermal precursors (Flamme and Risau, 1992). The growth and morphogenesis of angioblasts into the primitive plexus requires vascular endothelial growth factor (VEGF or VEGF-A), a polypeptide growth factor specific for endothelial cells (Ferrara and Henzel, 1989; Gospodarowicz et al., 1989). In vitro, VEGF-A can stimulate endothelial cell proliferation, migration, and sprouting activity. In vivo, VEGF-A is critical for vascular development in a dose-dependent manner. Lacking even one allele of the VEGF-A gene leads to embryonic lethality, with impaired vessel formation (Carmeliet et al., 1996; Ferrara et al., 1996). There is delay in endothelial cell development, as well as defective vessel sprouting, remodeling, and survival. Interestingly, too much VEGF-A also leads to impaired vascular development and embryonic lethality (Miquerol et al., 2000). Therefore, VEGF-A has to be critically regulated, spatially, temporally, and quantitatively.

Two high affinity VEGF-A receptors, belonging to the receptor tyrosine kinase family, have been identified, VEGFR-1 (flt-1, fems-like tyrosine kinase), and VEGFR-2 (flk-1, fetal liver kinase) (de Vries et al.,1992; Millauer et al., 1993; Quinn et al., 1993; Shibuya et al., 1990). A null mutation in VEGFR-2 (flk-1) results in lack of a vasculature and very few endothelial cells, suggesting that flk-1 functions in the differentiation and/or proliferation of endothelial cells (Shalaby et al., 1995). In contrast, mice deficient in VEGFR-1 (flt-1) have excess endothelial cells that are not organized into normal tubular networks (Fong et al., 1995). Therefore, flt-1 may function to down-regulate VEGF-A activity. Consistent with this model, mice with an engineered mutation in flt-1 that results in a truncated receptor lacking the intracellular domain develop normally (Hiratsuka et al., 1998). This is consistent with the model that only the extracellular ligand-binding domain of this receptor has a critical function in vascular development, and that this function is to bind and negatively regulate the level of VEGF-A that is available to activate flk-1.

b. The VEGF and VEGF receptor families

Other members of the VEGF family have been identified based on sequence homology to VEGF-A. There are four other mammalian members besides VEGF-A: placental growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D (Eriksson and Alitalo, 1999). PlGF and VEGF-B share more sequence homology with VEGF than do VEGF-C and VEGF-D. VEGF-C and –D share similar structures and may comprise a subgroup. Each VEGF family member binds to one or more of three VEGF receptors (Figure 1). PlGF and VEGF-B binds only to VEGFR-1. VEGF-A binds to both VEGFR-1 and VEGFR-2. VEGF-C does not bind to VEGFR-1, but binds to VEGFR-2 as well as to a third tyrosine kinase receptor, VEGFR-3, or flt-4 (Veikkola et al., 2000). Human VEGF-D binds to both VEGFR-2 and VEGFR-3, but mouse VEGF-D only binds to VEGFR-3 (Baldwin et al., 2001). The VEGF family members have non-overlapping functions. That lack of VEGF-A results in early embryonic lethality indicates that the other VEGF family members cannot compensate for its deficiency. VEGF-B does not have a critical developmental role. Mice deficient in VEGF-B develop normally, and only have a subtle cardiac phenotype after birth (Bellomo et al., 2000). These mice have reduced heart size and impaired recovery from ischemia. PlGF also does not have a critical developmental role. Mice with targeted inactivation of the PlGF gene show no developmental defect (Carmeliet et al., 2001). The function of VEGF-C and VEGFR-3 appears to be in the development of the lymphatics in late embryogenesis and in adults. VEGF-C can induce lymphatic formation in chicken chorioallantoic membrane (CAM) (Oh et al., 1997). Transgenic mice over-expressing VEGF-C or VEGF-D in the skin have hyperplasia of skin lymphatic (Jeltsch et al., 1997; Veikkola et al., 2001). Mutations in the gene encoding VEGFR-3 have been associated with human hereditary lymphedema (Irrthum et al., 2000, Karkkainen et al., 2000). However, VEGF-C, VEGF-D, and VEGFR-3 may also function in vascular development. VEGF-C and –D do bind to and activate VEGFR-2 (Eichmann et al., 1998; Achen et al., 1998). VEGF-C can stimulate vascular endothelial cell proliferation and migration in vitro and induces angiogenesis in mouse cornea and in chicken CAM assays (Joukov et al., 1996; Cao et al., 1998). VEGF-C can also induce angiogenesis in vivo in a model of limb ischemia (Witzenbichler et al., 1998). VEGF-D can promote neovascularization in the rabbit cornea assay as well as stimulate endothelial cell growth and morphogenesis in vitro (Marconcini et al., 1999). Expression of human VEGF-D in tumor cells stimulates

both angiogenesis and lymphangiogenesis in tumor xenografts (Stacker et al., 2001). In addition, VEGFR-3 is expressed in vascular endothelial cells in early embryogenesis, and is only restricted to lymphatic

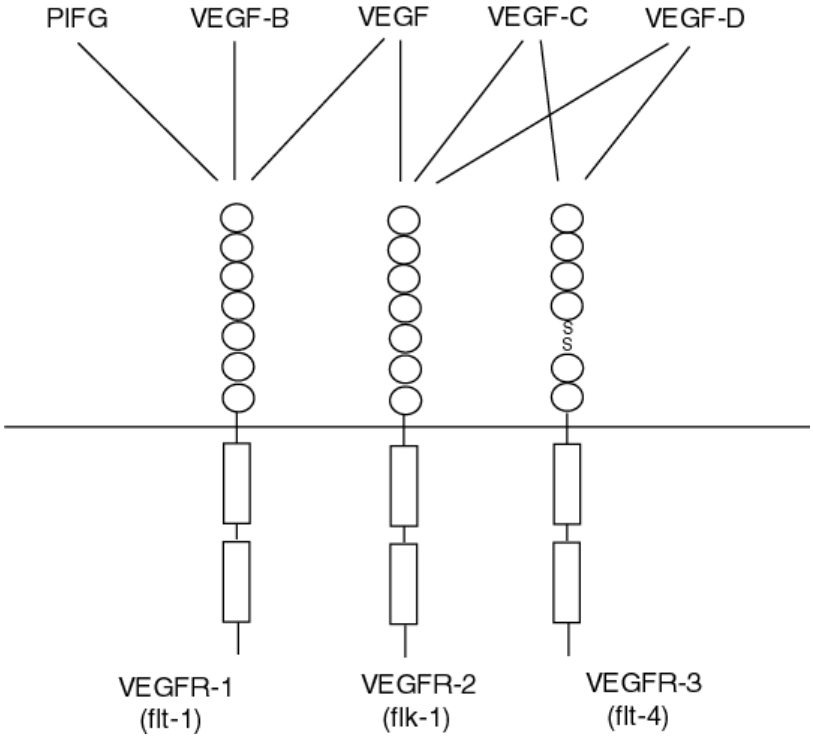

Figure 1. Diagram of VEGF and VEGF receptor family

endothelial cells after mid-gestation and in adults (Kaipainen et al., 1995, Kukk et al., 1996). In support of a role for VEGFR-3 and its ligands in embryonic vascular development, targeted inactivation of VEGFR-3 results in early embryonic lethality and defective vascular remodeling (Dumont et al., 1998). Primitive vascular networks form in these mice but they do not remodel into mature vessels. There is also defective vessel branching in some tissues. This suggests that besides its role in lymphatic development, VEGFR-3, and perhaps its known ligands, VEGF-C and VEGF-D, also function in vascular development in early embryogenesis. The phenotypes of mice deficient in VEGF-C or –D have not yet been reported.

Gene	**Bind to**	**Knockout phenotype (development)**
PlGF	VEGFR-1	Normal development
VEGF-A	VEGFR-1, VEGFR-2	Embryonic lethal in heterozygous state, delayed endothelial cell development, defective vascular remodeling and sprouting
VEGF-B	VEGFR-1	Normal development

VEGF-C	VEGFR-2, VEGFR-3	Not yet reported
VEGF-D	VEGFR-2, VEGFR-3	Not yet reported
VEGFR-1 (flt-1)	PlGF, VEGF-A, VEGF-B	Embryonic lethal, excess endothelial cells
VEGFR-2 (KDR, flk-1)	VEGF-A, VEGF-C, VEGF-D	Embryonic lethal, few endothelial cells
VEGFR-3 (flt-4)	VEGF-C, VEGF-D	Embryonic lethal, impaired vascular remodeling and sprouting

2) Development of the lungs

a. Development of the epithelium

Development of the lungs begins with endodermal budding from the ventral forgut at embryonic day 9.5 (E9.5) in the mouse and gestation week 4-5 in humans (Burri, 1984; Ten Have-Opbroek, 1991). Growth and repetitive branching of the epithelium give rise to the airways. The mesenchyme surrounding the endoderm gives rise to blood vessels, nerves, and interstitial cells. Four stages of lung development have been described, based mainly on airway morphology: (1) pseudoglandular stage, from E9.5-E16.5 in the mouse, (2) canalicular stage, from E16.5-E17.5, (3) saccular stage, from E17.5-P5 (postnatal day 5) in the mouse, and (4) alveolar stage, from P5-P30 (Boyden, 1977). The pseudoglandular stage is characterized by growth and branching of the epithelium to form all the conducting airways and the beginning of the pulmonary acinus, the respiratory (gas-exchange) unit of the lungs. The pattern of epithelial branching is stereotypical and is determined by epithelial-mesenchymal interactions. At the end of the pseudoglandular period, the airways terminate in a prospective acinus composed of the terminal bronchiole, 2-4 future respiratory bronchioles, and small clusters of short tubules and buds. During the canalicular stage, these clusters of tubules and buds develop by the lengthening and further peripheral branching and widening of the distal airspaces. The saccular stage is characterized by enlargement and further division of the distal airspaces into smooth walled structures, termed saccules. The wall of these saccules is thick and composed of epithelial lining on either side of a central core of connective tissue containing two capillary networks. These saccules can support gas exchange, but their function is not adequate for the metabolic needs of the organism. Alveolar formation starts after birth in the mouse by formation of secondary septa from the walls of the saccules to form pre-alveolar shallow sacs. Maturation of alveoli results from remodeling of the thick walls to form thin walls containing a single capillary network.

b. Development of the lung vasculature

During development of the epithelium, lung blood vessels develop concomitantly in the mesenchyme. Their development has to be tightly coordinated with the development of the epithelium to result in the intimate relationship between the two systems. In the mature lungs, pulmonary arterial branches mirror airway branching and capillaries form an intimate association with alveolar surfaces. Our knowledge of the development of lung blood vessels is quite rudimentary. Some morphologic features have been described (Boyden, 1977; Hislop and Reid, 1977). During the pseudoglandular stage, formation of the pulmonary vessels is likely initiated by vasculogenesis. Mesenchymal precursors differentiate in situ into endothelial cells. These endothelial cells coalesce and form a primitive network of interconnected channels. This was shown by classical embryological studies. When quail lung rudiments are grafted onto chick hosts, the vessels that subsequently develop in the graft are of graft origin, as determined by immunostaining for a quail-specific endothelial cell marker (Pardanaud et al., 1989). This is supportive of a vasculogenic process, with the presence of angioblasts intrinsic to the grafts. In contrast, grafting of a limb bud results in development of blood vessels in the grafts that are of host origin, indicating lack of angioblasts in the original graft, and angiogenic ingrowths from the host vasculature (Pardanaud et al., 1989). A recent study using subcutaneous allografts of lung rudiments in the mouse also found vessels developing from cells derived from the graft (Schwarz et al., 2000). The primitive vascular network that develops surrounding the airways presumably is subsequently remodeled into larger vessels. During the canalicular and saccular stages, as the distal airspaces lengthen, branch, enlarge, and subdivide to form saccules, there is extensive formation of capillaries in the surrounding mesenchyme as well as rearrangement of these vessels to form the double capillary network in the saccular walls. During alveolar development, the formation of the secondary septa is thought to result from invagination of one of the capillary beds in the saccular wall to form ridges that grow to divide the saccules into pre-alveolar sacs.

The double capillary beds in premature alveolar sacs need to be remodeled into the one capillary network on the mature alveolar walls. Active angiogenesis and remodeling of the vascular beds is therefore critical for alveolar morphogenesis.

A pioneering study of the early development of the lung vasculature in mouse embryos by light and electron microscopy of tissue sections and scanning electron microscopy of vascular casts shows both vasculogenic and angiogenic processes in the embryonic lungs (deMello et al., 1997). There is development of vascular spaces surrounding the airways as early as E10. This is most likely a vasculogenic process. Sproutings from central vessels by angiogenesis were noted at day E12, the earliest day that a vascular cast could be made. Connections between the central and peripheral systems were seen starting on E13-E14. Taken together, these data support a model of early lung vascularization by both angiogenesis and vasculogenesis: the de novo differentiation in the mesoderm surrounding the airways of a primitive plexus by vasculogenesis in concert with airway formation. The primitive plexus is subsequently remodeled into mature vessels. In addition, angiogenic extensions arise centrally from the developing aorta and left atrium, grow into the mesenchyme surrounding the lungs, and subsequently establish connections to the peripheral systems to complete the pulmonary circulation. A study of mice carrying a beta-galactosidase transgene under the control of an endothelial cell specific promoter for easy identification of endothelial cells shows more clearly the morphology of vessels during lung development (Schachtner et al., 2000). Endothelial cells are found in the mesenchyme surrounding invading epithelial buds at all stages of lung development. These form initially primitive capillary networks that then remodel into more definitive vascular structures. An unexpected finding from this study is the presence of connections between the aortic sac and the proximal primitive vascular network in lung mesenchyme as early as E10.5, suggesting that the central pulmonary vessels may also be formed by vasculogenesis. Using organ cultures and tissue recombination, Gebb and Shannon showed that development of endothelial cells in lung mesenchyme is dependent on the presence of lung epithelium. Lung mesenchyme cultured alone degenerates and lacks cells expressing flk-1, a marker of endothelial cells, whereas lung mesenchyme cultured together with epithelium contains a large number of flk-1 positive cells (Gebb and Shannon, 2000).

Despite the importance of the vasculature as a critical functional component of the mature lungs, and the potential role of angiogenesis in regulating the morphogenetic processes of lung development, the molecular mechanisms that regulate lung vessel formation are poorly understood. Very few studies have characterized molecular factors that function in lung vascular development. An anti-angiogenic molecule, Endothelial Monocyte Activating Polypeptide II (EMAP II), has been implicated in lung vascularization. Its expression level is decreased in late fetal stage, a period of increased vascularization in the embryonic lungs (Schwarz et al., 1999). Treatment of subcutaneous lung allografts with exogenous EMAP II results in decreased vascularity of the lung grafts (Schwarz et al., 2000). Many questions still remain. In particular, what are the factors that stimulate lung vessel development and how the pattern of vessel formation is coordinated with airway development are not entirely clear. Our hypothesis is that lung vascular development is regulated by angiogenic growth factors, some of which may be produced by epithelium, and that paracrine interactions may serve to coordinate the development of airways and blood vessels. In addition, the development of vessels may be critical for the continuing development of epithelium, and this feedback mechanism would also serve to ensure the concomitant development of the two systems. The family of VEGF seems particularly suited to serve as regulators of lung vascular development due to their known function in regulating endothelial cell growth and differentiation and their expression during lung development.

c. The VEGF family in lung vascular development

In the lungs, a role for VEGF-A and the other members of the VEGF family in vascular development is suggested by their pattern of expression. Previous studies have shown expression of VEGFR-1 (Flt-1) in mesenchymal cells surrounding the lung bud in E10.5 mouse embryos (Peters et al., 1993), expression of VEGFR-2 (Flk-1) in E12.5 lungs (Dumont et al., 1995; Maisonpierre et al., 1997), and of both VEGFR-1 and –2 in E17.5 embryonic lungs (Breier et al., 1995). However, the cellular location of these factors were not reported, except for one study reporting VEGFR-2 expression in endothelial cells in E14.5 lungs (Millauer et al., 1993). In our own studies (see below) we found high level of expression of VEGF-A by lung epithelium from E14.5 on. Earlier embryonic lungs express much lower levels, detectable only by RT-PCR. We also found that both VEGFR-1 and –2 are expressed at high levels by mesenchymal cells as early as E12.5, the earliest time point in our study. This pattern of expression, VEGF-A by

epithelium and its receptors by mesenchymal cells, suggests that VEGF-A participate in the inductive interactions between epithelium and mesenchyme in the formation of blood vessels. It has not been possible to test this hypothesis directly, however, since even one null allele of the VEGF-A gene results in early embryonic lethality (at E10.5-E11.5). However, one study suggests that regulated expression of VEGF-A is critical for lung morphogenesis. Over-expression of VEGF-A in lung epithelium in a transgenic mouse model led to abnormal epithelial development (Zeng et al., 1998). Saccules are not formed, instead the lungs are composed of dilated tubules with increased peritubular vascularity. The formation of vessels was not characterized in further details in this study. Since VEGF-A is not expected to have a direct effect on epithelium due to lack of epithelial expression of VEGF-A receptors, the epithelial abnormality observed suggests that perturbation of lung vascular development results in perturbation of airway development.

VEGF-B, -C, and –D, and VEGFR-3 are also expressed in embryonic lungs and therefore may also function in lung vascular development. VEGF-C and VEGFR-3 are expressed in the mesenchyme in E12.5 lungs (Kukk et al., 1996). VEGF-D is expressed at low level in E14 lungs and at much higher level in E17 lungs throughout the parenchyma (Farnebo et al., 1999). The function of these members of the VEGF and VEGF receptor families in lung development, especially in lung vascular development, is not known. Based on the non-overlapping function of each receptor as suggested by the phenotypes of the mice lacking them, one would expect that signaling through individual receptor might play a different role in the development of the lung vasculature. Since each member of the VEGF family interacts with more than one receptor, there may be competition in binding to the same receptor, and therefore the local levels of expression of the different factors may dictate which receptor signaling pathway is preferentially activated. The temporal and spatial expression of these factors may thus serve to regulate different aspects of vascular development. Our current research project seeks to identify the function of VEGF and VEGF receptor families in lung vascular development and to determine the effects of the perturbation of vascular development on epithelial development. We will determine the function of ligands by inactivating their expression or inhibiting their receptor binding, and of the receptors by selectively activating individual receptor or combination of receptors with receptor-specific ligands.

3) Significance

Even though much has been learned about the formation of blood vessels in general, very little is known about the formation of blood vessels during organogenesis such as during lung formation. The normal development of organs requires the formation of a functional blood supply. The pattern of vascular formation in each organ has to be tailored to each individual organ and therefore would require interactions between the vasculature and tissue itself. Studying the development of the lung vasculature not only addresses questions on these fundamental developmental processes but may also provide insights into the pathogenesis of diseases of lung development. Vascular malformations, pulmonary artery atresia, and pulmonary capillary atresia are obvious diseases resulting from abnormal vascular development. Yet diseases of airway maldevelopment such has pulmonary hypoplasia, cystic adenomatoid malformation, and bronchopulmonary dysplasia may also result from abnormalities in vessel formation, since normal airway development may depend on normal vascular development. In addition, most cancers in the lung result from neoplastic growth of epithelial cells, and these tumors depend on vascularization for their continuing growth. It is possible that neoplastic epithelium may turn on angiogenic mechanisms used during embryonic development to recruit a blood supply. Fibrotic lung diseases occurring either idiopathically or following injury may be due to abnormal lung repair, and normal lung repair may require the re-initiation of embryonic developmental pathways, including vascularization. The same may be true for the inability to repair areas of lung destruction such as in emphysema. Therefore, understanding the mechanisms of embryonic lung vascularization and how vascularization contributes to normal lung development may lead to the understanding of the pathogenesis of many developmental, neoplastic, destructive, and fibrotic lung diseases, and may identify therapeutic targets for these diseases.

C. Preliminary Studies and Any Results

1) Experience of the investigator relevant to the application

Dr. _____ has studied gene function in both animal models and in organ and cell cultures. The candidate has extensive experience with many approaches used to analyze tissue morphogenesis. The candidate also has expertise in angiogenesis, having studied the function of VEGF-A in skeletal development (_____). The candidate is currently the recipient of a Mentored Physician Scientist Award

(K08) to study epithelial-mesenchymal interactions in lung vascular development. Among the studies supported by this award, the candidate has succeeded in establishing the kidney graft model to study lung development (_____). The candidate has also carried out the studies on the role of VEGF-A in lung development using renal capsule grafts that form the preliminary results for the current research project. Since starting an independent position, the candidate continues to maintain interactions with Dr. _____ and other developmental and vascular biology groups at _____ for scientific interchange.

2) Expression of VEGF-A and VEGF-A receptors during lung development

To determine if VEGF may play a role in lung vascular development, we surveyed the expression pattern of VEGF and of its two receptors, VEGFR-1 (flt-1) and VEGFR-2 (flk-1) in embryonic lungs in different stages of development. We found that both VEGF-A and VEGF-A receptors are expressed in embryonic lungs, and that the expression pattern suggests paracrine interactions between epithelium and mesenchyme in vessel formation. VEGF-A expression is not seen in E12.5 and E13.5 lungs (Figure 2A and data not shown). However, we did detect expression of VEGF-A in these stages using RT-PCR (data not shown). VEGF-A expression becomes detectable by in situ hybridization at high level in epithelium on E14.5 (Figure 2B – not shown) and continues to be expressed in epithelial cells until E18.5. Interestingly, as development proceeds, the expression of VEGF becomes restricted to the epithelial cells of the distal airspaces at E16.5

Figure 2. Expression of VEGF-A in mouse embryonic lungs

Figure 3. Expression of VEGFR-1 in mouse embryonic lungs

(Figure 2C, arrows – not shown) and of the saccular walls at E18.5 (Figure 2D, arrows – not shown). At these stages, VEGF-A may function in the formation of the vessels of the pulmonary acinus.

Interestingly, we found that the two VEGF-A receptors, VEGFR-1 (flk-1) and VEGFR-2 (flt-1) are expressed at high levels earlier than VEGF-A. This suggests that these VEGF-A receptors may function through other ligands in the early period. VEGFR-1 is expressed in the mesenchyme throughout the lungs at the earliest time point in our survey, E12.5, and continues to be expressed in lung mesenchyme throughout development (Figure 3 – not shown). VEGFR-2 has similar pattern of expression (data not shown). These receptors are expressed in endothelial cells, both in those lining clearly identified large vessels, as well as in those lining capillaries. The expression pattern of VEGF-A and of its receptors is thus consistent with the model that epithelium directs vascular morphogenesis in the mesenchyme by producing VEGF-A to stimulate VEGF-A responsive mesenchymal cells to form blood vessels.

3) Lung renal capsule grafts as a model of lung development

To more expediently study gene function in lung development, we have generated in our laboratory a novel model of lung development that follows closely lung development in utero and that is readily accessible to experimental manipulation. We found that in E12.5 lung rudiments grafted underneath the kidney capsule of syngeneic or immunodeficient hosts, there is extensive development of both the epithelium and the vasculature up to the saccular stage. For details please see preprint _____ in Appendix 1.

4) Inhibition of VEGF-A leads to abnormal lung development

To investigate the function of VEGF-A in lung development, we inhibited VEGF-A function in the lung renal capsule graft model using a soluble form of VEGFR-1, mFlt-IgG. This is a chimeric molecule containing part of the extracellular domain of VEGFR-1 fused to the Fc portion of a mouse IgG. The extracellular domain portion of VEGFR-1 used in this construct is known to bind efficiently to VEGFR-1 ligands, including VEGF-A. Therefore, this soluble receptor can sequester VEGF-A and prevent its binding to and activating its cognate receptors. The particular mFlt-IgG that we used was provided by Dr. _____ at _____, and it has been used successfully in many applications to inhibit the function of VEGFR-1 ligands, including VEGF-A (Ferrara et al., 1998, Gerber at al., 1999).

Administration of mFlt-IgG to host mice harboring lung renal capsule grafts for one week caused alteration in the development of the grafts. The mFlt-IgG treated grafts are smaller and showed significantly different epithelial morphology (Figure 4A&B – not shown). The number of both bronchi (large airways lined by ciliated epithelium and surrounded by cartilage) and bronchioles (smaller airways lined by cuboidal nonciliated epithelium) are reduced. There are no definite saccules, instead, there are areas where the epithelium looks loosely arranged and areas where cells are compacted together (Figure 4C&D – not shown). The cells in these areas appear apoptotic. Indeed, TUNEL staining shows large numbers of apoptotic cells in the treated grafts (Figure 4E-G – not shown). Immunostaining with PECAM-1 antibody shows the presence of a few dilated vascular spaces, and no capillaries (Figure 5A-D – not shown). These results are consistent with the inhibition of vessel formation, with resulting alteration in epithelial development and global apoptosis, probably of both epithelial and mesenchymal cells. To determine when inhibition with mFlt-IgG exerts an effect, we analyzed lung grafts treated with mFlt-IgG for 3 and 6 days. After three days, there was no apparent effect on the morphologic development of the lung grafts (data not shown). After six days effect on the development of the grafts becomes apparent (Figure 5E&F – not shown). Assessment of proliferating cells by BrdU incorporation showed significant decrease in cell proliferation in the mFlt-IgG treated grafts at 6 days (Figure 5G-H – not shown).

5) Gene transfer into lung rudiments using adenoviral vectors

We have found that foreign genes can be introduced into lung rudiments by infection with recombinant adenovirus carrying the transgene. Following dissection from embryos, E12.5 lungs are mildly digested with a solution of 0.05 % dispase for 10 minutes at 37^0C to slightly disrupt the pleura. The lungs are then washed in medium containing EDTA to inactivate the dispase and then incubated for several hours with recombinant adenovirus. Following infection, the lungs are washed and can be grafted. Using β-galactosidase as a reporter gene, we found that we can transduce genes into cells of E12.5 lungs. Following mild dispase digestion and infection with adenovirus, the lung rudiment was cultured overnight

and tested for expression of the β-galactosidase transgene by substrate staining. Approximately 20-30% of cells in the lung rudiments, mostly mesenchymal cells close to the surface, were infected with the recombinant adenovirus in this protocol and expressed the β-galactosidase transgene, as evidenced by the blue substrate stain (Figure 6 – not shown).

Figure 4. mFlt-IgG treatment leads to abnormal airway development in lung renal capsule grafts

Figure 5. mFlt-IgG treatment inhibits vessel development in lung renal capsule grafts

Figure 6. Expression of the beta-galactosidase transgene mediated by adenovirus infection of lung rudiments

6) Appendices

Appendix 1:
Appendix 2:
Appendix 3:
Appendix 4:
Appendix 5:

D. Research Design And Methods

1) Ongoing Specific Aim 1: We will determine the function of Vascular Endothelial Growth Factor A (VEGF-A) in lung development.

a. Rationale.

From our preliminary studies, the expression of VEGF-A and VEGF-A receptors in the embryonic lungs suggests that VEGF-A may play a major role in lung vascular development. In addition, the complementary pattern of expression of VEGF-A and its receptors suggest that these molecules may participate in the coordinated development of the two systems. VEGF-A is expressed by epithelial cells, and VEGF-A receptors are expressed by surrounding mesenchymal cells, suggesting that epithelium induces the formation of the vasculature in the surrounding mesenchyme by secreting VEGF-A. Our preliminary studies showed that inhibition of VEGF-A leads to inhibition of lung vessel development and results in extensive cell death. This study is limited, however, in that the inhibitor of VEGF-A that we used, mFlt-IgG, is not specific to VEGF-A, and may bind to molecules other than VEGF-A. For example, it is known to also bind to PlGF and VEGF-B. It may also bind to as yet unidentified ligands. Thus the effects observed cannot be attributed entirely to inhibition of VEGF-A. In order to isolate the effects due to VEGF-A alone, the most direct study is to eliminate VEGF-A expression in vivo by inactivating the VEGF-A gene. Since global inactivation of the VEGF-A gene results in early embryonic lethality, we seek to inactivate this gene only in lung epithelium and in an inducible manner. We will study lung vascular and epithelial development following VEGF gene inactivation during specific periods of lung development. To accomplish spatial and temporal control of gene inactivation, we take advantage of two methodologies, the Cre-lox method of tissue specific gene inactivation, and the technique of tetracycline regulatable gene expression. Fortunately, all the transgenic mouse lines necessary for this set of experiments have been generated and are available to us.

b. Generation of mice with inducible lung-specific VEGF gene inactivation

To generate mice with inducible lung-specific VEGF-A gene inactivation, we will use the Cre-lox and tetracycline-inducible gene expression systems (Mansuy and Bujard, 2000; Ray et al., 2000). When a gene is flanked by loxP sites (“floxed” gene), it can be excised by the Cre recombinase. The excision, and therefore inactivation, of a gene is thus controlled by the expression of the Cre recombinase. The expression of Cre recombinase can in turn be regulated in an inducible manner by placing it under the control of a promoter containing a tetracycline responsive element (TRE). This promoter element is activated when a reverse tetracyline activator (rtTA) protein binds to it in the presence of either tetracycline or a derivative, doxycycline (Dox). The expression of rtTA can be regulated in a tissue-specific manner by placing it under the control of a tissue specific promoter. Therefore, temporal and spatial regulation of expression of Cre recombinase can be achieved by Dox treatment and tissue-specific expression of rtTA. Regulated temporal and spatial expression of Cre recombinase lead to regulated temporal and spatial inactivation of a “floxed” gene.

Mice whose VEGF-A gene has flanking loxP sites have been generated by Dr. _____ (VEGF-loxP mice). These mice have been used successfully to generate mice with both inducible global VEGF-A gene inactivation and tissue-specific VEGF-A gene inactivation using Cre-mediated excision (_____). In all these cases the VEGF-A gene is reproducibly silenced when the Cre recombinase is expressed. Dr. _____has provided us with these mice for our proposed studies (see letter). Mice with transgenes expressing Cre recombinase in lung epithelium in a tetracycline-inducible manner have been generated by Dr. _____ and have been provided to us for our proposed studies (see letter). These are double transgenic mice carrying the Cre recombinase gene under the control of a TRE containing promoter as well as the rtTA activator gene under the control of either the human SP-C (surfactant protein C) or rat

CCSP (Clara cell specific protein) gene promoter. The promoters are active in lung epithelium only and have been used to generate mice expressing a variety of transgene in lung epithelium, including the rtTA gene (_____). The double transgenic mice will be referred to as SPC/Cre or CCSP/Cre mice. Administration of tetracycline or doxycycline to these mice results in Cre recombinase expression and activity in lung epithelium that is seen as early as E9.5 with the human SP-C promoter and E14.5 with the rat CCSP promoter (_____).

To obtain mice with inducible VEGF-A gene inactivation in lung epithelium, we will use the following breeding scheme. Other breeding schemes are also possible. We will generate the following mouse lines by crossing: homozygous TRE-Cre/homozygous VEGF-loxP, heterozygous SPC-rtTA (or CCSP-rtTA)/wild type VEGF, and heterozygous SPC-rtTA (or CCSP-rtTA)/homozygous VEGF-loxP. Mice will be genotyped by PCR of tail DNA. None of these mouse lines carry all the three transgenes together, therefore they should not have inactivated VEGF-A gene and therefore can be maintained as breeding adults.

c. Studies of lung development following VEGF inactivation in vivo

Inactivation of VEGF-A during embryonic development. We will study the effects of both heterozygous and homozygous VEGF-A gene inactivation in lung development. To inactivate one allele of VEGF-A during embryonic development, we will administer Dox through drinking water to pregnant heterozygous SPC-rtTA (or CCSP-rtTA)/wild type VEGF female mice that had been mated with homozygous TRE-Cre/homozygous VEGF-LoxP male mice. All the embryos will be heterozygous for VEGF-loxP and heterozygous for TRE-Cre. Among these half of them will be heterozygous for SPC-rtTA or CCSP-rtTA. Administration of Dox will therefore result in excision of one allele of VEGF-A gene in the 50% embryos that are heterozygous for the SPC-rtTA (or CCSP-rtTA). The other 50% of embryos can be used as controls. To inactivate both alleles of VEGF-A we will administer Dox to pregnant heterozygous SPC-rtTA (or CCSP-rtTA)/homozygous VEGF-loxP female mice that had been mated with homozygous TRE-Cre/homozygous VEGF-LoxP male mice. All the embryos will be homozygous for VEGF-loxP and heterozygous for TRE-Cre. Among these half of them will be heterozygous for SPC-rtTA or CCSP-rtTA. Administration of Dox will therefore result in excision of both alleles of VEGF-A gene in the 50% embryos that are heterozygous for the SPC-rtTA (or CCSP-rtTA). The other 50% of embryos can be used as controls. It has been observed that following the administration of Dox recombination can be seen as early as after 48 hours, and that treatment with Dox starting at E6.5 and continuing throughout gestation results in expression of Cre recombinase activity throughout the entire lung epithelium (_____). Therefore, we will administer Dox during several stages of embryonic development: starting at E6.5 to inactivate the VEGF-A gene as early as the start of lung formation (E9.5); at E11.5 to inactivate VEGF-A gene by E13.5-14.5 (which in our preliminary studies is the period when high expression level of VEGF-A begins); and E14.5 to inactivate VEGF-A gene by E16.5-E17.5, the start of saccular formation during which VEGF-A is expressed in the distal epithelium, suggesting that it may be important for saccularization. With this design, we can study the effect of VEGF-A deficiency in different stages of lung development. After Dox administration, mice will be sacrificed at various time points thereafter and lung harvested for analyses.

Inactivation of VEGF-A gene during postnatal development. To inactivate the VEGF-A gene postnatally we will perform the crosses as described above and wait for the pups to be born before treating them with Dox in the drinking water. For pups still nursing, Dox can be transmitted to the pups through the mother's milk. We will treat newborn pups and evaluate the effects on lung development after one or two weeks of treatment, to determine the effects of VEGF-A deficiency on the alveolization process.

d. Analyses of lungs

To analyze the effects of VEGF-A gene inactivation on lung development, lungs will be harvested at several time points post VEGF-A gene inactivation with Dox treatment and analyzed for epithelial and vascular development. Lungs will be processed for RNA and protein isolation, frozen sections, and paraffin sections. VEGF-A mRNA expression will be analyzed by in situ hybridization of tissue sections and by quantitative RT-PCR of total lung RNA to determine the degree of success of gene inactivation. VEGF-A protein expression will be analyzed by Western blotting of whole lung lysates and by immunostaining of tissue sections with commercially available antibodies. Lung morphology will be evaluated by hematoxylin and eosin staining of paraffin sections. Differentiation of epithelium will be assessed by immunostaining with epithelial differentiation markers such as SP-C (available commercially)

and CCSP (gift of Dr. _____). Development of the vasculature will be assessed by immunostaining for endothelial cell markers such as flk-1 (VEGFR-2), PECAM-1, and CD34 using commercially available antibodies.

e. Potential difficulties and alternatives

We do not anticipate significant problems with this set of experiments. All relevant transgenic mouse lines have been made available to us by Dr. _____ and Dr. _____. The crosses to generate the mice needed for the study are straightforward. The SPC-rtTA, CCSP-rtTA, and TRE-Cre mice have been tested extensively in Dr. _____'s laboratory for the temporal and spatial expression of Cre recombinase in response to Dox. A potential difficulty, however, is that VEGF-A gene inactivation in the lungs will not be complete, and that there is still low level of VEGF-A expression in the triple transgenic mice in the presence of Dox. This low level of VEGF-A may be sufficient to promote lung vessel formation and therefore we will not detect any abnormalities in lung development. This is unlikely since the level of VEGF-A expression is very critical for normal vascular development; inactivation of even a single allele of the VEGF-A gene results in embryonic lethality. Therefore, we expect that any decrease in VEGF-A expression would result in discernable alteration in lung vascular development. Another potential difficulty is the "leakiness" of the promoters used, so that we may have expression of the Cre recombinase even in the absence of Dox. This should not be a significant problem, however. The TRE promoter will not be active in the absence of rtTA, therefore having mice carrying both TRE-Cre and VEGF-loxP transgenes should not have inactivation of the VEGF-A gene. Mice carrying both SPC-rtTA (or CCSP-rtTA) and VEGF-loxP genes should also not have the VEGF-A gene inactivated, since there is no Cre transgene. According to our breeding schemes, the only time that we have all three transgenes together are in our experimental embryos or pups. Therefore, we may get inactivation of the VEGF-A gene prior to administration of Dox if the expression level of rtTA from the SP-C or CCSP promoter is so high that the rtTA would be active even in the absence of Dox. This has only been observed at the later stages (after E17.5) with the SP-C promoter and in very few epithelial cells (less than 1%) (_____). We will determine whether there are problems with low level VEGF-A gene inactivation in the triple transgenic embryos or pups by studying their lung development in the absence of Dox, compared to littermate controls that are not triple-transgenic.

2) Ongoing Specific Aim 2: We will determine the function of other members of the VEGF family in lung development.

a. Rationale

Among the other VEGF family members, PlGF and VEGF-B individually do not appear to play critical roles in lung development, as mice with targeted inactivation of either of these genes show no developmental defects (Bellomo et al., 2000; Carmeliet et al., 2001). However, VEGF-C and VEGF-D may play a role in lung development, as both are expressed at high levels in embryonic lungs (Kukk et al., 1996, Farnebo et al., 1999). In the mice, VEGF-C binds to both VEGFR-2 and VEGFR-3, and VEGF-D binds only to VEGFR-3 (Baldwin et al., 2001). Binding to VEGFR-2 may stimulate angiogenesis. Indeed, VEGF-C is angiogenic in several angiogenesis assays (Joukov et al., 1996; Cao et al., 1998; Witzenbichler et al., 1998). Binding to VEGFR-3 stimulates lymphangiogenesis in later embryonic development, but with unclear effects in early embryonic development (Jeltsch et al., 1997; Veikkola et al., 2001). Inactivating the VEGFR-3 gene results in defective vascular remodeling and early embryonic lethality, suggesting that in early embryogenesis the binding of VEGF-C and/or VEGF-D to VEGFR-3 may function in the remodeling of the primitive plexus into mature vessels (Dumont et al., 1998). Vessel sproutings are also defective in the VEGFR-3 deficient embryos, indicating that activating this receptor may also be important in angiogenic vessel growth. The phenotypes of VEGF-C and VEGF-D deficient mice have not been reported, therefore their in vivo function is not clear. We expect that VEGF-C and –D also play a role in lung vascular development, either in remodeling of the primitive plexus into mature vessels or in vessel sproutings. We will assess the function of VEGF-C and VEGF-D in lung vascular development by studying the effects of inhibiting them with a soluble receptor in our lung renal capsule graft model. Since VEGF-C and VEGF-D may regulate aspects of vessel development different from those regulated by VEGF-A, these experiments will allow us to determine the consequences of perturbing different aspects of lung vessel development on epithelial morphogenesis. For example, if VEGF-C and –D play a role in maturation of the primitive plexus, we can determine whether maturation of vessels is important for normal maintenance of adjacent airways. This may also serve as a mechanism to ensure proper parallel development of the two systems.

b.Temporal and spatial expression of VEGF-C and –D and of VEGFR-3 during lung development

Previous studies have reported the expression of VEGF-C, VEGF-D, and VEGFR-3 in embryonic lungs at some stages of development. However, the temporal, spatial, and cellular expression patterns of these genes are not completely defined. In order to gain more insights into the potential function and mechanisms of action of these molecules, we will carry out a systematic study of their expression pattern during lung development using in situ hybridization on tissue sections, which allows the detection of mRNA within specific cell populations. Adjacent tissue sections of embryonic lungs at E10.5, 12.5, 14.5, 16.5, and 18.5 will be hybridized with S^{35}-labeled anti-sense probes against VEGF-C, VEGF-D, and VEGFR-3. This allows for the comparison of the populations of cells that express each of the ligands versus those that express the receptor. The candidate has available VEGF-C and VEGFR-3 probes as a generous gift of Dr. _____. We will use for VEGF-D probe either a VEGF-D specific oligonucleotide (Farnebo et al., 1999), or we will generate a probe by subcloning a VEGF-D cDNA fragment obtained by RT-PCR of embryonic lung RNA with VEGF-D specific primers.

Cells expressing VEGFR-3 in embryonic lungs may be either vascular or lymphatic endothelial cells. The candidate will determine whether VEGFR-3 positive cells are vascular or lymphatic endothelial cells as follows. We will identify cells expressing VEGFR-3 in mouse embryonic lungs using immunohistochemistry with VEGFR-3 antibody (commercially available) and observe whether channels lined by VEGFR-3 positive cells contain red blood cells. We will do immunostaining of adjacent sections with antibody against VEGFR-3 and with antibody against laminin, which is only found in blood vessel basement membrane and not that of lymphatic (Skobe et al., 2001). We will carry out in situ hybridization of adjacent sections with antisense probe against VEGFR-3 and with that against LYVE-1, a recently identified specific cell surface marker of lymphatic endothelium (Banerji et al., 1999; Prevo et al., 2001). We will generate antisense probe against LYVE-1 by subcloning a LYVE-1 cDNA fragment using LYVE-1 specific primers and RT-PCR of adult mouse lung RNA, which expresses high levels of LYVE-1. Antibody specific to mouse LYVE-1 that can be used for immunostaining has been reported (Prevo et al., 2001), however, this is not currently available to us.

c. Functional analysis of VEGF-C and –D in lung development

To determine the function of VEGF-C and VEGF-D in lung development, we will inhibit VEGF-C and –D function using soluble VEGFR-3 in the lung renal capsule graft model. Soluble VEGFR-3 would bind to and sequester VEGF-C and VEGF-D, preventing them from binding to endogenous receptors. Recombinant plasmid expressing soluble VEGFR-3 consisting of the first three immunoglobulin homology domains of the extracellular portion of VEGFR-3 fused to the Fc tail of human IgG1 has been made in Dr. _____'s laboratory. The recombinant chimeric protein has been shown to be effective at inhibiting the action of VEGF-C on responsive cells in vitro (Hamada et al, 2000). When expressed in vivo as a transgene in late embryogenesis using the Keratin-14 promoter, this protein inhibits lymphangiogenesis (Makinen et al, 2001). We have available the plasmid expressing this recombinant soluble VEGFR3-Ig as a generous gift of Dr. _____. We will inhibit VEGF-C and VEGF-D in lung grafts using several complementary approaches. We will produce recombinant solubleVEGFR-3 protein and administer it systemically to the host mice. We will generate recombinant adenovirus expressing soluble VEGFR-3 and use it to infect the lung rudiments prior to grafting to express soluble VEGFR-3 locally in the lung graft. We will infect the host mice harboring the lung grafts to express the soluble VEGFR-3 systemically in the host.

Production of recombinant soluble VEGFR-3. We will produce recombinant protein using the high recombinant protein expression system of Drosophila S2 cells. We will transfect Drosophila S2 cells with the VEGFR3-Ig expressing plasmid and select for stable expressing cells. These cells will be grown in high volume and secreted recombinant proteins will be purified from conditioned medium using protein A affinity chromatography. The Drosophila S2 cell system has been shown to produce large amount of secreted proteins, in the order of 20 mg/L. This is the same expression system used to produce soluble VEGFR-3 by Dr. _____'s laboratory (_____). Therefore, we expect to be able to produce sufficient amount of proteins for the experiments proposed below. The recombinant protein produced will be purified from the conditioned medium by protein A affinity chromatography, since it contains the Fc portion of an Ig molecule. We will test the activity of the recombinant protein by assaying its ability to inhibit VEGF-C induced tyrosine phosphorylation of VEGFR-3. VEGFR-3 expressing endothelial cells will be stimulated with VEGF-C in the presence or absence of soluble VEGFR-3. After stimulation, the receptor will be immunoprecipitated using

receptor specific antibody and assayed for the presence of tyrosine phosphorylation by Western blotting with an antiphosphotyrosine antibody.

Treatment of lung renal capsule grafts with recombinant soluble VEGFR-3. We will inhibit VEGF-C and –D function using soluble VEGFR-3 in the lung renal capsule graft model. E12.5 lung rudiments will be dissected out of embryos and grafted underneath the kidney capsules of syngeneic mice. Host mice will be treated systemically with either vehicle or recombinant soluble VEGFR-3 by daily intraperitoneal injection. When expressed as a transgene, the VEGFR-3 is detected in serum at approximately 100 ng/ml level. This apparently was sufficient to cause an effect on the embryonic development of the lymphatics. In the adults this level of soluble VEGFR-3 causes no discernable effects. Therefore the administration of soluble VEGFR-3 to the mouse hosts should not cause any untoward effects to the hosts. We will empirically determine the optimal dose of soluble VEGFR-3 to inhibit VEGF-C and –D in vivo as follows. We will inject mice daily with doses ranging from 5-25 μg/g/d. These are doses that have been found to be effective for mFlt-IgG. We will determine the serum levels of soluble VEGFR-3 daily before the next injected dose to determine the trough levels. Determination of VEGFR-3 level will be done using ELISA with antibodies against human IgG1-Fc. Doses that give serum trough levels close to 100 ng/ml will be used initially to treat hosts harboring lung grafts. We will also use lower and higher doses to determine the optimal dose response to the soluble VEGFR-3.

Treatment of lung renal capsule grafts with adenovirus expressing soluble VEGFR-3. We will construct replication defective adenoviral vectors expressing soluble VEGFR-3 using the efficient and rapid method of recombinant adenovirus production using homologous recombination in bacteria (available commercially from Stratagene). We will then use the recombinant adenovirus to produce soluble VEGFR-3 either locally in the lung grafts or systemically in the hosts. To produce soluble VEGFR-3 locally in the lung grafts, the embryonic lung rudiments will be infected with recombinant adenovirus expressing soluble VEGFR-3, according to the protocol described in our preliminary data, prior to grafting. The 20-30% infection rate that we obtain in this protocol should produce sufficiently high levels of soluble VEGFR-3 locally in the lung grafts to inhibit endogenous ligands. To produce soluble VEGFR-3 systemically in the host mice, after grafting of lung rudiments the host mice will be infected with recombinant adenovirus by a single intravenously injected dose. This results in the recombinant virus being taken up by the host cells (most likely liver endothelium) and the exogenous gene carried by the virus expressed and secreted into the circulation at high levels for several weeks. This technique has been used successfully to express a variety of secreted proteins systemically in mice, including soluble VEGF receptors (Hattori et al., 2001, Kuo et al., 2001). The serum level of the exogenous proteins depends on the quantity of adenovirus injected, but serum levels of up to 3 μg/ml – 8 mg/ml after a single dose of 10^9 viral particles have been reported (Kuo et al., 2001). The serum levels of the exogenous proteins persist for several weeks. Injection of empty adenovirus causes no untoward side effects to the mice (Hattori et al., 2001). We will determine the amount of recombinant adenovirus to inject the host mice by first assaying the serum levels of soluble VEGFR-3 obtained after injecting different doses of recombinant virus into adult mice. Determination of VEGFR-3 level will be done using ELISA with antibodies against human IgG1-Fc. We will then use different doses of virus to inject into host mice harboring the lung grafts to determine the dose response. Injection of empty adenovirus will be used as controls.

Analysis of the effects of soluble VEGFR-3 on lung development. Lung grafts will be dissected at various time points after treatment with soluble VEGFR-3 and analyzed. Inhibition of VEGFR-3 will be assessed by assaying for the amount of phosphorylated receptors. VEGFR-3 will be immunoprecipitated with receptor-specific antibody and assayed for the presence of phophotyrosine by Western blotting. Morphological development will be assessed by hematoxylin and eosin staining of paraffin sections. Large vessels do form in our lung renal capsule grafts, presumably from formation and remodeling of primitive capillary plexus. We will therefore be able to determine whether inhibition of VEGF-C and –D affects capillary plexus formation and/or its remodeling and maturation by the presence or absence of capillaries and large vessels. Differentiation of epithelial cells will be assayed by immunostaining for epithelial cell markers such as SP-C and CCSP. Vascular development will be assessed by immunostaining for endothelial cell markers such as flk-1, CD34, and PECAM. Since inhibition of VEGF-C and VEGF-D may also inhibit development of lymphatics, we will distinguish between blood vessels and lymphatics by their differential expression of LYVE-1 and laminin, as discussed above in section 3.2.b. We can also distinguish

blood vessels from lymphatic by the presence of red blood cells, and by labeling blood vessel endothelial cells with intravenously injected biotinylated lectin.

d. Potential difficulties and alternatives

A potential problem in this set of experiments is production of sufficient amount of recombinant soluble VEGFR-3 for systemic administration. However, our other methods, using adenovirus to express the protein locally in the lung grafts or systemically in the host should be sufficient. An alternative approach is to inhibit VEGF-C or VEGF-D using neutralizing antibodies or to inhibit their expression using anti-sense oligonucleotides. Currently neutralizing antibodies against VEGF-C or VEGF-D are not available. We can attempt to raise polyclonal neutralizing antibodies against VEGF-C or VEGF-D in rabbit. A rabbit plasmacytoma cells line, 240E, can be transiently transfected with a plasmid expressing a foreign protein and injected into rabbits (Spieker-Polet et al., 1995). The cells continue to express the foreign proteins in vivo, which elicits an immune response. Dr._____, an investigator in the _____ at _____, has expertise in this technique and is available to assist us. Antisense nucleotides have been widely used to inhibit gene expression both in vitro and in vivo (Lebedeva and Stein, 2001). Even though they do not work reliably, and it is difficult to predict a priori which gene will be amenable to antisense inhibition, many studies have reported successful inhibition of gene expression by antisense oligonucleotides in vivo (Arora and Iversen, 2000; Waters et al., 2000; Lowry et al., 2001). We can attempt to inhibit expression of VEGF-C and VEGF-D in the lung renal capsule grafts by treating the host mice with antisense nucleotides complementary to different regions of VEGF-C and VEGF-D mRNAs.

3)Ongoing Specific Aim 3: The candidate will determine the function of VEGF receptors in lung development

a. Rationale

The VEGF family members act by binding to and activating transmembrane receptors. Binding to ligand results in tyrosine phosphorylation of the receptors and activation of downstream signaling events, leading to changes in cell behavior, including cell proliferation, differentiation, or migration. Therefore the effects of each ligand depend on the types of cells that express the receptors and the types of signal transduction events that are initiated. Studies in mice with null mutations in each of the VEGF receptor underscore the non-overlapping function of these receptors. Mice lacking VEGFR-1 die in early embryogenesis, with increased numbers of endothelial cells that do not properly form vascular channels (Fong et al., 1995). Thus this receptor may serve as a negative control of endothelial cell growth and differentiation. In support of this, mice whose VEGFR-1 gene has been engineered to express a receptor with a non-active kinase domain develop perfectly normally with no apparent defects (Hiratsuka et al., 1998). Another study showed that VEGFR-1 kinase activity may actually antagonize the signaling activity of VEGFR-2. The ligand-activated kinase activity of VEGFR-2 is decreased in the presence of ligand-activated VEGFR-1 (Rahimi et al., 2000). Mice lacking VEGFR-2 also die in early embryogenesis, but with few endothelial cells, implicating a critical function for this receptor in endothelial cell growth and/or differentiation (Shalaby et al., 1995). VEGFR-3 deficiency also results in early embryonic lethality (Dumont et al., 1998). In this case, endothelial cells seem to develop normally and form normal primitive plexus, but these do not remodel and mature into normal vessels. There is also defective vessel sproutings in some tissues. Mice with null mutations in any of the VEGF receptors die early, precluding analyses of the function of individual VEGF receptor on vascular development in the lungs. Since many VEGF family members bind to more than one receptor, inhibiting ligands as proposed in our Aims 1 and 2 do not distinguish the function of individual receptor. In this Aim, we use a gain of function approach to study the function of individual receptor and combination of receptors in lung vascular development. Our method is to inhibit endogenous ligands with combination of soluble receptors, then selectively activate individual receptor or combination of receptors with exogenous receptor-specific ligands.

b. Receptor-specific ligands

Variants of human VEGF-A that bind preferentially to either VEGFR-1 or VEGFR-2 have been generated using site directed mutagenesis and phage-display selection (Keyt et al., 1996; Li et al., 2000). Two variants of human VEGF-A, one binding selectively to VEGFR-1, and the other selectively to VEGFR-2, were identified. The VEGFR-1 selective variant has the following mutations: I43A/I46A/Q79A/I83A. The VEGFR-2 selective variant has the following mutations: D63S/G65M/L66R. Binding to VEGFR-2 of the VEGFR-1 selective variant is reduced 100-fold. Binding to VEGFR-1 of the VEGFR-2 selective variant is

reduced 2000-fold. The two variants also show differential biological effects. Only the VEGFR-2 selective variant is able to induce downstream substrate phosphorylation, cause cell migration, and have angiogenic activity in the cornea pocket assay (Gille et al., 2001). The cornea pocket assay was done in the rat, indicating that the human VEGF-A variant can bind and activate rodent VEGFR-2 (Gille et al., 2001).

A variant of human VEGF-C that binds only to VEGFR-3, and not VEGFR-2 has been characterized. Mutation analysis of the conserved cysteine residues in VEGF-C showed that when cysteine 156 is replaced by a serine residue, the resulting VEGF-C mutant can no longer bind VEGFR-2 and induce its autophosphorylation (Joukov et al., 1998). However, it retains its capacity to bind and activate both human and mouse VEGFR-3 (Veikkola et al., 2001).

c. Functional analyses of activation of VEGF receptors on lung development

We will take advantage of the receptor-specific ligands to selectively activate individual VEGF receptor or combination of receptors in lung renal capsule grafts. We will treat the grafts with a combination of soluble receptors to inhibit endogenous ligands and an exogenous receptor-specific ligand to activate specific receptor. We will treat the lung grafts by either expressing these molecules in the grafts via infection with recombinant adenovirus and/or infecting the host mice with recombinant adenovirus to have the host cells express the recombinant proteins and secrete them into the circulation. Following treatment of lung renal capsule grafts with combination of soluble receptors and receptor-specific ligands, the grafts will be removed at various time points and analyzed. We will analyze for epithelial and vascular development as outlined in the previous Aims.

Adenovirus expressing receptor-specific ligands. Full-length cDNAs encoding human VEGF-A and VEGF-C will be obtained using specific primers and RT-PCR of human lung RNA (available commercially) and subcloned into plasmid vectors fused to a C terminal 6 X His tag and an HA tag. The mutations required to make receptor-specific variants will be introduced by site-directed mutagenesis (Kunkel et al., 1991). The resulting cDNAs encoding the variant forms will be subcloned into transfer vectors to be used for the construction of recombinant adenovirus. Following adenoviral construction we will test for the activity of the recombinant proteins by expressing them in cells in culture and then test the conditioned medium for the presence of activity that stimulates the tyrosine phosphorylation of the appropriate receptors. Cells expressing receptors will be stimulated with conditioned medium and the receptors immunoprecipitated with receptor specific antibodies and tested for the presence of tyrosine phosphorylation by Western blotting with anti-phosphotyrosine antibody. To use the recombinant adenovirus in lung grafts experiments, we will first determine the level of expression of the recombinant protein in host mice by injecting different doses of the recombinant virus into mice and then assaying for the level of exogenous proteins by ELISA with VEGF-A or VEGF-C antibodies or with anti-His and anti-HA antibodies. For the treatment of lung grafts, we will use viral doses that result in serum levels up to 1 ng/ml. Expression of VEGF-A systemically in mice using adenovirus up to this level was shown to be sufficient for an effect. Expression level higher than this leads to toxicity (Hattori et al., 2001). We will study different doses to determine a dose response.

Adenovirus expressing soluble VEGFR-1 and VEGFR-2. In addition to adenovirus expressing soluble VEGFR-3 that we will construct as described in Specific Aim 2, we will also construct adenovirus expressing soluble VEGFR-1 and VEGFR-2. We have full-length cDNA for mouse VEGFR-1 and VEGFR-2 in the laboratory. We will use PCR and specific primers to amplify the first three immunoglobulin domains of the extracellular regions of these receptors to make fusion construct to a murine IgG Fc fragment. The first three immunoglobulin domains of these receptors have been shown to be sufficient for ligand binding, and have been used for the construction of soluble receptors by many investigators (Ferrara et al., 1998; Kuo et al., 2001). These fusion constructs will then be subcloned into transfer vectors to be used for the construction of recombinant adenovirus. Following adenoviral construction, we will test for the activity of the recombinant soluble receptors by expressing them in cells in culture and then test the conditioned medium for activity that blocks the appropriate ligands. For example, we will test the ability of soluble VEGFR-1 to block VEGF-A stimulation of the tyrosine phosphorylation of VEGFR-2 in cells in culture. For lung graft experiments, we will first determine the level of expression of the recombinant proteins in host mice by injecting different doses of the recombinant virus into mice and then assay for the level of exogenous proteins using Western blotting or ELISA with antibodies against VEGFR-1 or VEGFR-2. To treat lung grafts, we will use doses of virus that will result in sufficient serum levels of soluble receptors. For VEGFR-

3 that will have been determined by the experiments in Specific Aim 2.. For VEGFR-1 and VEGFR-2 we will start with levels that have been used successfully by other investigators (Kuo et al., 2001).

Selective activation of individual VEGF receptor. We will first treat lung renal capsule grafts with receptor-specific VEGF-A and VEGF-C variants to determine whether further stimulation of each receptor by exogenous ligands would lead to changes in vascular morphogenesis. There may be no effect, however, since endogenous ligands may not be limiting. We will then selectively activate individual receptor after inhibiting endogenous ligands. We will selectively activate VEGFR-1 during lung development in renal capsule grafts as follows. We will infect the host mice with adenovirus expressing soluble VEGFR-2 and soluble VEGFR-3 to have these proteins expressed by the host and secreted into the circulation. Soluble VEGFR-2 and –3 will bind to VEGF-A, VEGF-C, and VEGF-D. Therefore only PlGF and VEGF-B are free to activate VEGFR-1. We will analyze the development of the lung grafts and determine the effects of having only VEGFR-1 activated by endogenous ligands. This may not be too informative however, since PlGF and VEGF-B acting through VEGFR-1 may not have any critical function, and the deficiency in VEGF-A may result in such significant defects in lung development, so that the effect of PlGF and VEGF-B may not be apparent. We will also selectively activate VEGFR-1 in this case with VEGF-A variant that binds only to VEGFR-1 and not VEGFR-2. This will tell us whether VEGF-A acting through VEGFR-1 has any consequential effect. We will introduce VEGF-A variant into the lung grafts by infecting them with adenovirus expressing this variant prior to grafting. Alternatively, the adenovirus expressing VEGF-A variant can also be given to the host.

To selectively activate VEGFR-2 in our lung renal capsule graft model, we will infect the host mice with adenovirus expressing soluble VEGFR-1 and soluble VEGFR-3. These will bind to all of the known endogenous ligands. PlGF, VEGF-A, and VEGF-B will bind to soluble VEGFR-1; VEGF-C and VEGF-D will bind to VEGFR-3. We will then treat the grafts with the variant of VEGF-A that is specific to VEGFR-2 by either infecting the lung rudiments prior to grafting or by infecting the hosts with recombinant adenovirus expressing this variant.

To selectively activate VEGFR-3, we will infect the host mice with soluble VEGFR-1 and VEGFR-2. These will bind to all the endogenous ligands, except for VEGF-D, which does not bind to either of these two soluble receptors. By studying the effects of treatment with these two soluble receptors on lung graft development, we can discern the function of VEGF-D alone. In addition, we can further activate VEGFR-3 by treating lung grafts with the variant of VEGF-C that binds only to VEGFR-3 and not to VEGFR-2. We can do this by either infecting the lung rudiments prior to grafting or by infecting the hosts with recombinant adenovirus expressing this variant.

Selective activation of combination of receptors. Taking advantage of soluble receptors and receptor specific ligands, we can also study the combined function of two receptors. Treatment with soluble VEGFR-1 will inhibit binding of endogenous ligands to VEGFR-1, as well as binding of VEGF-A to VEGFR-2. Therefore combined treatment with soluble VEGFR-1 and VEGFR-2 specific VEGF-A will allow activation of both VEGFR-2 and VEGFR-3 by endogenous ligands as well as activation of VEGFR-2 by the exogenous VEGF-A. Similarly, treatment with soluble VEGFR-2 will prevent binding of endogenous VEGF-A to VEGFR-1 and VEGFR-2, as well as the binding of endogenous VEGF-C to VEGFR-3. Therefore treatment with soluble VEGFR-2 and VEGFR-1 specific VEGF-A and VEGFR-3 specific VEGF-C will allow activation of both VEGFR-1 and VEGFR-3.

d. Potential difficulties and alternatives

A potential difficulty in this set of experiments is production of sufficient quantities of either soluble receptors or receptor-specific ligands using either systemic or local infection with adenovirus. We think that this will not be a problem, since high levels of expression of both VEGF-A and soluble VEGF receptors using systemic infection with adenovirus have been reported (Hattori et al., 2001; Kuo et al., 2001). Alternatively, we can produce recombinant proteins in Drosophila cells as discussed in Specific Aim 2 to administer systemically to the mice. We did not propose this as a first approach, since it involves producing and purifying a large number of proteins, which can be quite labor-intensive. It is feasible, however, should it become necessary. An alternative approach to study the function of individual receptor is to inhibit their function or their expression. We can attempt to raise polyclonal neutralizing antibodies to the receptors to inhibit their function, or we can try to inhibit their expression using antisense oligonucleotides, as discussed in Specific Aim 2.

4) Summary

The above peer-reviewed proposal focuses on the family of VEGF and VEGF receptors in lung vascular development. Even though these may not be the only factors that play a role in vessel development in the lungs, they are clearly the critical ones based on previous studies. At the end of our proposed experiments, we will be able to dissect and understand the function of, and interactions among, members of this important family of angiogenic factors and their receptors in lung vascular development. The VEGF and VEGF receptor families have many members that share mutual interactions, and the particular function of individual member in vascular development is not clear. We expect that the interaction of each ligand with its particular receptor lead to specific cellular responses, and binding of one ligand to more than one receptor may serve as a means to fine tune these specific responses. Thus the spatial and temporal expression of all the ligands and the receptors serve as fine controls of cell morphogenetic events. Our lung renal capsule graft model and our experimental design of both inhibiting specific ligands and activating specific receptors individually and in combination provide a novel means of dissecting the different physiologic function of and interplay among all these ligands and receptors. We will gain understanding as to the function of this family of angiogenic factors not only in the development of the vascular system of the lungs but our findings may extend to vascular development in general. In addition, by determining the effects of perturbations in vascular development on epithelial development, we can gain insights as to the interdependence of the development of the two systems, whose formation has to be regulated exquisitely in parallel.

C. New Additional Research Project

1) Statement Of Hypothesis And Specific Aim

Our hypothesis is that there are reciprocal inductive interactions between a tissue and its vasculature during organogenesis. The above project aims at determining the roles of the VEGF and VEGF receptor families in these interactions during lung development. However, to directly test the hypothesis of epithelial-endothelial cell interactions, it is important to be able to ablate one cell type and determine the consequences on the other cell type. We therefore propose to ablate in vivo either epithelial cells or endothelial cells during different stages of lung formation and determine the consequences of the absence of one cell type on the development of the other cell type.

2) Background and Significance

a. Vascular formation and patterning in organogenesis

The vasculature is an integral part of all tissues. In each tissue, blood vessels have a distinct local pattern specific to each organ. The development of the vasculature has been extensively studied, and many genes that regulate the formation, proliferation, migration, and morphogenesis of endothelial cells have been identified. However, it is less clear how blood vessels develop within the context of an organ. Evidence is emerging that vascular formation and patterning may not be self-determined by the endothelial cells but by extrinsic local environmental cues. In most vertebrates, the trunk axial vessels form in a constant relationship with the surrounding tissues. The dorsal aorta forms adjacent to and ventral to the notochord, and the posterior cardinal vein forms adjacent to and dorsal to trunk endoderm. In zebrafish mutants lacking axial mesoderm (notochord), the formation of the dorsal aorta is defective (Fouquet et al., 1997; Sumoy et al., 1997). Dorsal endothelial cells (Flk-1 expressing cells) normally present below the notochord are absent. Transplantation of wild-type axial mesodermal cells induces the formation of Flk-1 expressing cells immediately beneath the transplanted cells. These data suggest that the notochord is necessary for the formation of the dorsal aorta. Of note the formation of the posterior cardinal veins, and Flk-1 expressing cells ventral to trunk endoderm, are not affected in these mutants, suggesting that the formation of these vessels may be regulated by other local structures. In the embryonic mouse limb skin, arteries are aligned with the peripheral nerves and follow their branching pattern. During embryogenesis, induction of arterial markers occurs in skin vessels that are associated with the invading nerves. In mutant mice that lack peripheral nerves, expression of arterial markers is reduced and the branching pattern is disrupted (Mukouyama et al., 2002). Similarly, in mutant mice with a disorganized pattern of peripheral nerves, the arterial pattern is also disrupted, with arterial markers expressed only in those vessels associated with nerves. The XX and XY gonads have identical primary vasculature until the expression of male-specific genes in the XY gonad, at which time formation of testis cords commence coincidence with

the establishment of a male-specific arterial system. In explant cultures, only the XY gonads are able to induce the migration of endothelial cells from the adjacent mesonephros to form the male arterial system (Brennan et al., 2002). These examples support the idea that tissue cells may specify its own vascular patterning. This may be accomplished by either secreted or cell surface associated factors. An angiogenic factor selective for endocrine gland endothelium has recently been isolated (LeCouter et al., 2001). The expression of this growth factor is restricted to steroidogenic tissues such as adrenal, ovary, testis, and placenta.

Besides the role of the tissue in determining its own vascular patterning, it is also becoming increasingly appreciated that blood vessels may do more than provide the tissue's metabolic needs. Indeed, the vasculature may have an inductive role in tissue morphogenesis. The early development of the liver and pancreatic buds may depend on adjacent endothelial cells (Lammert et al., 2001; Matsumoto et al., 2001). In Xenopus embryos, blocking the formation of the dorsal aorta results in reduction of pancreatic differentiation (Lammert et al., 2001). Co-culturing mouse pre-pancreatic endoderm with the dorsal aorta or other tissues containing endothelial cells leads to pancreatic differentiation. Structures resembling pancreatic buds are formed that express pancreatic-specific genes such as Pdx1 and insulin. Ectopic expression of VEGF in the areas of future pancreatic formation results in hyperplasia of pancreatic tissues. In flk-1 null mice that lack endothelial cells, the liver buds do not form (Matsumoto et al., 2001). In liver bud explants grown in culture from flk-1 homozygous embryos, hepatic cells constitute 5% of the cell mass compared to 20% in heterozygous liver bud explants. Treatment of the wild type explants with an angiogenic inhibitor also inhibits hepatic cell development. In the higher vocal center (HVC) of the adult songbird neostriatum, testosterone induces angiogenesis and neurogenesis to mediate the seasonal hypertrophy of the HVC. Inhibition of angiogenesis results in inhibition of neurogenesis (Louissaint et al., 2002). The authors showed that testosterone induces the expression of brain-derived neurotrophic factor (BDNF) by cultured primary HVC microvascular endothelial cells. BDNF is capable of promoting the outgrowth of neurons from HVC in culture. Thus testosterone may act by inducing angiogenesis in the HVC as well as the production of a neurotrophic factor by endothelial cells to promote neurogenesis.

Evidence is thus accumulating to support the hypothesis that there are reciprocal inductive interactions between a tissue and its vasculature during organogenesis. Tissue cells may induce the formation and specify the patterning of its blood vessels, and conversely, endothelial cells in each tissue may regulate the growth and morphogenesis of the tissue. This interdependence in their development would serve to ensure the proper architectural relationship between a tissue and its vasculature that is unique to each organ and necessary for its proper function. The lungs exemplify this intimate functional relationship between a tissue and its vasculature. Lung blood vessels form parallel to the conducting airways and form capillary networks surrounding the terminal gas-exchange air sacs, or alveoli (Please see sections above for more detailed background on lung epithelial and vascular development). The spatial proximity and the close temporal development of the airways and the vasculature lead me to hypothesize that the epithelium and blood vessels each have inductive roles on the differentiation, morphogenesis, and/or survival of the other. To test this hypothesis I propose to generate transgenic mice in which cells can be ablated in a spatially and temporally regulated manner. I will then use these mice to ablate either lung epithelial or endothelial cells during different stages of lung development to determine the roles of one cell type on the development of the other.

b. Significance

Studying the interdependence of airway and blood vessel development in the lungs will help not only our understanding of lung morphogenesis but also of blood vessel formation within an organ and of the role of endothelial cells in organ formation. This knowledge may have broad therapeutic implication. It may help us understand the pathogenesis of developmental diseases and may lead to both their treatment and the ability to stimulate tissue regeneration in the adult. In addition, since neoplastic diseases may recapitulate some developmental processes, the knowledge gained may contribute to therapy for these diseases as well.

3) Research Design And Methods

In order to ablate specific cells in vivo, we will generate a transgenic mouse line carrying a regulatable transgene encoding a proapoptotic molecule whose expression results in cell death. The proapoptotic gene is placed under a promoter containing a tetracycline-response element (TRE). This promoter is normally silent and is activated either by the tetracycline-controlled transactivator (tTA) in the

absence of doxycycline (Dox), or by the reverse tTA (rtTA) in the presence of Dox. Cell-type specific expression of the gene of interest is therefore achieved by placing tTA or rtTA under the control of a cell type specific promoter, and the removal or administration of Dox achieves temporal regulation of gene expression.

a. Bax as a molecule for cell ablation

Apoptosis is an integral part of vertebrate development. Apoptotic cells are eliminated without subsequent induction of an inflammatory response. Therefore, expression of a proapoptotic molecule is a great means of cell ablation. One excellent candidate is Bax, a member of the Bcl-2 protein family. Bax is predominantly cytosolic and translocates to the mitochondrial outermembrane following several apoptotic signals. Once on the mitochondrial membrane, Bax forms oligomeric pore structures that lead to the release of cytochrome C and apoptosis (Desagher and Martinou, 2000). The translocation of Bax to the mitochondrial membrane is an essential step in the execution of apoptosis by Bax. This process requires a conformational change in the Bax molecule that causes the disengagement of the N-terminal BH-3 death domain from the C-terminal α9 helix mitochondrial-targeting domain (Desagher et al., 1999). Forced overexpression of Bax can induce Bax translocation, probably through similar conformational change induced by dimerization (Gross et al., 1998; Pastorino et al., 1998). Deletion of the amino acid 184 serine in the α9 helix of human Bax also disengages the BH-3 domain and leads to the translocation of Bax to mitochondria and apoptosis (Nechushtan et al., 1999; Suzuki et al., 2000). Expression of this serine 184 deleted form (BaxΔS184) has been shown to effectively cause cell apoptosis (Nechushtan et al., 1999). We will therefore express this constitutively active BaxΔS184 using a TRE containing promoter to generate transgenic mice in which cell apoptosis can be regulated.

b. Generation of mice with conditional cell ablation

We will generate BaxΔS184 cDNA by deletion of the amino acid serine 184 in wild type human Bax cDNA (provided by Dr._____) by PCR using specifically designed primers. BaxΔS184 cDNA will then be subcloned into pEGFPC1 (Clontech) to add an N-terminal EGFP tag, then placed downstream of a TRE regulated promoter in the pTRE2 vector (Clontech). The construct is then tested for expression and for the ability to cause cell apoptosis by co-transfection with a plasmid expressing rtTA into various cell types, including epithelial cells. Expression of BaxΔS184 in response to Dox will be assessed by expression of the EGFP tag. Apoptosis of transfected cells will be determined by staining for annexin V, a marker for apoptotic cells, and by the MTT- cell viability assay. Once function and inducibility are verified the construct will be injected into fertilized eggs to generate transgenic mouse lines (TRE-BaxΔS184 mice). Injection of fertilized eggs will be done by transgenic core facility at _____. Genotyping of transgenic mice will be carried out by PCR using upstream and downstream sequences of human BaxΔS184 cDNA in the transgene as primers and confirmed by Southern blotting using human Bax cDNA sequences as probe. The different lines of TRE-BaxΔS184 mice will be screened for inducible expression of BaxΔS184 that results in cell apoptosis. This is done by cross breeding with transgenic mouse lines expressing either tTA or rtTA such as the TgN(tTAhCMV)3Uh mice that express tTA in many tissues under the human CMV promoter (available from Jackson Labs). Pregnant mice will be treated with Dox (in rtTA lines) or withdrawn from Dox treatment (in the tTA lines) to induce BaxΔS184 expression. Double transgenic embryos (carrying both BaxΔS184 transgene and rtTA/tTA transgene) will be analyzed for the expected cellular expression of BaxΔS184 and cell apoptosis. Expression of BaxΔS184 will be assessed by the expression of the EGFP tag and cell apoptosis by TUNNEL staining on tissue sections. Before using in the experiments described below, the TRE-BaxΔS184 mice will be analyzed for the absence of any morphological abnormalities caused by the presence of the transgene. The mice will be observed for any gross phenotypic changes. Tissue sections will be made from different organs and analyzed histologically to discern any abnormal morphology.

c. Study of the role of epithelial cells in embryonic lung vessel development

We will ablate lung epithelial cells during different stages of embryonic development in vivo and determine the consequences of their absence on different aspects of vascular development. The TRE-BaxΔS184 mouse line will be bred to the SPC-rtTA mouse line that expresses rtTA under the control of the human SPC promoter (the same line used in Ongoing Specific Aim 1 described in section B.3.1; the mice are provided by Dr. _____). This promoter is active in lung epithelial cells throughout embryonic development. The SPC-rtTA mice have been used to express a number of transgenes in lung epithelium in a Dox-regulatable manner (_____). We will breed heterozygous or homozygous TRE-BaxΔS184 mice with

heterozygous SPC-rtTA mice to generate littermates of embryos heterozygous for both transgenes or for only the TRE-BaxΔS184 transgene, which can be used as controls. We will ablate cells by adding Dox to the drinking water of pregnant dams. Transgene expression in this system was shown to be induced by 6 hour after Dox treatment and reach maximum after 16 hour (_____). Therefore we will start Dox treatment 1-2 days before the gestational stage of interest to achieve significant BaxΔS184 expression and cell ablation by that stage. After treatment, embryonic lungs will be removed for analyses of epithelial cell death and of the consequences of epithelial cell ablation on vessel development. We are interested in the role of the epithelium in the following stages of vascular development:

(1) Vasculogenesis, or the proliferation, differentiation, recruitment and assembly of endothelial cells into primitive vascular networks: the lung buds form by outgrowths of the ventral foregut into the surrounding mesenchyme around E9.5. The epithelial buds subsequently undergo growth and repetitive branching to form the airways. During the first few days of lung development (E9.5-12.5) vasculogenesis occurs in the mesenchyme to form a primary vascular plexus surrounding the branching epithelial tubes. This occurs by the in situ development of endothelial cells from precursors already present or recruited into the lung mesenchyme, and the organization of these endothelial cells into an interconnecting tubular network. Since vasculogenesis occurs in proximity to the branching epithelial tubes, we ask whether epithelial cells are necessary for the development of the primary vascular plexus. We will treat pregnant mice with Dox on E9.5 or E10.5 to achieve sufficient epithelial cell ablation by E10.5-12., during which time vasculogenesis is normally actively occurring around the epithelium. We will analyze endothelial cell development in the lungs 1 or 2 days after start of Dox treatment. We are particularly interested in determining whether the density of endothelial cells, and in particular the percentage of proliferating cells, is altered after epithelial cell ablation, which would imply that their formation, growth, and/or recruitment have been affected. We are also interested in determining whether the organization of endothelial cells into a vascular plexus is impaired in the absence of epithelium. Extent of epithelial cell ablation will be assessed on hematoxylin & eosin stained tissue sections for tissue morphology and by TUNNEL staining for apoptotic cells. Epithelial cells are easily recognizable on histological sections. Endothelial cell development will be assessed by immunostaining for the endothelial cell markers Flk-1, PECAM-1, or CD34. Immunostaining will be done either on tissue section to visualize individual cells or on whole mount to visualize vascular networks in three dimensions. The proliferative status of endothelial cells will be determined by BrdU labeling.

(2) Remodeling of the primitive vascular networks into more mature vessels: as lung development proceeds during E13.5-15.5, vasculogenesis continues to occur around the distal branching epithelial buds. However, the primitive vascular plexus that has formed around the more proximal airways remodels into larger-caliber and more mature vessels. We will determine whether the remodeling of the primitive vascular plexus requires the presence of the adjacent epithelium. We will ablate epithelial cells during this stage by treating pregnant mice with Dox starting on E12.5 or E13.5 and analyze the lungs 1 or 2 days after treatment. Epithelial cell ablation will be assessed as above. Endothelial cell development will be assessed both by morphology on H&E stained sections and by immunostaining for endothelial cell markers. We are particularly interested in whether there are larger caliber vessels present adjacent to proximal airways in which epithelial cells have been ablated.

(3) Continuing growth and maintenance of remodeled vessels: as lung development progresses during E16.5-E18.5, the remodeled vessels around the proximal airways continue to mature and grow in size. We will determine if the growth and survival of these vessels depend on the continuing presence of the adjacent epithelium. We will therefore start Dox treatment on E15.5 or E16.5 and analyze the lungs 1 or 2 days after treatment for the presence and morphology of the vessels accompanying the large proximal airways.

d. Study of the role of endothelial cells in embryonic lung epithelial development

We will ablate endothelial cells during different stages of lung embryonic development in vivo by cross breeding the TRE-BaxΔS184 mice with mice that express rtTA in endothelial cells. The best promoter to express rtTA in endothelial cells for our purpose is the Flk-1, or VEGFR-2 promoter. Flk-1 is expressed

by the endothelial cells found around the early branching lung epithelial tubes, and it continues to be expressed in endothelial cells throughout development and in the adults. There are currently no available transgenic mice that express rtTA under the flk-1 promoter; therefore, we propose to generate these mice in order to carry out endothelial cell ablation. Another approach to study the role of endothelial cells in organ development is to use the flk-1 null mice that have defect in endothelial cell development. However these mice die in early embryogenesis, between E8.5 to E9.5, precluding their use in the study of lung development.

(1) Generation of mice with endothelial cell expression of rtTA

Our plan is to "knock-in" the rtTA transgene into the flk-1 gene locus. A previously generated flk-1 targeting vector containing a lacZ transgene inserted into exon 1 has been used to generate mice with targeted mutagenesis of the flk-1 gene (Shalaby et al., 1995). Mice heterozygous for the targeted allele show expression of lacZ in endothelial cells. We will replace the lacZ sequences in exon 1 in this vector (provided by Dr. _____) with rtTA sequences. The construct will then be electroporated into ES cells. ES clones with a recombined flk-1 locus will be selected and screened by PCR and confirmed by Southern blotting using an external probe. Correctly targeted clone will be injected into mouse blastocysts to generate chimeras. ES cell work and blastocyst injection will be done by transgenic core facility. Chimeras will be tested for germline transmission of the targeted locus and used to generate mice heterozygous for a targeted flk-1 allele expressing rtTA under the flk-1 promoter (FLK1-rtTA mice). Expression of rtTA in endothelial cells will be confirmed by cross breeding with reporter mice such as the TgN(tetoplacZ)2Mam mice that express LacZ under TRE promoter (available from Jackson Labs). Pregnant mice will be treated with Dox to induce expression of LacZ in double transgenic embryos. Expression of LacZ in endothelial cells will be assessed by substrate staining.

(2) Endothelial cell ablation

We will use the same breeding scheme as above, replacing SPC-rtTA mice with FLK1-rtTA mice to ablate endothelial cells at different stages of embryonic development. We will ablate endothelial cells only in the early stages of lung development before vascular function becomes necessary, so as not to confound an effect due to the endothelial cells themselves vs. a metabolic effect due to lack of blood circulation. We are interested in the role of endothelial cells in lung bud formation and in epithelial branching morphogenesis. Around E9.5, lung buds form by the local protrusion of the foregut endoderm into the surrounding mesenchyme. There are endothelial cells (flk-1 positive cells) in the mesenchyme around the lung buds at this time (Schachtner et al., 2000). We will determine the role of these endothelial cells in lung bud formation by ablating them with Dox treatment starting on E7.5 or E8.5. After Dox treatment the embryos will be dissected on E9.5 to determine if the lung buds have formed. Histological sections will also be prepared from whole embryos to determine the presence of lung buds. Extent of endothelial cell ablation will be assessed by immunostaining for endothelial cell markers and by TUNNEL staining. During E10.5-12.5 the lung buds continue to grow and form many branches. Primitive vascular networks form around the branching tubules, but these do not seem to be connected to the central vessels, and therefore presumably, the circulation has not yet been established (deMello et al., 1997; deMello and Reid., 2000). We will determine the role of endothelial cells on epithelial branching by ablating them with Dox treatment starting on E9.5 or E10.5 and analyze the lungs 1 or 2 days later. Gross morphology of lungs dissected from embryos will be compared as well as morphology of lungs on tissue sections. Number of branches and terminal buds will be counted to determine the effects on branching. Endothelial cell ablation will be assessed by immunostaining for endothelial cell markers and by TUNNEL staining. To confirm the effect of endothelial cells aside from vascular function, we will also study the role of endothelial cells in epithelial branching morphogenesis in organ cultures. E11.5 lung rudiments go through limited branching morphogenesis in vitro. We will culture E11.5 double transgenic lungs and compare the complexity of branching in these lungs in the presence of or absence of Dox. Endothelial cell ablation in the treated explants will be assessed by whole mount immunostaining for endothelial cell markers.

e. Potential difficulties and alternatives

We do not expect any significant difficulties generating the proposed transgenic mice. Transgenic mice expressing a variety of molecules have been successfully generated. Obtaining lines with high level of expression will depend on integration sites, and screening through many lines may be necessary. One potential difficulty in the tetracycline regulatable gene system is the residual affinity of rtTA to the TRE promoter in the absence of Dox, resulting in a low degree of transgene activation and phenotype induction

in the animals that are not receiving Dox. To overcome this problem, the tetracycline-controlled transcriptional silencer (tTS) has been developed (Freundlieb et al., 1999). tTS binds to TRE only in the absence of Dox and completely inhibits the expression of the downstream gene, even in the presence of rtTA. Dox causes the dissociation of tTS from TRE to relieve the transcriptional suppression. This system has been shown to eliminate transgene leakage in transgenic mice model (Zhu et al., 2001). In case transgene leak occurs and causes unplanned cell death, we will utilize the tTS system to suppress the leak. Another potential problem is the timing of our Dox treatment and the expected time of cell death. We may need to modify our treatment and analysis time points depending on our preliminary results once we get the mice. The studies of endothelial cells ablation may be problematic, if vascular function is necessary early, and the effects that we observe are due to lack of oxygen and nutrients and not due to the endothelial cells themselves. However, we are restricting our studies to earlier lung developmental stages to avoid this possibility, and we are also planning to do organ cultures to separate the effect of the circulation, if present.

4. Summary

This project aims to determine the interdependence in development between epithelial and endothelial cells in the lungs by cell type-specific ablation in vivo. The results obtained would be of great interest as they give us better understanding of the role of the tissue in its own vascular patterning and conversely, the role of endothelial cells in tissue morphogenesis. The TRE-BaxΔS184 mice will also be a very useful tool for other studies. They can be used to study the role of any particular cell type in any processes, as long as mice expressing a Tet transactivator (tTA or rtTA) in the cells of interest are available. Thus they can be used in the studies of many developmental and disease processes. The FLK1-rtTA mice will also be very useful in other studies. They can be used to express any molecules of interest in endothelial cells in a temporally-regulated manner. Thus they can be used to study many aspect of endothelial cell function.

D. Research Design and Method

Timetable: The first 1-2 years will be devoted to the generation, characterization, and analyses of mice with inducible VEGF-A gene inactivation in lung epithelium. We will study the effect of VEGF-A deficiency on lung morphogenesis at different developmental stages, both embryonic and postnatal. We will also initiate the production of purification of soluble VEGFR-3. During the next 1-2 years we will initiate the construction and testing of recombinant adenovirus expressing soluble VEGFR-1, VEGFR-2, and VEGFR-3. We will also start the construction of recombinant adenovirus expressing receptor-specific ligands. We will also start the generation of the TRE-BAX and FLK1-rtTA mice. The subsequent years will be devoted to studying the functional effects of soluble receptors and receptor-specific ligands in the lung renal capsule graft models and carrying out the cell ablation studies.

E. Human Subjects:

None

F. Vertebrate Animals

Description of Animals and Procedures Involving Animals

All the necessary animal procedures will be performed either in the Animal Care Facility located at _____, _____, or in the Small Animal Surgery room in the _____.

Species and Strains. The C57Bl6, C3H inbred strains of mice will be used for renal capsule grafting. Transgenic mouse lines are in C57Bl6 or 129 background. For developmental studies, mice of both sexes will be used, either as adults (2-6 month old) or as pups (up to one month old). Renal capsule grafts will be done on adult female mice.

Husbandry of transgenic lines. Transgenic mice will be mated to propagate the lines or to generate new lines with combination of the transgenes. Mice will be genotyped by analysis of DNA isolated from tail biopsy. At the time of tail biopsy, animals will be marked with an ear tag with a unique number for identification.

Generation of timed-pregnant mice. Adult female mice will be mated with adult male mice. Plugs will be checked daily for evidence of mating.

Renal capsule grafting. Embryonic lung rudiments will be dissected out of embryos from timed-pregnant females. They will then be grafted underneath the kidney capsule of adult mouse hosts. The surgery will

be done using aseptic techniques under general anesthesia following standard protocol approved by the Animal Care Committee at _____.
Tissue collection. For tissue collection, mice will be sacrificed by cervical dislocation and tissue collected at autopsy.

Justification

Use of Animals. This project uses mice as model of human development. We use both transgenic mice to study lung development in utero, as well as a lung allograft model to study lung development in an environment free from the placental barrier. The complex nature of organ development, with interactions of multiple cell types and the three-dimensional extracellular matrix requires the study of whole organ in situ or in an environment that closely resembles embryonic development. In addition, the study of the development of the vasculature requires the presence of a functional circulatory system. Therefore, neither in vitro cell cultures nor organ cultures could be adequate.

Species used. The mouse is the best-characterized laboratory animal used in biomedical research. Some of the studies that we proposed can only be done on transgenic mice. Mice are also easy to maintain and keep in reasonable number.

Numbers used. Our studies require the maintenance of several transgenic lines. The need for triple transgenic lines requires a large number of breeding in order to obtain adequate numbers of animals for use. We try to minimize the number used but still have adequate number for statistical significance. In sum we estimate an average of approximately 60 cages of mice in an ongoing basis, both for line maintenance, and to generate adequate number for experimentation.

Veterinary Care

The _____ Animal Care Facility complies with PHS policy on the use of laboratory animals. Animals are maintained in accordance with the applicable portions of the Animal Welfare act and the DHHS Guide for the Care and Use of Laboratory Animals. Veterinary care is under the direction of a consulting veterinarian boarded by the _____ College of _____. Additional veterinary nursing staff and veterinary technicians provide a complete comprehensive program of diagnostics, preventive, and clinical medicine at our facility. The consulting veterinarian has authority to terminate all animal research that does not comply with current government regulation or university policy.

Animal Concerns

Mice will be anesthetized during all surgical procedures. They are also monitored during and after procedures for signs of distress. All mice showing distress will be euthanized. Anesthetic used is 2.5% Avertin, administered intraperitoneally. 100% Avertin is prepared by dissolving 10 gm of tribromoethyl alcohol in 10 ml of tertiary amyl alcohol. This drug is the anesthetic of choice for many procedures involving mice and is recommended at our institution.

Euthanasia

Euthanasia will be by CO_2 anesthesia followed by cervical dislocation or by bilateral thoracotomy. This is the method of choice for quick and painless euthanasia.

G. Literature Cited

H. Consortium/Contractural Arrangements

None.

I. Consultants

Not applicable.

7. Checklist

8. Appendix