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THE EFFECTS OF VEGF-A ON sFLT-1 EXPRESSION IN THE DEVELOPING LUNG

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Lynette Maria Mendoza

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Committee: Ann Akeson, PhD, chair
James Greenberg, MD, co-chair
John Shannon, PhD
Katherine Yutzey, PhD

ABSTRACT

Vasculogenesis and angiogenesis are two processes responsible for the development of the vascular plexus in the lung. These two processes have been shown to be regulated by vascular endothelial growth factor-A, (VEGF-A). This potent effector acts through its receptors, Flt-1, Flk-1 and neuropilin-1, to induce endothelial cell differentiation, migration and proliferation. Studies have shown evidence that VEGF-A concentrations must be tightly regulated for proper development to occur. Because VEGF-A is a known regulator of angiogenesis and vasculogenesis, it is of interest to analyze the factors that coordinate its regulated processes. Recent reports have identified a soluble form of the Flt-1 receptor (sFlt-1), which has been implicated as an important negative regulator of VEGF-A activity. In this study, we examine the temporal and spatial expression patterns of sFlt-1 and Flt-1 in the developing lung and how their expression is affected by increased VEGF-A production. We report both sFlt-1 and Flt-1 receptors localized predominantly to endothelial cells. In addition, sFlt-1 mRNA was upregulated during increased production of VEGF-A, suggesting that VEGF-A may be regulating expression of its receptors in a paracrine fashion as a mode of its regulation.

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LIST OF SYMBOLS AND ABBREVIATIONS

μCi	microcurie
μg	microgram
μm	micrometer
bp	basepair
cDNA	complementary deoxyribonucleic acid
CMV	cytomegelo mosaic virus
ELISA	enzyme-linked immunosorbent assay
H&E	hematoxylin and eosin staining
kDa	kilodalton
mg	milligram
ml	milliliter
mRNA	messenger ribonucleic acid
RNase	ribonuclease
^{35}S-CTP	radioactive sulfur conjugated to cytidine triphosphate
^{35}S-UTP	radioactive sulfur conjugated to uridine triphosphate

INTRODUCTION

Pulmonary branching morphogenesis and vascular development

Pulmonary vascular morphogenesis is a fundamental aspect of normal lung organogenesis. Vascular development must parallel development of the airways for proper alignment, and effective gas exchange. Early in lung development, epithelial tubules are formed and are surrounded by thick mesenchyme as the conducting airways are being formed. Lung mesenchymal cells undergo multiple divisions and differentiation, giving rise to the various components in the lung vasculature. The epithelium also undergoes multiple changes, where columnar cells lining the airways differentiate to become the cuboidal cells in the airways, and later epithelial cells differentiate to become the surfactant producing type I and type II cells (Schwartz, et al., 2000). Formation of the pulmonary vasculature arises from extrapulmonary vessels via angiogenesis. Through vasculogenesis, these vessels together with distal capillary networks form the pulmonary vascular network (DeMello and Reid, 1991). Various signaling molecules influence lung morphogenesis and differentiation, for the developing vascular plexus and the conducting airways. Our lab is interested in the processes relating to pulmonary vasculature. One key player we focus on is vascular endothelial growth factor-A (VEGF-A), and how its spatial and temporal regulation is important in proper pulmonary vascular development. Figure 1 represents a simple model of how cross talk between the epithelium and mesenchyme directs VEGF-A signaling in the endothelium, via its receptors, and how these signals may determine endothelial cells to the vasculature.

VEGF-A and vascular patterning in the developing lung

Vasculogenesis is the process by which angioblast precursors differentiate to form endothelial cells. Angiogenesis is the process by which formation of new capillaries arise from pre-existing blood vessels. Vascularization in the lung is dependent on both processes and has been show to be regulated by VEGF-A (Carmeliet et al 1999; Shalaby et al., 1995). VEGF, originally known as vascular permeability factor (Ferrara et al., 1989; Keck et al., 1989) is a potent effector for endothelial cells through induction of their proliferation, migration and differentiation in both vasculogenesis and angiogenesis (Shalaby et al., 1995; Millauer et al., 1993). This heparin-binding growth factor is a member of the platelet-derived growth factor (PDGF) family. Four different family members, VEGF-A, -B, -C, and –D, are known to exist as a disulfide-linked homodimeric proteins between 24 and 46 kDa (Houck et al., 1991; Charnock-Jones et al., 1993).

VEGF-A in the mouse is expressed as three different isoforms via alternative splicing, VEGF-120 (V120), VEGF-164 (V164) and VEGF-188 (V188). Each isoform, has unique properties suggesting that they may play different roles during development (Ferrara et al., 1992). V120 and V164 are the predominant diffusible isoforms that are highly expressed early in lung development, while the non-diffusible, heparin-binding V188, is expressed later in development, just before birth (Ng et al., 2001). It has been suggested that this localized and temporal expression of these isoforms is what creates a spatial gradient of VEGF-A in the lung and allows it to act as a chemoattractant for developing capillaries (Ng et al., 2001; Akeson, et al., submitted 2003).

VEGF-A is expressed by the mesenchyme at low, but constant levels throughout most of lung development. Expression by the epithelium is detected at embryonic day (E) 14.5 and by

E16.5, VEGF-A expression is predominantly in distal respiratory epithelial cells (Gebb and Shannon, 2000; Greenberg et al., 2002). VEGF-A is absolutely required during development, as loss of a single allele results in an embryonic lethality due to severe vascular defects (Carmiliet et al., 1996; Ferrara et al., 1996). Studies also show the importance of VEGF-A concentration and how it must be tightly regulated for normal vascular patterning and branching morphogenesis to occur during embryonic lung development. Previously, increased levels of VEGF-A disrupted embryonic development causing heart defects and resulted in embryonic lethality by E14 (Miquerol et al., 2000). Overexpresssion of VEGF-A isoform V164 throughout lung development also led to disruption of the vasculature in addition to defects in branching morphogenesis (Zeng et al, 1998). We have studied overexpression of VEGF-A, using the V164 isoform, in the lung, controlled by the surfactant protein C promoter (SP-C) or the Clara cell promoter (CC10), specific to distal and proximal epithelium, respectively. Consistent with the previous findings, Figure 2 shows altered vasculature and smooth muscle patterning, as well as disrupted airways when VEGF-A is overexpressed during development, further suggesting the importance of VEGF-A concentration for proper lung development (Akeson et al., submitted 2003).

VEGF receptors and their role in signaling

VEGF-A acts through its tyrosine-kinase receptors, (RTKs), vascular endothelial growth factor receptor-1 (VEGFR-1/ Fms-like tyrosine kinase (Flt-1), VEGFR-2/ Fetal liver kinase (Flk-1), and co-receptor neuropilin-1, (Nrp-1) represent the VEGF-A receptor family. Because these receptors have been assigned various names, they are referred to here as Flt-1, Flk-1 and Nrp-1. Flt-1 binds to and is activated by VEGF-A, -B, and placental growth factor (PGF) (Kendall et al., 1994; Sawano et al., 1996; Olofsson et al., 1996). The function of this receptor is still not clear.

Knock-out studies of Flt-1 resulted in embryonic lethality at E9.0 as a result of overgrowth of abnormal endothelial-like cells appearing as disorganized blood vessels (Fong et al., 1999). Among the receptors that bind VEGF-A, Flt-1 has the highest affinity for VEGF-A, but displays weak tyrosine kinase activity, usually one-tenth of Flk-1 (Seethaaram et al., 1995; Shibuya, 2001). Initial studies showed that Flt-1 lacking the tyrosine domain was sufficient for normal development and angiogenesis (Hiratsuka et al., 1998), and since Flt-1 deficient mice exibit an overgrowth in endothelial cells, it has been suggested that Flt-1 may be acting in a negative fashion in regulation of VEGF-A. Despite these views, there is evidence that Flt-1 has a role in signaling and a role in hematopoiesis and recruitment of endothelial progenitor cells (Hattori et al., 2002; LeCouter et al., 2003).

Soluble Flt-1 is generated by alternative splicing of the pre-mRNA encoding Flt-1, between exon 13 and 14. Both the soluble and full-length Flt-1 mRNAs contain the consensus AG donor splice-site nucleotides immediately 5' of the site of divergence (see Figure 3). The identical donor splice-sites are followed by guanine and adenine which are the most and second most common nucleotides on the acceptor side of spliced sequences (Mount, 1982; Kendall and Thomas, 1993). Interestingly, the sequence 3' of the sFlt-1 mRNA splice site (GUGAGC), resembles an unprocessed donor site (GURAGU), which suggests that this splicing event is a specific read-through process of intron 13, which can switch to splice out this sequence and generate the full length Flt-1 receptor. This alternatively spliced receptor encodes an additional 31 amino acids derived from the intron sequence. Furthermore, sFlt-1 has been shown to act as a potent antagonist of VEGF-A (Kendall and Thomas, 1993). This soluble form of Flt-1 binds to both VEGF and PGF-2 with high affinity, and can heterodimerize to Flk-1, acting in a dominant negative fashion (Park et al., 1994; Kendall et al., 1996). Recent studies implicated sFlt-1 as an

important negative regulator of VEGF-A activity in tumor biology and placental function (Boocock et al., 1995; Clark et al 1996; He et al., 1999).

Knockout studies for Flk-1 also resulted in embryonic lethality by E9.0, due to lack of vasculature and failure to organize blood vessels (Shalaby et al., 1995). Recent evidence demonstrates that Flk-1 is not required for development of hematopoietic and endothelial cell progenitors, but rather for endothelial cell migration and proliferation (Fong et al., 1999). Since then, it has been agreed that Flk-1 is a major mediator of mitogenic, angiogenic and permeability-enhancing effects of VEGF-A (Ferarra et al., 2003).

Nrp-1 deficient mice have abnormal neural vascularization and disrupted cardiovascular development. These animals lack major defects in endothelial cell differentiation or in vessel formation (Kawasaki et al., 1999). While this co-receptor has been shown to enhance V164 binding to Flk-1, there is no evidence that this receptor directly participates in signaling after VEGF binding. (Kawasaki et al., 1999).

Importance of VEGF-A regulation in the developing lung

VEGF-A acts through its tyrosine-kinase receptors (RTKs) to induce endothelial cell differentiation, migration and proliferation, all of which are critical during pulmonary vascular development. Disruption of the vasculature has been associated with neonatal pulmonary diseases such as congenital diaphragmatic hernia (CDH) and bronchopulmonary dysplasia (BPD) (Coalson et al., 1999). Because VEGF-A plays such a major role in angiogenesis, as well as vasculogenesis, it is necessary to identify the factors regulating VEGF activity as wells as understand the mechanism(s) involved. Understanding this regulation may provide therapeutic implications for vascular diseases in the future.

Rationale: Soluble Flt-1 is necessary for VEGF-A regulation in the developing lung

Published studies provide evidence for the importance of VEGF-A regulation in vascular development. While previous studies demonstrate how VEGF-A functions in both vasculogenesis and angiogenesis, little is known about the mechanisms and factors involved in coordinating such a tightly regulated process. Based on the reports of sFlt-1 and its role in VEGF-A regulation, we hypothesize that VEGF-A regulates its expression in the developing lung by inducing sFlt-1 expression. Our approach was to determine spatial and temporal expression of sFlt-1 in the developing lung, with respect to the full length receptor, Flt-1. We then sought to determine whether VEGF-A regulates the expression of sFlt-1 as a feedback inhibition mechanism. Both Flt-1 and sFlt-1 mRNA levels were assessed in VEGF-A overexpressing animals (SP-CrtTA/tetO_7 VEGF) and their single transgenic littermates.

METHODS

Animals

In the experiments described here, FVB/N mice were either non-transgenic or transgenic mice hemizygous for the SP-CrtTA transgene mated to homozygous transgenic mice containing the $(tetO)_7$ VEGF transgene (Figure 4). The rtTA/tetO transgene system operates when the rtTA protein binds to tetracycline operator (tetO) DNA binding sequence, only in the presence of doxycycline. Following this binding, the downstream transgenes are activated by the minimal CMV promoter. Because the rtTA transgene is controlled by the surfactant protein C (SP-C), VEGF-A expression is exclusive to epithelial cells (Tichelaar et al., 1998). Conditional overexpression of VEGF-A was induced at specific time points by administering the pregnant dams with doxycycline-treated food pellets. Vaginal plugging was defined as day E0.5. The dams were euthanized by CO_2 asphyxiation followed by removal of the embryos. All embryos were weighed and measured (crown-rump) to accurately determine day of gestation. Fetal lungs were removed for morphological and molecular analysis. All experiments were conducted with the approval of the Animal Care and Use Committee of the Cincinnati Children's Hospital Research Foundation (Cincinnati, OH).

RNA Isolation from Lung epithelium and mesenchyme and whole lung

Lungs for epithelial and mesenchymal RNA isolation were obtained from E12.5 fetuses of FVB/N mice, where lung epithelium and mesenchyme were separated as described (Shannon et al., 1998). Lung tissue from embryos at E12.5, was harvested and homogenized in guanidium isothiocyanate for RNA isolation. Whole lung RNA was isolated from older animals (E14.5 and E16.5) with TRIzol (GibcoBRL) and a Rapid Total RNA Isolation Kit (Eppendorf-5 Prime).

DNase treatment of the isolated RNA was done as described in Ambion's DNA-free protocol. To test the purity of isolated epithelial and mesenchymal tissue pools, analysis by reverse transcriptase and polymerase chain reaction (RT-PCR) using surfactant protein C (SP-C) and vascular endothelial growth factor receptor-2 (Flk-1) as markers was done. Analysis of sFlt-1, Flt-1, Flk-1 and neuropilin-1 (Nrp-1) mRNA was performed on purified epithelial and mesenchymal cDNA. First strand cDNA was synthesized using Superscript First-Strand Synthesis system for RT-PCR (Invitrogen). Amplifications were carried out using Taq DNA polymerase (Fisher). For SPC there were 30 cycles at 94°C, 5 seconds; 58°C, 10 seconds; 72°C, 15 seconds; for Flk-1, β actin, Flt-1 and sFlt-1 there were 30 cycles at 94°C, 30 seconds; 56.5°C, 45 seconds; 72°C 45 seconds. For Nrp-1 PCR was performed with the following conditions: 94°C for 3 minutes and 5 cycles at 94°C for 30 seconds; 55°C for 45 seconds; 72°C for 3 minutes and 5 cycles again at 94°C for 30 sec; 52°C for 45 seconds; 72°C for 3 minutes and 25 more cycles at 94°C for 30 seconds; 49.5°C for 45 seconds; 72°C for 3 minutes and a final extension at 72°C for 7 minutes. Primers and product sizes in nucleotides (nt) were:

SP-C	Fwd: 5' CGCCTTCTCATCGTGGTTGTGG 3' Rev: 5' TTGGCCCTGAAGTTCTGGAGTTTT 3' Product: 337 nt
Flk-1	Fwd: 5' TGAAGCCATCAACAAAGCGG 3' Rev: 5' TGTAATGTGTGGGGTAGGATTTCC 3' Product: 508 nt
β actin	Fwd: 5' TGGAATCCTGTGGCATCCATGAAAC 3' Rev: 5' TAAAACGCAGCTCAGTAACAGTCCG 3' Product: 348 nt
Flt-1	Fwd: 5' ATTCTGTCTCAACAAGGACGCAG 3' Rev: 5' ATTTCAGGTGTGGCATACTCCG 3' Product: 603 nt
sFlt-1	Fwd: 5' GAAACGCTCCTCCCAAAATGAG 3' Rev: 5' CAGGTAGACACCCGACACAGATTC 3' Product: 385 nt
Nrp-1	Fwd: 5' AGGTTTCTCAGCCAACTACAGTGTG 3'

Rev: 5' CCTACAGCAGTAACGAATCGCAG 3'
Product: 252 nt

All samples were electrophoresed on a 1.2% agarose gel, where samples without RT were used as the negative control.

In Situ Hybridization

Preparation of Probes:

In situ probe templates were prepared using RNA from postnatal day 6 lungs as template and primer pairs for sFlt-1 and Flt-1 for RT-PCR. The products were purified with Qiaquick PCR Purification system (Qiagen) and ligated into a TA Cloning vector system, pCRII (Invitogen). Nucleotide sequencing was done to assess orientation of the insert for each clone. The plasmids were linearized by restriction digest and purified once more using Qiaquick Nucleotide Removal Kit (Qiagen). RNA probes were then synthesized with SP6 and T7 polymerases from a Riboprobe Combination System Kit (Promega) and radiolabeled using 300 μCi of ^{35}S-UTP (New England Nuclear) for Flt-1 riboprobes and 300 μCi of ^{35}S-UTP and 300 μCi of ^{35}S-CTP (Amersham) for double-labeled sFlt-1 riboprobes.

Hybridization

Lung tissue from non-transgenic mice was fixed in 4% paraformaldehyde in phosphate buffered saline (1 X PBS) overnight. The fixed tissue was dehydrated in an ethanol series and paraffin embedded for sectioning. Sections were cut at 5μm, (2 per silane-coated slide) in a RNase-free treated water bath. Slides were deparaffinized in xylene, rehydrated in ethanol and post-fixed in 4% paraformaldehyde for 20 minutes. Following fixation, the slides were treated with proteinase K (20μg/ml), refixed and dehydrated once more through an ethanol series. All solutions were treated with diethyl pyrocarbonate (DEPC) to neutralize residual RNase activity.

Antisense and sense probes for Flt-1 were hybridized at 55°C, and for sFlt-1 at 47.5°C. High stringency washes for Flt-1 and an intermediate stringency wash for sFlt-1 were done to remove any unhybridized RNA, followed by RNase A treatment (10mg/ml). Slides were dehydrated by ethanol and air dried for 20 minutes. Following an overnight Kodak Omat x-ray film exposure, the slides were dipped in Kodak NTB2 emulsion and stored at 4°C. Exposure times for Flt-1 and double-labeled sFlt-1 ranged from 3 to 6 weeks. After developing in Kodak D-19 developer, the slides were fixed in 4% paraformaldehyde, dehydrated through an ethanol series and coverslipped with permount. Slides were counterstained in 0.02% toluidine blue in 1X PBS.

Qualitative analysis of mRNA

Doxycycline-treated dams (SPCrtTA/tetO$_7$VEGF) for timed periods, E10.5 to E16.5; E12.5 to 14.5 and E10.5 to E12.5 were sacrificed and fetal mouse pups were removed for each given time point. All litters were genotyped by PCR, using primers to specific for the SPCrtTA transgene and primers specific for the tetOVEGF transgene. Mice generated from the initial crosses were either: bitransgenic (SPCrtTA/tetOVEGF) or single transgenic for either the SPCrtTA transgene or the tetOVEGF transgene (Figure 4). RNA was isolated from the fetal lung tissue and reverse transcribed as described above. RT-PCR analysis was performed to assess differences in sFlt-1, Flt-1 and Flk-1 mRNA transcripts in overexpressing animals with respect to their wild type littermate controls. Conditions for the amplifications were the same for sFlt-1, Flt-1, Flk-1 and β–actin as described above with the exception of using 25 cycles instead of 30. Samples without RT were used as the negative control and all samples were electrophoresed on a 1.2% agarose gel.

Real Time RT-PCR

Total RNA was isolated and reversed transcribed from fetal lung tissue from doxycycline treated animals as described above. The cDNAs were amplified using the DNA Engine Opticon 2 fluorescence detection system (MJ Research) with 1 X SYBR Green I (Molecular Probes, Inc.), where the PCR products were detected in real time. Amplifications for β-actin, sFlt-1, and Flt-1 were performed using the previously mentioned primers and their conditions for 40 cycles. Relative mRNA levels were calculated after obtaining the standard curves for β-actin. Values for sFlt-1 and Flt-1 relative mRNA were obtained by dividing the initial quantity value (obtained from the standard dilution) by that of the β- actin quantity value for each given sample. This allowed the samples to be normalized against β-actin. VEGF-A mRNA levels were also assessed using the following primers:

Fwd: 5' GTGCACTGGACCCTGGCTTTACTG

Rev: 5' –ATGTGCTGGCTTTGGTGAGGTTTG.

The conditions for VEGF-A were: 94°C for 5 minutes following 40 cycles at 94°C for 30 seconds; 61°C for 40 seconds; 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes and holding at 4°C. Three transgenic litters were used as samples for doxycycline experiments with VEGF-A induction from E10.5-16.5, and two transgenic litters were used as samples for doxycycline experiments with VEGF-A induction from E10.5-12.5 and E12.5 -14.5.

Statistical Analysis

Statistical analysis was performed to determine the standard deviation within samples along with performing a Student's t-test to determine the significance of any differences observed. Differences were considered significant at $P < 0.05$.

RESULTS

Flt-1, sFlt-1, Nrp-1 and Flk-1 are expressed in early lung mesenchyme.

Previous studies have shown that localization for VEGF receptors is predominantly on endothelial cells (Peters, et al., 1993; Millauer, et al., 1993; Breier et al., 1995; Fong et al., 1996; Gebb, and Shannon, 2000). Contrary to earlier studies, recent data suggest that Flt-1 and Flk-1 are also being expressed in the epithelium of various tissues such as kidney, retinal pigment cells, mammary glands, and more interestingly in the lung (Kanellis, et al., 2000; Ohno-Matsui, et al., 2003; Hovey, et al., 2001; Lassus, et al., 2001). Expression of Flt-1 and Flk-1 in lung epithelium has been reported in both Type II pneumocytes and bronchiolar cells by immunohistochemistry (Klekamp, et al., 1999; Kaner, et al., 2001; Compernolle, et al., 2002).

The conflicting reports of where VEGF receptors are localized were of interest to our study to understand how VEGF-A activity is regulated. Previous studies demonstrate that overexpression of VEGF-A results in dysplastic epithelium and disrupted vasculature. These findings lead us to ask whether VEGF-A is acting in an autocrine fashion on epithelial cells. If acting in an autocrine fashion, then epithelial cells must be expressing the VEGF-A receptors. However, if acting in a paracrine fashion, then these receptors expressed in the endothelium would lead to indirect effects on the epithelium.

To address our first question of whether sFlt-1, Flt-1 and the other VEGF receptors are expressed specifically in the endothelium or in both the epithelial and endothelial compartments in the developing lung, RT-PCR analysis was performed on RNA purified from E12.5 lung tissue. This experiment was performed to assess compartmental localization in early development. Epithelium and mesenchyme were separated using microsurgery and RNA was isolated from the respective pooled tissue. RT-PCR analysis shown in Figure 5A was performed

on both pools of RNA. To test the purity of each tissue isolation, SP-C and Flk-1 primers were used as markers for the epithelium and the mesenchyme, respectively. SP-C primers amplified product in RNA from epithelium, but not the endothelium. Flk-1 primers amplified product in the endothelium, but not the epithelium. This experiment demonstrated no detectable cross contamination of the tissue pools.

To assess compartmental expression of sFlt-1 and Flt-1 in the developing lung, primers for RT-PCR were designed to regions in the Flt-1 gene specific for sFlt-1, in the unique intron 13 sequence. For Flt-1, primers corresponded to sequence in exon 14 (Figure 3). We also examined mRNA expression for Nrp-1, a co-receptor for Flk-1. Figure 5B shows sFlt-1, mFlt-1, and Nrp-1 are expressed in the mesenchyme in the pseudoglandular stage (E12-16 in the mouse). sFlt-1, Flt-1 and Nrp-1 message is detected in the epithelium at this stage, but the signal for sFlt-1 and Flt-1 is much weaker in intensity with respect to the signal observed in the mesenchyme.

We next asked the timing of sFlt-1 and Flt-1 expression during lung development. RT-PCR was performed on RNA from whole lung tissue taken at the various stages of development. Figure 6 shows that both sFlt-1 and Flt-1 are expressed in the lung from early pseudoglandular stage (E12.5-16) to the saccular stage (E18.5). Although a weak signal was observed for sFlt-1 at post-natal day 8.5, and no signal was observed for Flt-1 at this stage, both receptors had detectable transcripts in adult lung.

Characterization of sFlt-1and Flt-1 in the developing lung

To assess localization of these receptors during development, a more region specific assay, *in situ* hybridization, was performed on mouse lung tissue at specific developmental

stages. Previous studies reported that Flt-1 localized to the epithelium in Type II pneumocytes, and our RT-PCR analysis showed some traces of expression in the epithelium. However, by *in situ* hybridization, we show that Flt-1 was detected in the mesenchyme and not the epithelium at E12.5 and E14.5 (Figure 7, 8.). By E16.5, Flt-1 has mostly localized to vascular endothelial cells and then specifically localizes to the vasculature by postnatal day 1 (PND1, Figure 7, 8.). Because it is difficult to distinguish a specific signal at the earlier stages of lung development (E12.5 and E14.5), higher magnifications were shown to demonstrate the specific endothelial signal observed (Figure 8). In addition to this, we did not detect a signal in either proximal or distal epithelium from E12.5 to PND1, contrast to published reports (Figure 8).

In situ hybridization was performed to assess expression of sFlt-1 in the lung, using a construct based on the region used for primer design (Figure 3). Specificity of the probes was designed in regions of the Flt-1 gene that corresponded to either sFlt-1 (intron 13 sequence) or Flt-1 (exon 14 sequence). Because Flt-1 was detected predominantly on the endothelium, sFlt-1 was predicted to have a similar expression pattern. However, after a 6 week exposure, signal for sFlt-1 was undetectable.

Several explanations could account for our failure to detect signal for sFlt-1 expression by *in situ* hybridization. One possibility would be that the size of the probe, which was relatively small (385 bp), would result in a longer exposure time and cause difficulty to distinguish a low signal from background. Another possibility would be the specific activity of the probe. To increase specific activity, the probe was double labeled with both ^{35}S-UTP and ^{35}S-CTP. However, this still did not produce a detectable signal for sFlt-1 in our *in situ* analysis.

Increased VEGF-A induces upregulation of sFlt-1 and Flt-1

We hypothesized that VEGF-A autoregulates its activity by inducing expression of sFlt-1, as a feedback mechanism. To address our question of whether sFlt-1 is upregulated with increased levels of VEFG-A, we performed RT-PCR analysis of our VEGF-A overexpressor (SPCrtTA/tetOVEGF) transgenic animals. Overexpression of VEGF-A by doxycycline treatment at E12.5-14.5 and E10.5-16.5 resulted in sFlt-1 upregulation in the bitransgenic (bTG) animals in comparison with their single transgenic (sTG) litter-mate controls (Figure 9). Flk-1, mFlt-1 mRNA transcripts showed no observable differences in message in relation to the litter-mate controls. Doxycycline experiments later in development (Dox E16-18) show no apparent differences in mRNA for any of the receptors suggesting that this upregulation may be important earlier in development, but not in late development.

To further assess this possible mode of VEGF-A regulation, a more sensitive, Real time RT-PCR methodology was used. Relative mRNA levels of both sFlt-1 and Flt-1 in our overexpressing VEGF-A animals were normalized against β-actin. Samples were done in duplicate and averaged. We found upregulation of both sFlt-1 and Flt-1 in bitransgenic animals (*SPCrtTA/tetO VEGFA*), in comparison to their single transgenic (*SPCrtTA* or *tetOVEGFA*) littermates for both time-points of the doxycycline experiments. Bitransgenic animals VEGF-A induced from E10.5- 16.5, show a 5-fold increase in sFt-1 mRNA levels and about a 2-fold increase for Flt-1 mRNA levels in comparison to their single transgenic littermates (Figure 10A). Increased VEGF from E12.5-14.5, show a 23-fold increase in sFlt-1 and a 3.3-fold increase in Flt-1 mRNA for bitransgenic animals (Figure 10B). Although it was of interest to analyze the effects of increased VEGF-A on sFlt-1 and Flt-1 mRNA levels at an earlier time point in development (E10.5-12.5), animals had inconsistent values for this time point (data not shown).

VEGF-A mRNA levels were also assessed to address the question of different levels of VEGF-A transgene expression in our bitransgenic animals. This was done to determine if differences observed within our transgenic animals for sFlt-1 and Flt-1 mRNA levels were based on whether the animals had lower or higher levels of the VEGF-A transgene. The bitransgenic animals were separated into two groups based on VEGF-A mRNA levels (Table 1). We assumed that animals in the lower expression group were hemizygous and those in the higher expression group were homozygous. This assumption was based on the way crosses were set up for our transgenic animals (Figure 4), where bitransgenic animals (SPCrtTA/tetOVEGFA) generated could either be hemizygous or homozygous for the tetOVEGFA transgene. Single transgenic animals were considered to have endogenous levels of VEGF-A mRNA expression due to ELISA analysis done previously by our lab, which demonstrated that single transgenic animals had equivalent levels of VEGF-A to non-trangenic animals (Akeson et al., submitted 2003). In Table 1, relative VEGF-A mRNA levels are given for individual and combined samples.

Statistical analysis was performed to assess whether the differences in sFlt-1 and Flt-1 upregulation were significantly different. Increased VEGF-A from E10.5-16.5, showed experimentals for sFlt were significantly upregulated with a p-value of 0.008. However, upregulation for Flt-1 mRNA levels was not significant (Figure 10A). Although increased VEGF-A from E12.5-14.5 shows a trend for sFlt-1 and Flt-1 mRNA upregulation in bitransgenic animals, p-values for these experimentals resulted in non-significant values (Figure 10B). Following our assessment of which animals were low or high for the VEGF-A transgene, we looked at combined VEGF-A mRNA levels for our hemi- and homozygous bitransgenic animals to determine a significant difference of the transgene expression (Figure 11A). Bitransgenic

animals with VEGF-A induction from E10.5-16.5 show a significant difference ($p= 0.006$) between the low and high VEGF-A animals. Differences between single transgenics and low VEGF-A animals for this timed induction were found not significant ($p= 0.440$), whereas differences between single transgenics and high VEGF-A animals were found highly significant ($p=0.001$, Figure 11). We then assessed sFlt-1 and Flt-1 mRNA levels for our grouped animals to determine if the previous differences in mRNA levels for bitransgenics correlated to VEGF-A transgene expression. Differences for sFlt-1 mRNA levels were found significant between single transgenics and high VEGF-A animals ($p=0.008$), as well as low VEGF-A and high VEGF-A animals ($p= 0.032$, Figure 11). Although we found trends for Flt-1 upregulation previously, differences between any of the groups for Flt-1 mRNA levels were found not significant (Figure 11). Based on the analysis, we conclude that sFlt-1 mRNA is upregulated with increased VEGF-A, and that this upregulation is dosage dependant.

DISCUSSION

The purpose of this project was to attain a better understanding of how VEGF-A is regulated in the developing lung and whether sFlt-1 participates in this regulation. VEGF-A has been shown to be regulated in various ways, either by means of pro-angiogenic factors including hypoxia-inducible factor (HIF-1 alpha) and protein tyrosine phosphatase inhibitors, or by anti-angiogenic factors such as HCPTPA, a protein tyrosine phosphatase (Huang, et al., 1999) and sFlt-1. Nonetheless, VEGF-A activity is a tightly regulated process, where haploinsufficiency results in embryonic lethality and increased levels of VEGF-A results in disruption of vascular patterning in development. Collectively, these data suggest that VEGF-A levels must be maintained in a narrow concentration range for proper development. Although the literature has presented the importance of VEGF-A and its receptor system in vasculogenesis, little is known about the mechanism outlining its regulation in pulmonary development.

We sought to investigate the role of sFlt-1 in VEGF-A regulation. The actual function of Flt-1 has been under debate for several years. Although studies have shown Flt-1 to act either positively or negatively in VEGF-A signaling, sFlt-1 is identified as a negative VEGF-A regulator (Kendall and Thomas, 1993). Generally, generation of alternatively spliced soluble receptors functions as a biological mechanism for inhibiting a particular cellular response. Soluble Flt-1 acts as a negative regulator by sequestering VEGF-A from its other signaling receptors, or by heterodimerizing with Flk-1 acting as a dominant negative to further prevent signaling (Kendall, et al., 1996). Previous studies have shown that the alternative splicing of Flt-1 is important in regulating VEGF-A activity in placenta and that this form of physiological regulation may be of significance in other organs as well (Clark et al., 1998; He et al., 1999).

Based on these observations and our interest in pulmonary vasculature, we hypothesized that VEGF-A regulates the expression of sFlt-1 in the developing lung.

Our first set of experiments were aimed to characterize spatial and temporal expression patterns sFlt-1 in the lung and whether its expression was correlated with the expression of other VEGF receptors, Flt-1, Flk-1 or the co-receptor, Nrp-1. By RT-PCR we show that both sFlt-1 and Flt-1 are expressed throughout most of development and that this expression is also present in adult lung. We also show that early in development, in the pseudoglandular stage, mRNA for all the VEGF receptors is expressed predominantly in the mesenchyme, with the exception of Nrp-1, which is found in both mesenchyme and the epithelium.

Both Flk-1 and Flt-1 were initially reported to localize to the vascular endothelium, but recent reports show by immunohistochemistry that in addition to their expression in the vasculature, these receptors are found in the epithelium in both Type II pneumocytes and bronchiolar cells (Compernolle et al., 2003; Lassus et al., 2001). Our data shows no transcripts for Flk-1 in isolated epithelial tissue at E12.5, which is consistent with the earlier reports. However, traces of Flt-1 and sFlt-1 were detected in the epithelium by RT-PCR, which seemed to agree with the recent findings. We report by *in situ* hybridization that Flt-1 localized to the mesenchyme early in development, and by E16.5 it is predominantly localized to vascular endothelial cells and continues to be by postnatal day 1 (Figure 7,8). Although contrary to the recent reports, we believe that our findings are accurate. What has been recently reported by immunohistochemistry may be subject to the quality of the antibody used. Despite our observation of Flt-1 and sFlt-1 in the epithelium by RT-PCR, the data shown by *in situ* hybridization was considered a more valid result due to lack of signal detected in the epithelium, suggesting that any expression in the epithelium may not be biologically significant.

Based on the previous findings of sFlt-1, our hypothesis led us to test the possibility of sFlt-1 mRNA upregulation with increased levels of VEGF-A in the developing lung. In addition to observing a possible upregulation for sFlt-1, we also wanted to assess whether Flt-1 mRNA levels changed. Because both sFlt-1 and Flt-1 are generated from the same pre-mRNA, we expected the following possibilities: (1) Increased levels of VEGF-A would result in upregulation of sFlt-1, but no change in Flt-1; (2) Increased levels of VEGF-A would result in no change in sFlt-1 levels, but an increase in Flt-1 or (3) Increased levels of VEGF-A would result in an increase for both sFlt-1 and Flt-1 levels. For these experiments, increased VEGF-A was induced at different time points of development (E10.5-16.5 and E12.5-14.5). RT-PCR of sFlt-1 and Flt-1 was assessed to obtain a more qualitative than quantitative answer. As the results indicate, there was an increase in sFlt-1 transcripts, but not Flt-1 or Flk-1 in our VEGF-A induced bitransgenic animals at both time points of development, which led us to believe that VEGF-A levels may be regulating expression of sFlt-1 (Figure 9).

To interpret these results in a more quantitative fashion, real time RT-PCR was performed on sFlt-1 and Flt-1. Like the data from RT-PCR, real time RT-PCR also showed an upregulation in sFlt-1 transcripts in response to increased VEGF-A. Unexpectedly, upregulation trends were also observed for Flt-1 mRNA in response to increased VEGF-A at both E10.5-16.5 and E12.5-14.5 times of doxycycline induction.

Because of variability within samples for our bitrangenic animals, determining levels of VEGF-A transgene expression for the given samples was necessary. The initial crosses set up (Figure 4), allowed conditional induction of VEGF-A only in animals with both the SP-CrtTA transgene and the $tetO_7$VEGF transgene. However, what was not known was whether the animals generated were hemizygous or homozygous for the tetOVEGF-A transgene. To

account for differences observed within bitransgenic animals resulting from low versus high levels of the transgene, VEGF-A mRNA levels were assessed. This was done to determine whether sFlt-1 and Flt-1 mRNA differences observed correlated to the amount of VEGF-A transgene expression. Table 1 shows the relative VEGF-A mRNA levels observed for transgenic animals which were grouped as endogenous for single transgenic animals, and assumed hemizygous or homozygous for bitransgenics. Animals that were grouped as homozygous for the VEGF-A transgene, had at least twice the levels of VEGF-A mRNA than the hemizygous animals, which is consistent with the assumption that when twice the transgene present, at least twice as much mRNA is expressed.

The significance of these differences was analyzed by performing a Student's T-test. Analysis shows that animals with increased VEGF-A, induced from E10.5-16.5 had significant upregulation of sFlt-1 levels (Figure 10A). However, upregulation observed for Flt-1, was found not significant (Figure 10A). Following this assessment, statistics were then performed on our grouped animals to establish whether upregulation differences, dependent on VEGF-A transgene expression were statistically significant. As Figure 11 demonstrates, upregulation of VEGF-A and sFlt-1 mRNA is highly significant between single transgenic animals and animals with high VEGF-A. Upregulation differences between low VEGF-A animals and high VEGF-A animals were also found significant, yet differences between single transgenics and low VEGF-A animals were not significant. Although Flt-1 mRNA levels show a trend for upregulation, differences between any of the groups were found not significant. Together, these findings indicate that while Flt-1 upregulation is not significant, sFlt-1 upregulation is significant VEGF-A dose responsive.

Statistical analysis was also performed on animals VEGF-A induced from E12.5-14.5. Although the upregulation trends for these animals were observed, they were found statistically not significant (Figure 10B). Based on the VEGF-A mRNA levels observed for these animals (Table 1), we believe that this trend in upregulation, is also representative of a VEGF-A dose response. Although samples were performed in duplicate and for one litter in triplicate, addtional trial runs might yield a more significant result for this time period. Our data provides evidence that VEGF-A may play a role in regulating alternative splicing of Flt-1 for autoregulation, based on a significant and greater upregulation for sFlt-1 and not full length Flt-1. Together, these and the above results demonstrate the following: 1) sFlt-1 mRNA is predominantly expressed in lung mesenchyme early in development, and Flt-1 is expressed in lung endothelium; 2) increased levels of VEGF-A results in a significant increase in sFlt-1 mRNA levels, but not in a significant increase in Flt-1 mRNA. This upregulation suggest that sFlt-1 may participate in regulating increased levels of VEGF-A as an inhibition feedback mechanism. Finally, localization of the VEGF-A receptors and upregulation of sFlt-1 in response to increased VEGF-A, may be indicative that VEGF-A regulation is mediated in a paracrine fashion (through the endothelium), rather than an autocrine (through the epithelium) fashion.

Future Studies

These findings lead us to ask how is sFlt-1 functioning in the developing lung with respect to VEGF-A. VEGF-A has been shown to be tightly regulated in the lung, where proper development of the vasculature is limited to a narrow range of VEGF-A levels. In addition to this, we have learned that in other organ systems, sFlt-1 is an important regulator of angiogenesis

and is needed for sustaining VEGF-A levels for proper vascular networking. Previous studies have shown the importance of soluble receptors such as soluble fibroblast growth recptor-2 (FGFR-1), interleukin-4, -5 and -7 receptors (Il-4 receptor, IL-5 receptor and IL-7 receptor) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in mediating biological responses of a cell (Johnson et al., 1991; Mosley et al., 1989; Taverier et al., 2000; Goodwin et al., 1990; Prevost, et al., 2002). Given these findings, we would like to address the following questions for future studies: 1) If VEGF-A is not directly involved in the alternative splicing of Flt-1 as a regulation mechanism, then is it involved indirectly in activating downstream targets to induce alternative splicing factors in endothelial cells? 2) What is the true function of Flt-1 in signaling given that: (a) sFlt-1 and Flt-1 are generated from the same primary transcript, (b) our results indicate a significant upregulation for sFlt-1 mRNA, but not Flt-1 mRNA, despite trends for upregulation, in response to increased levels of VEGF and (c) both receptors have been implicated in the inhibition of VEGF-dependent endothelial proliferation and chemotaxis (Park et al. 1994; Zeng, et al., 2001, Kendall and Thomas, 1993; He et al., 1999).

To address the first question of whether splicing of Flt-1 is of importance mechanistically to VEGF-A regulation, it is necessary to examine the possible factors involved in alternative splicing of endothelial cells. We would hypothesize that increased levels in VEGF-A would indirectly induce upregulation of spliceosome proteins. Splicing of mRNA in eukaryotic cells is a necessary step in gene expression and ultimately in generating the appropriate protein. First and foremost, it would be of great interest to assess by Western blot, protein levels of both sFlt-1 and Flt-1 in overexpression of VEGF-A. This experiment would provide evidence of whether the upregulation observed in real time RT-PCR is the same at the protein level in the lung. Western blots would be performed using the antibodies and protocol described by Hornig et al.,

2000. Samples used would be from our VEGF-A overexpressing animals, generating the same crosses as was done for mRNA analysis. If the findings for this experiment are consistent with our previous findings of upregulation, then our next set of experiments would focus on assessing protein levels of components of the spliceosome machinery.

Leung et al., (2003) recently showed the factors involved in the alternative splicing of the glucocorticoid receptor (GCR), which generates two isoforms of the receptor, GCRα, and GCRβ. GCRβ acts as a dominant inhibitor of GCRα, yet both receptors are generated from the same pre-mRNA transcript. It is of interest to understand which factors are involved in generating sFlt-1, by means of a direct read-through of the contiguous intron sequence and more importantly, the switch to disable or turn off processing of the splice site. Major components of the spliceosome such as the serine-arginine rich protein, also known as the SR proteins, would be the first factors to analyze. SR proteins are key players of both constitutive and alternative splicing, which mediate protein interactions with other components of the splicing machinery by means of a N-terminal RNA recognition motif (RRM) and a C-terminus rich in arginine/serine dipeptide repeats, (RS domain) (Zuo and Maniatis, 1996).

Western blot analysis of the SR proteins, as were analyzed in Leung's study, would provide insightful data on which of these proteins is predominantly present in lung tissue. Next, we would like to assess whether the predominantly expressed SR protein expressed in lung shows any changes in levels, with respect to increased VEGF-A levels. If this SR protein would show an increase in its levels, we would then assess its correlation to sFlt-1 protein levels, in addition to Flt-1 during overexpression of VEGF-A. Next, to determine functional evidence of this SR protein, inhibition assays would be performed, by *in vitro* studies. Using our cell lines, mouse fetal lung mesenchyme cells (MFLM-4 and MFLM-91U), we would conduct assays in

which we would attempt to inhibit function of the SR protein by using a blocking peptide or an antisense oligonucleotide to inhibit function. If inhibition was achieved, we would expect to see an increase in Flt-1 levels, thereby revealing that this protein is directly involved in the alternative splicing of Flt-1 and provide new evidence for VEGF-A regulation.

It would also be of interest to generate a conditional overexpressing sFlt-1 mouse to assess any morphological changes in the vasculature upon induction of the transgene. In addition, this mouse would allow us to assess localization of upregulated proteins, such as SR proteins. This mouse would be generated in a similar fashion to our *tetOdnR2* transgene, only the construct would encode sFlt-1 gene (Kendall and Thomas, 1993). The animals generated would be bred to our SPCrtTA transgenic animals provided by our division (Tichelaar et al., 1998; Glasser et al., 1990) using the SPC promoter to direct expression of the transgene in airway epithelium. As mentioned above, overexpression of sFlt-1 would only occur in the presence of doxycycline, where rtTA would then bind to the tetO promoter and thus induce the expression of sFlt-1. This would allow the expression of sFlt-1 to be spatially specific and in a temporal fashion. In addition, this mouse model would allow us to test the hypothesis that VEGF-A regulation by sFlt-1 is essential in lung development for maintaining proper levels of VEGF-A and ultimately proper pulmonary vasculature for a functional lung.

Our second question for future studies addresses the role of Flt-1 signaling in lung development. As mentioned earlier, the function of Flt-1 is still under debate. Although studies have shown that this receptor can act in both a positive and negative manner, it is of interest to understand what dictates the different functions. Previously, placental growth factor (PGF) was shown to amplify VEGF driven angiogenesis (Carmeliet et al, 2001). More recently, Carmeliet et al., suggest that this amplification is a result of inter- and intramolecular cross-talk between

Flt-1 and Flk-1. Upon activation by PGF, Flt-1 is able to switch on intermolecular crosstalk and indirectly transactivate Flk-1, thereby enhancing response to VEGF-A.

Both PGF and VEGF-A bind to similar domains of Flt-1, but elicit different biological responses due to different tyrosines being phosphorylated. Park et al. (1994) reported that PGF could displace VEGF from sFlt-1 as a means for VEGF-A to activate Flk-1 homodimers or Flk-1/Flt-1 heterodimers and enhance angiogenic signals. These findings provide an interesting insight on the complexities of VEGF-A signaling. Although we know that sFlt-1 can heterodimerize with Flk-1 to inhibit VEGF-A actvity, there is no evidence that shows whether sFlt-1 can heterodimerize with Flt-1 as well, to further inhibit VEGF-A activity. If Flt-1 acts as a reservoir for VEGF-A during increased levels of VEGF-A expression, does it act to inhibit Flk-1 binding to VEGF-A and thus limit VEGF-A activity? If this is the case, it would explain why both sFlt-1 and Flt-1 are upregulated during VEGF-A overexpression.

Given these data, we would hypothesize that increased levels of PFG results in an increase of endothelial cell response to VEGF-A and in turn upregulate Flt-1 and sFlt-1 to maintain proper VEGF-A levels within the cell. To test our hypothesis, we would first assess whether sFlt-1 can heterodimerize to Flt-1. Using an invitro system, heterodimerization would be assessed by using our mouse cell lines (MFLM-4/MFLM-91U) and stimulating the cells with increased amounts of $VEGF_{164}$, mouse PGF-2 or heterodimers of human $VEGF_{165}$ and PGF-1 (R&D Systems) and then immunoprecipitating Flt-1 and then detect sFlt-1 by immunoblots as described (Auterio et al, 2003). Next we would assess levels of PGF, Flt-1 and sFlt-1 in our VEGF-A overexpressing transgenic animals. We would expect that if heterodimerization occurred for Flt-1 and sFlt-1 when stimulated with increased amounts of VEGF-A and/or PGF and not under normal conditions, then this would indicate that upregulation of Flt-1 is a result of

upregulation of VEGF-A activity within a cell. Flt-1 would then be acting in a negative manner in response to overstimulation of VEGF-A activity within the cell, aiding sFlt-1 in neutralizing the effects of excessive VEGF-A activity. These results would provide another level of understanding VEGF-A regulation via signaling through its receptors. This form of regulation is not only helps to understand VEGF-A regulation at the molecular level, but may also provide possible therapeutic applications in the future to VEGF-A associated vascular diseases.

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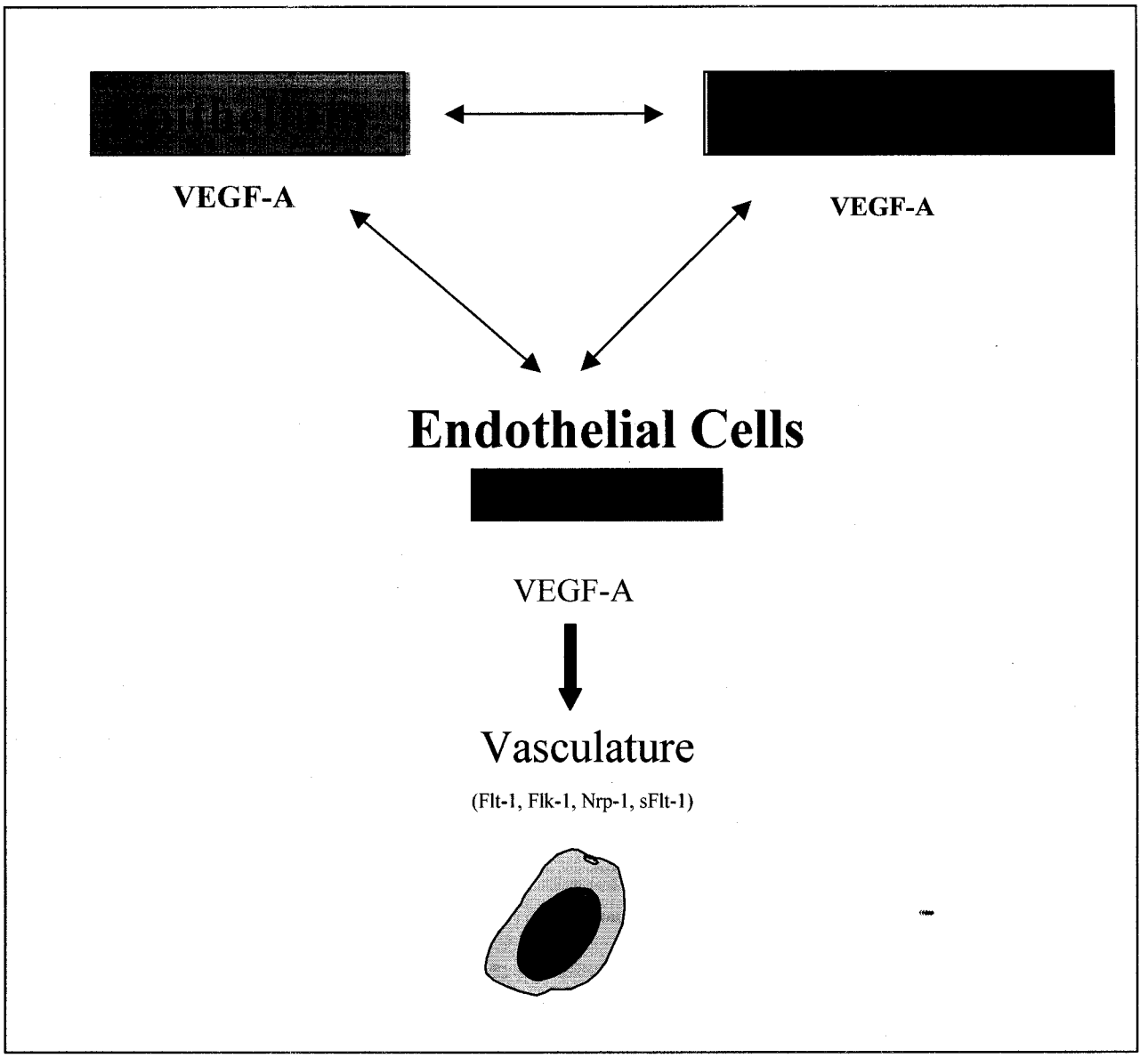


Figure 1. Model of lung inter-compartmental cross talk. Vascular endothelial growth factor (VEGF-A) is diffusely expressed early in lung mesenchyme. Midway through lung development, VEGF-A is expressed in the epithelium and then predominantly in distal respiratory epithelial cells. VEGF-A acts through its tyrosine kinase receptors, VEGF receptor-1 (Flt-1), VEGF receptor-2 (Flk-1) and the co-receptor neuropilin-1 (Nrp-1) and binds to the inhibitory soluble receptor, sFlt-1, which are predominantly localized to the endothelium. Endothelial cells are derived from the mesenchymal population of cells. By cross-talk from the epithelium and mesenchyme, VEGF-A is spatially and temporally expressed to signal through its receptors in the endothelium and ultimately form the vasculature of the developing lung. In addition, we believe the endothelium also cross-talks to both the epithelium and mesenchyme to influence proper airway development. Lack of cross-talk between these compartments, disrupts proper VEGF-A activity and thus results in a disruption of the vasculature in the lung. Disruption of the vasculature

hinders proper airway development and results in a dysfunctional lung. Thus, VEGF-A is a key player in the coordination of proper vasculature development and airway development, which is crucial for a functional lung.

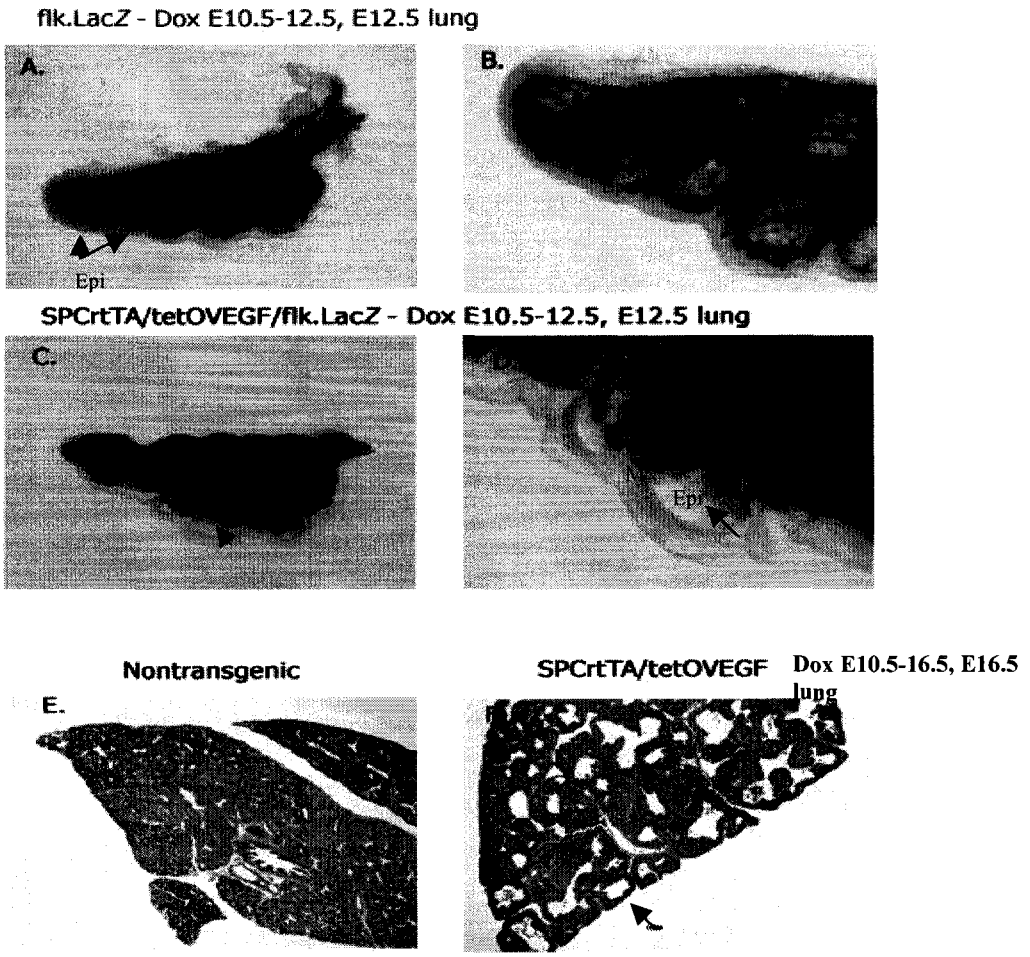


Figure 2. Increased levels of VEGF-A disrupts pulmonary vasculature and airways. Pulmonary vasculature was analyzed by detection of β-gal activity, which detects β-gal + endothelial cells (**A**, **B**, **C**, **D**). Nontransgenic animals show proper vasculature surrounding the epithelial (Epi) tubules (A,B). In transgenic mice *SPCrtTA/tetOVEGF/flk.LacZ*, the vasculature is absent around the epithelial tubules and distal mesenchyme (M) (**C**, **D**) as indicated by the arrows. In **E**, **F**, H&E staining was done on nontransgenic lungs collected at E16.5 (**E**), and *SPCrtTA/tetOVEGF* lungs doxycycline treated from E10.5-16.5, collected at E16.5 (F). Arrows show dilated airways in distal and proximal regions of the lung as a result of VEGF-A overexpression(**F**).

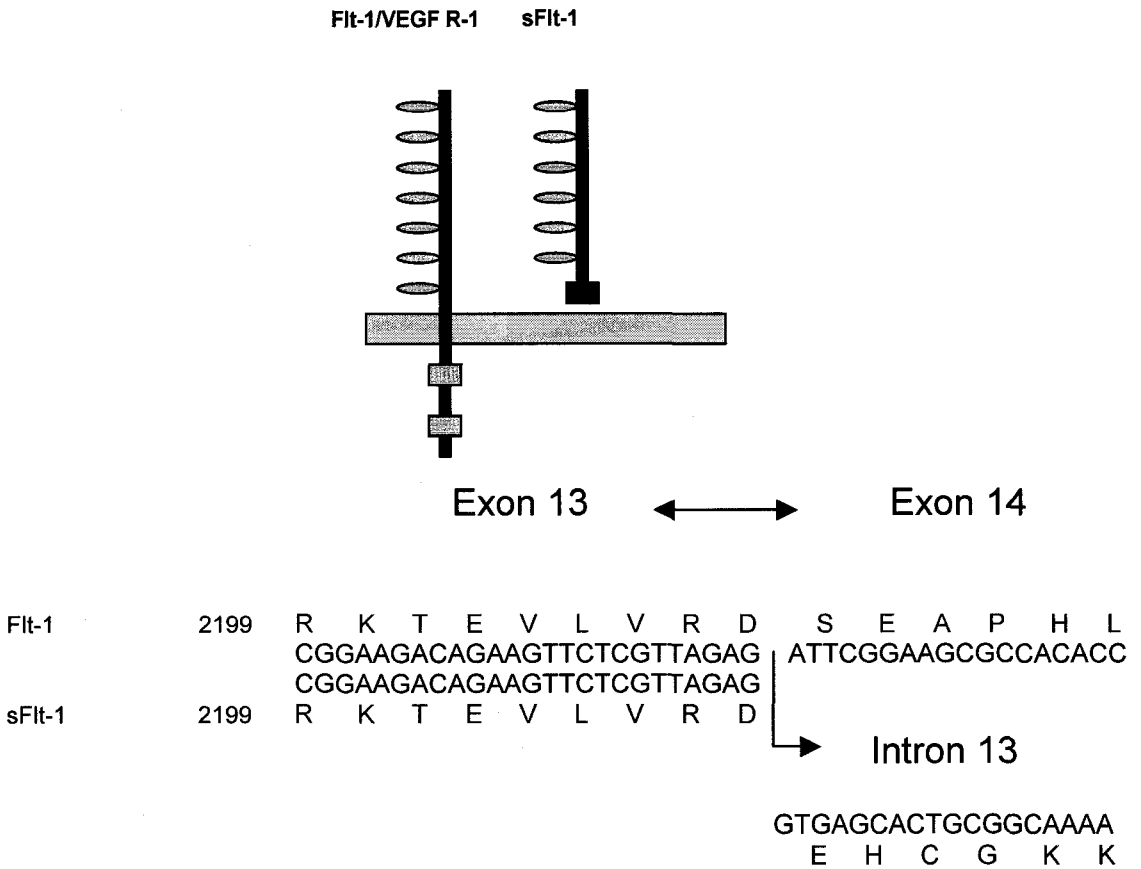


Figure 3. Splicing of the Flt-1 gene. Primers for sFlt-1 were specifically designed in the unique intron 13 region (highlighted in red), which is not present in Flt-1 due to alternative splicing of the pre-mRNA. Primers for Flt-1 were exclusive to exon 14 (downstream of what is shown here), which sFlt-1 lacks due to a stop codon within the unique intron sequence. Arrow indicates the divergent site of membrane-bound Flt-1 and sFlt-1. Amino acid sequence (in blue) is shown to illustrate the unique and distinct coding sequence following the splicing event.

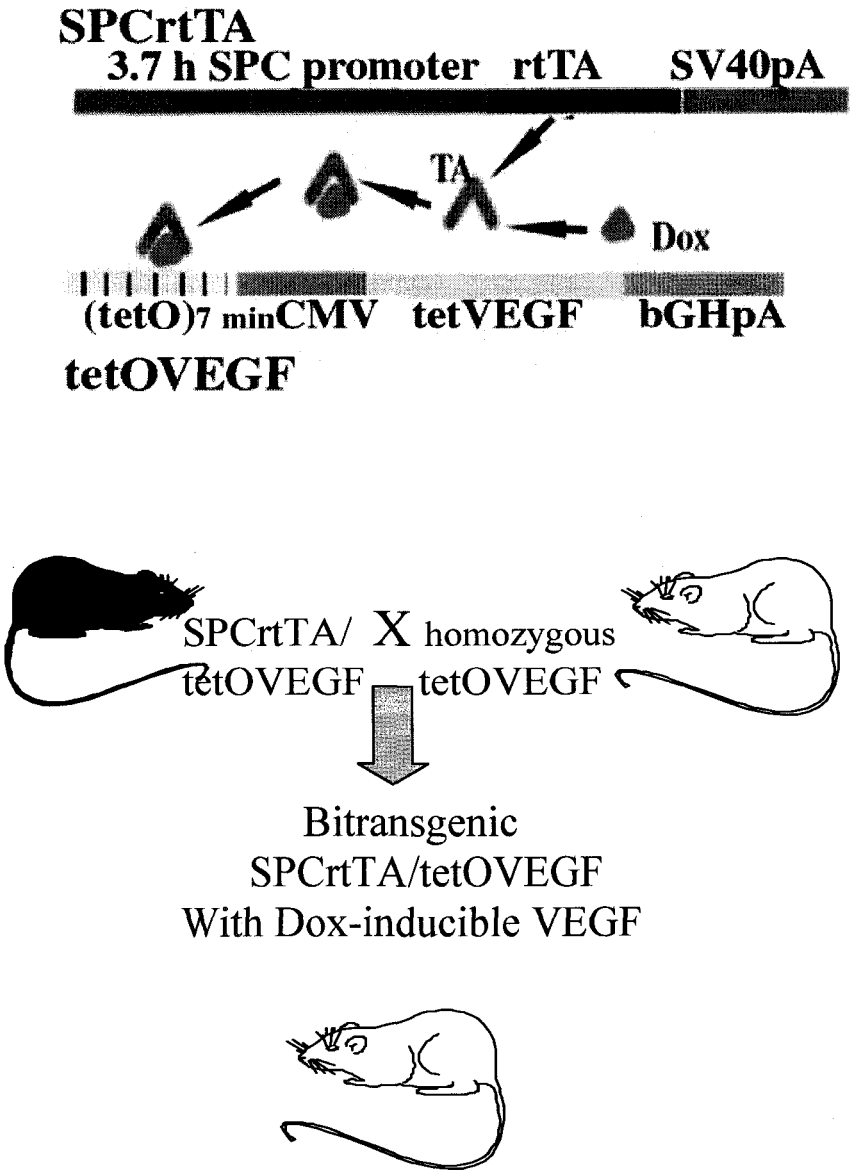


Figure 4. Model of the regulated conditional VEGF-A overexpression mouse. Transgenic mice used for the experiments described here were SP-CrtTA/(tetO) mice crossed with a homozygous tetO VEGF mouse. This cross produced either the bitransgenic SPCrtTA/tetOVEGF mouse or mice that were single trangenic for either the SPCrtTA transgene or the tetOVEGF transgene. The rtTA/tetO transgene system operates when the rtTA protein binds to the tetracycline operator (tetO) DNA binding sequence, but only in the presence of doxycycline. Following this binding, downstream transgenes are activated by the minimal CMV promoter. The rtTA transgene is driven off the surfactant protein C (SP-C) promoter to direct expression of VEGF-A exclusively to epithelial cells.

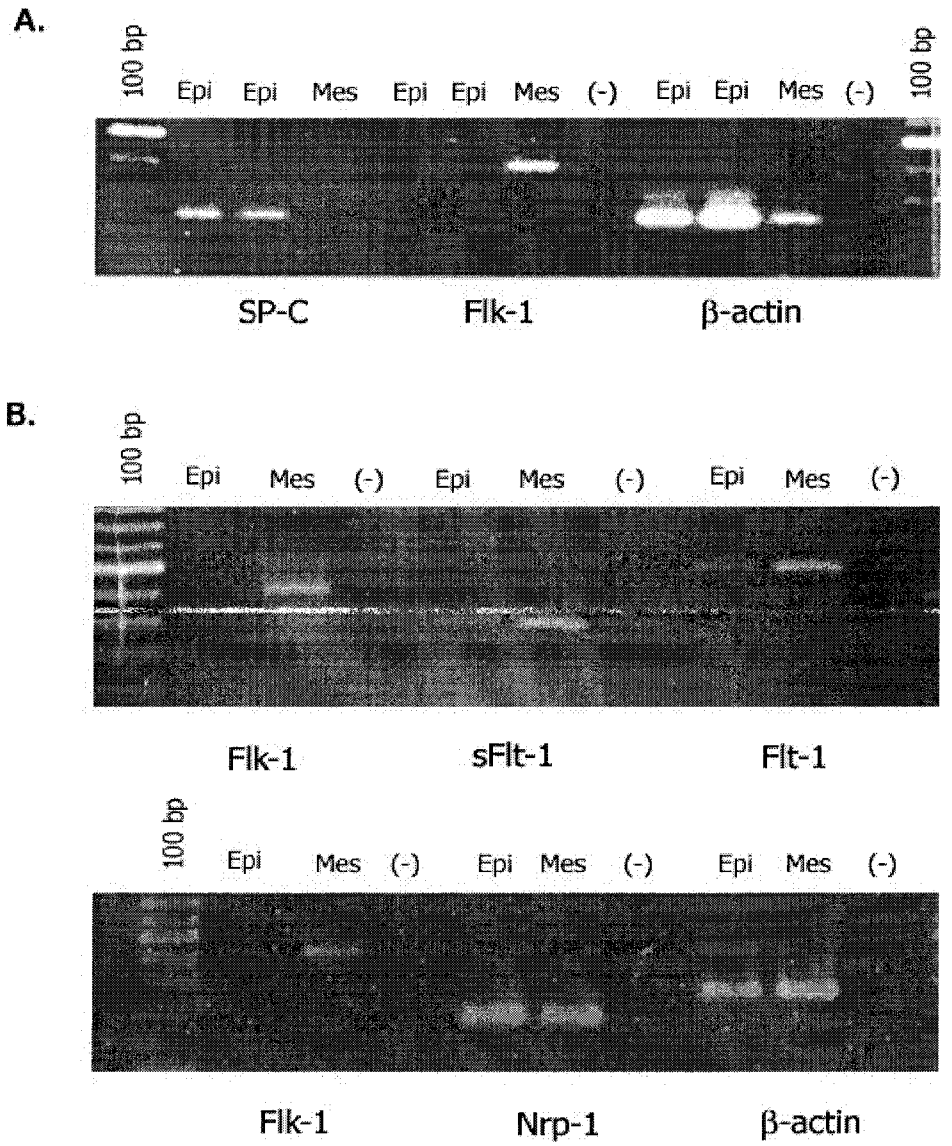


Figure 5. RT-PCR analysis of VEGF receptor expression in E12.5 lung epithelium and mesenchyme. **A:** Epithelial (Epi) and mesenchymal (Mes) RNA were isolated by microsurgery and used as template for reverse transcriptase-polymerase chain reaction (RT-PCR). SP-C is restricted to epithelial cells and Flk-1 is restricted to the mesenchyme, which were used to show purity of each pool of tissue. β-actin was used as the positive control for the template and samples with no template served as the negative control (-). **B**: RT-PCR analysis of the VEGF receptors sFlt-1, Flt-1, Flk-1 and Nrp-1 in E12.5 lung epithelium and mesenchyme.

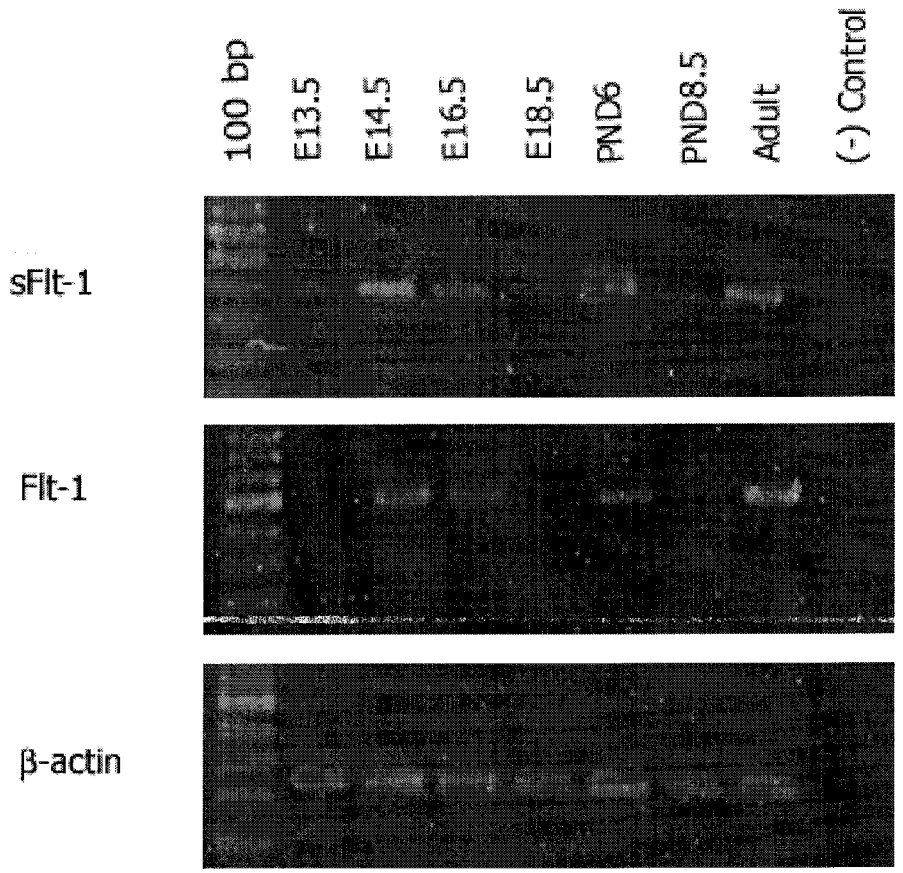
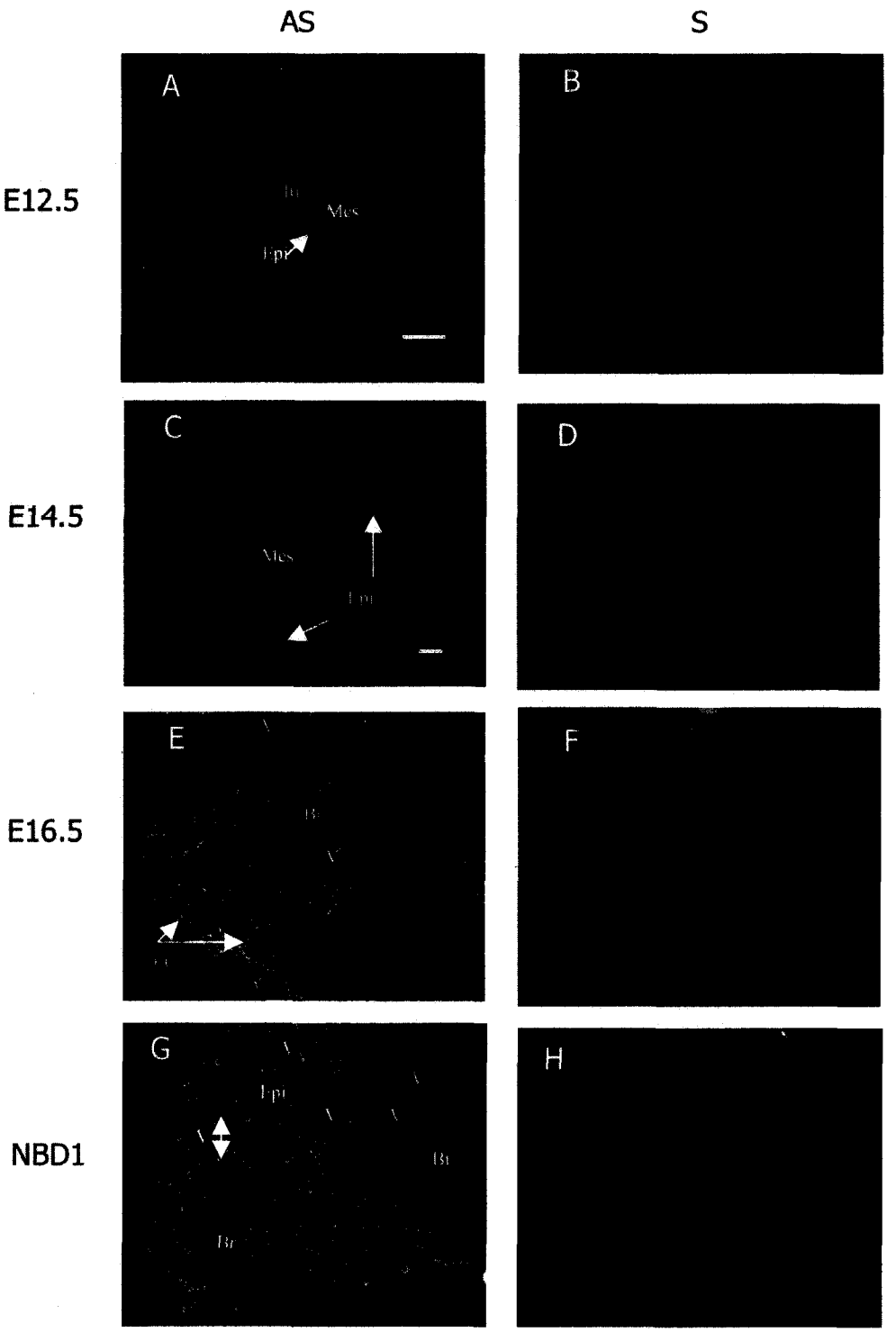


Figure 6. RT-PCR of sFlt-1 and Flt-1 mRNA expression in the developing lung. Transcripts are observed throughout lung development for sFlt-1, but not for Flt-1. β-actin was used as a positive control for cDNA template and as a measure of template concentration differences. Samples without cDNA/RT were used as the negative control.

Figure 7. *In situ* analysis for Flt-1 in the developing mouse lung. Sections from embryonic day 12.5 (E12.5), E14.5, E16.5 and postnatal day 1 (PND1) lung were hybridized with antisense (AS) and sense (S) probes for Flt-1. Flt-1 mRNA was detected throughout mouse lung development and localization was detected early in the mesenchyme (M) at E12.5 and again at E14.5 surrounding the epithelial airways (Epi) (A,C). By E16.5, Flt-1 mRNA is localized to endothelial cells (EC) within lung parenchyma and on vascular endothelial cells (V), and then specifically to the vascular endothelial cells by PND1 (E,G). No signal was detected in the major bronchi (Br). Scale bar = 500 μm for A, B, and E-H. For E14.5, (C and D), scale bar = 100 μm.

ISH for Flt-1



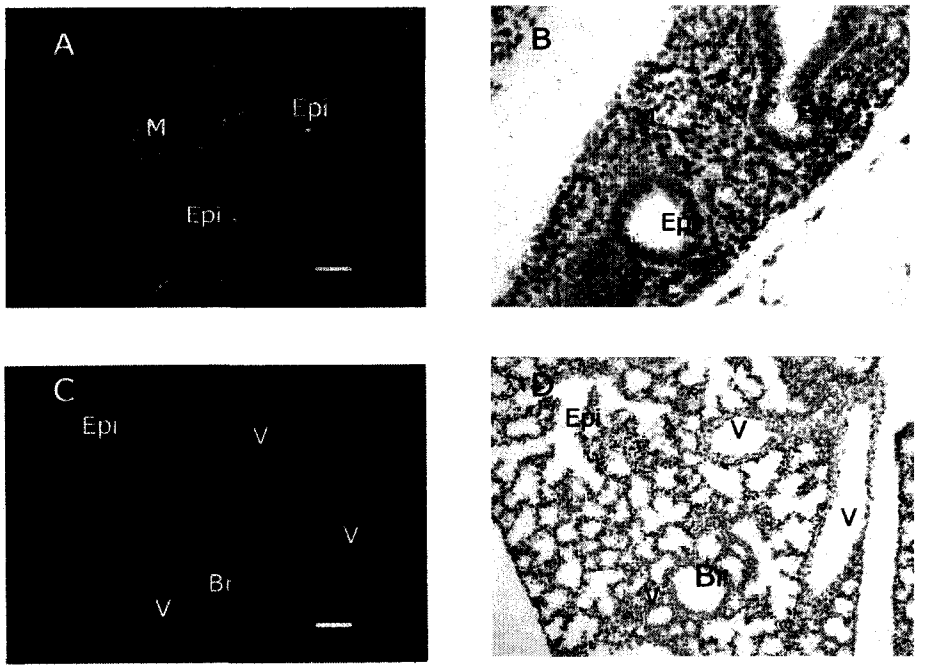


Figure 8. Flt-1 mRNA is detected in lung endothelium and not in epithelial cells. Flt-1 mRNA was detected throughout mouse lung development and localization was detected early in the mesenchyme (M) at E12.5 and then specifically to the vascular endothial cells by PND1 (Figure 7). Higher magnifications were shown to demonstrate that mRNA signal early in development (E12.5) was restricted to endothelial cells in the mesenchyme (A, B). By PND1, signal is restricted to the vascular endothelial cells in the lung (C, D). No signal was detected in the epithelium (Epi) at any stage of development in either distal epithelium cells or bronchiolar cells (Br) (A- D). Slides

were counterstained in 0.2% touididine blue and are shown to aid in orientation. Scale bar = 50μm for A, B, 100μm for C, D.

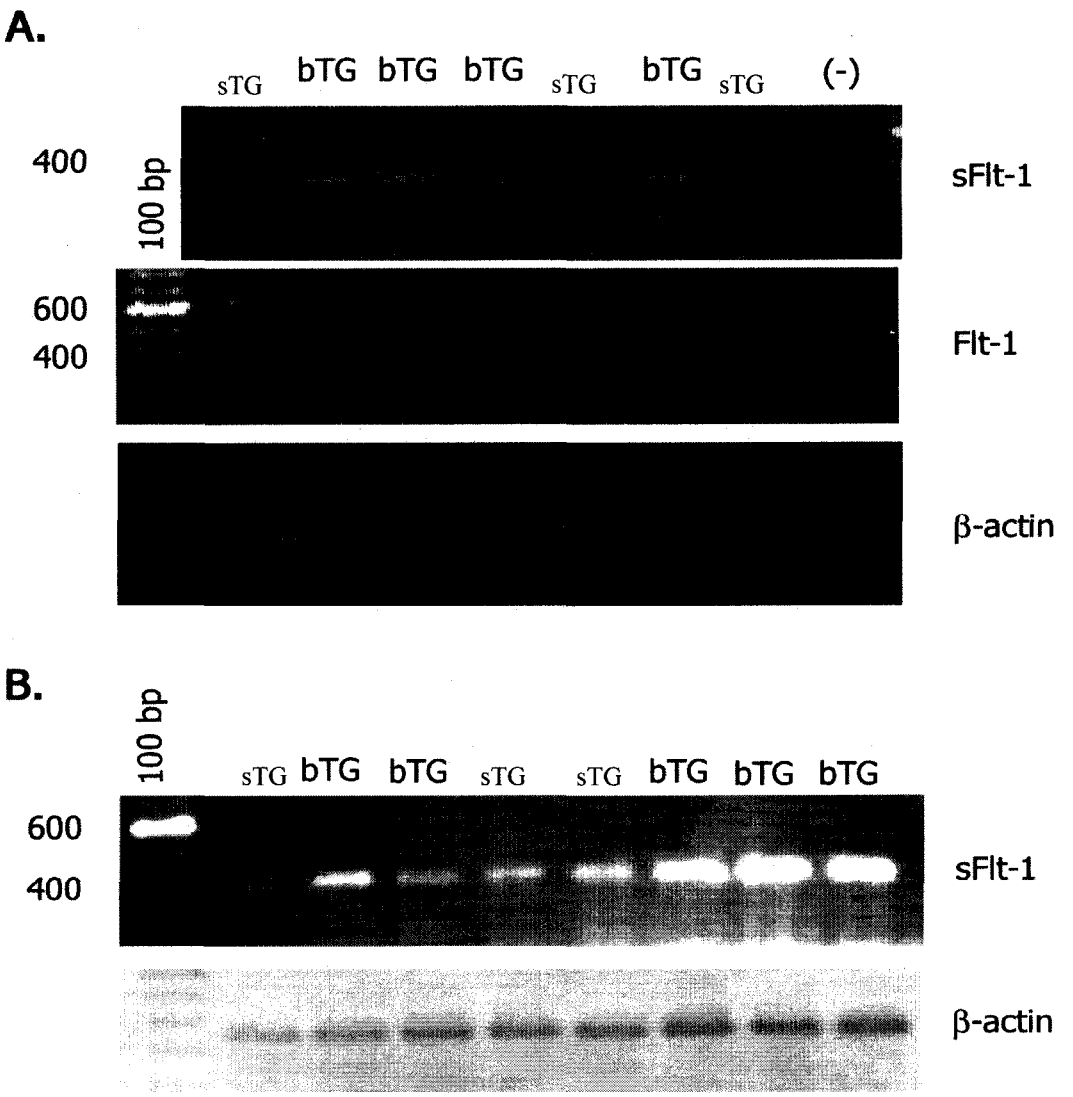
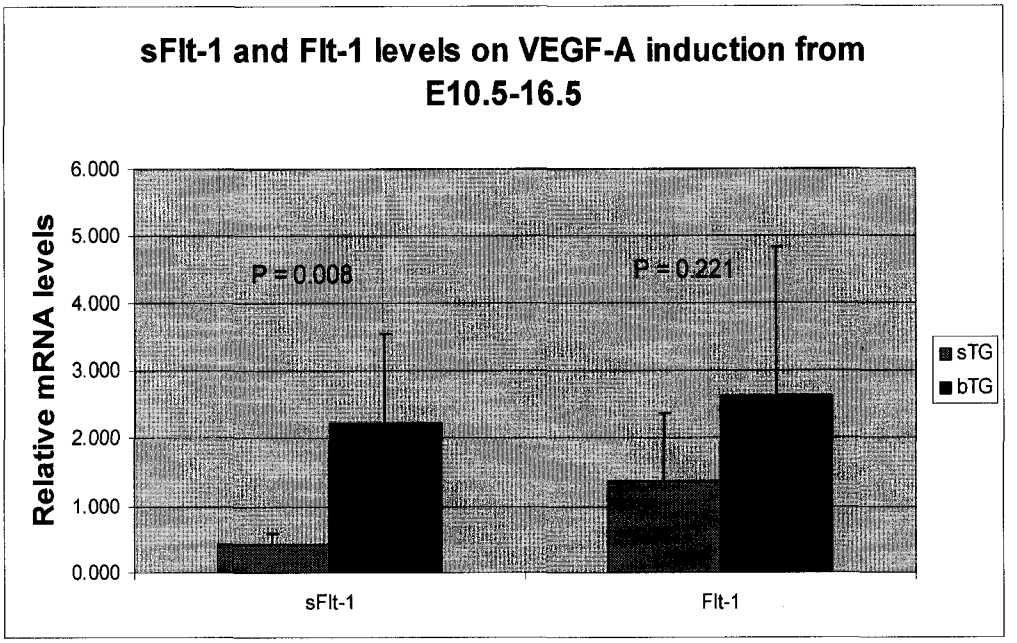


Figure 9. RT-PCR analysis of sFlt-1 in VEGF-A over-expressing mice. **A**: RNA was isolated from VEGF-A over-expression mice as described in the Methods section, and reverse transcribed for amplification by sFlt-1, Flt-1 and β-actin. VEGF-A over-expression was induced from E10.5-16.5 in these animals. **B**: RT-PCR analysis was also performed on VEGF-A induced mice earlier in development, where VEGF-A was induced at E12.5-14.5. Samples were prepared with

250 nanograms (ng) of cDNA. Samples with no template were used as the negative (-) control. bTG, bitransgenic animals, sTG single transgenic animals.

A.



B.

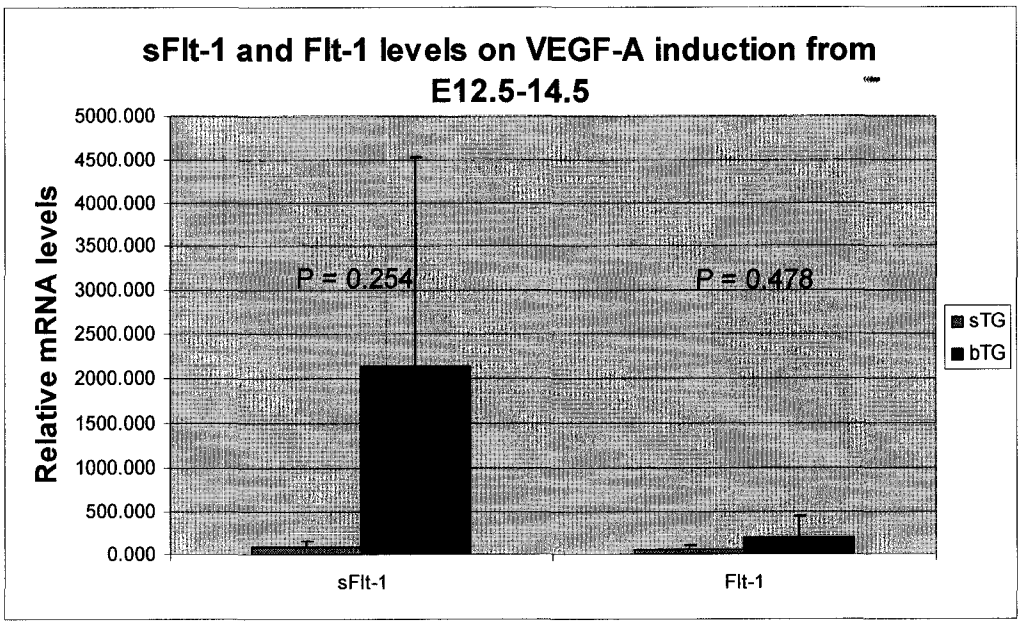


Figure 10. sFlt-1 and Flt-1 mRNA levels are upregulated with increased VEGF-A. Real time RT-PCR was conducted using RNA from VEGF-A induced mice as described for RT-PCR

analysis. **A**: sFlt-1 and Flt-1 mRNA levels were assessed in single transgenic (sTG) and bitransgenic (bTG) animals VEGF-A induced from E10.5-16.5. **B**: sFlt-1 and Flt-1 mRNA levels were assessed for sTG and bTG animals VEGF-A induced from E12.5-14.5. Differences in upregulation were considered significant for $P < 0.05$.

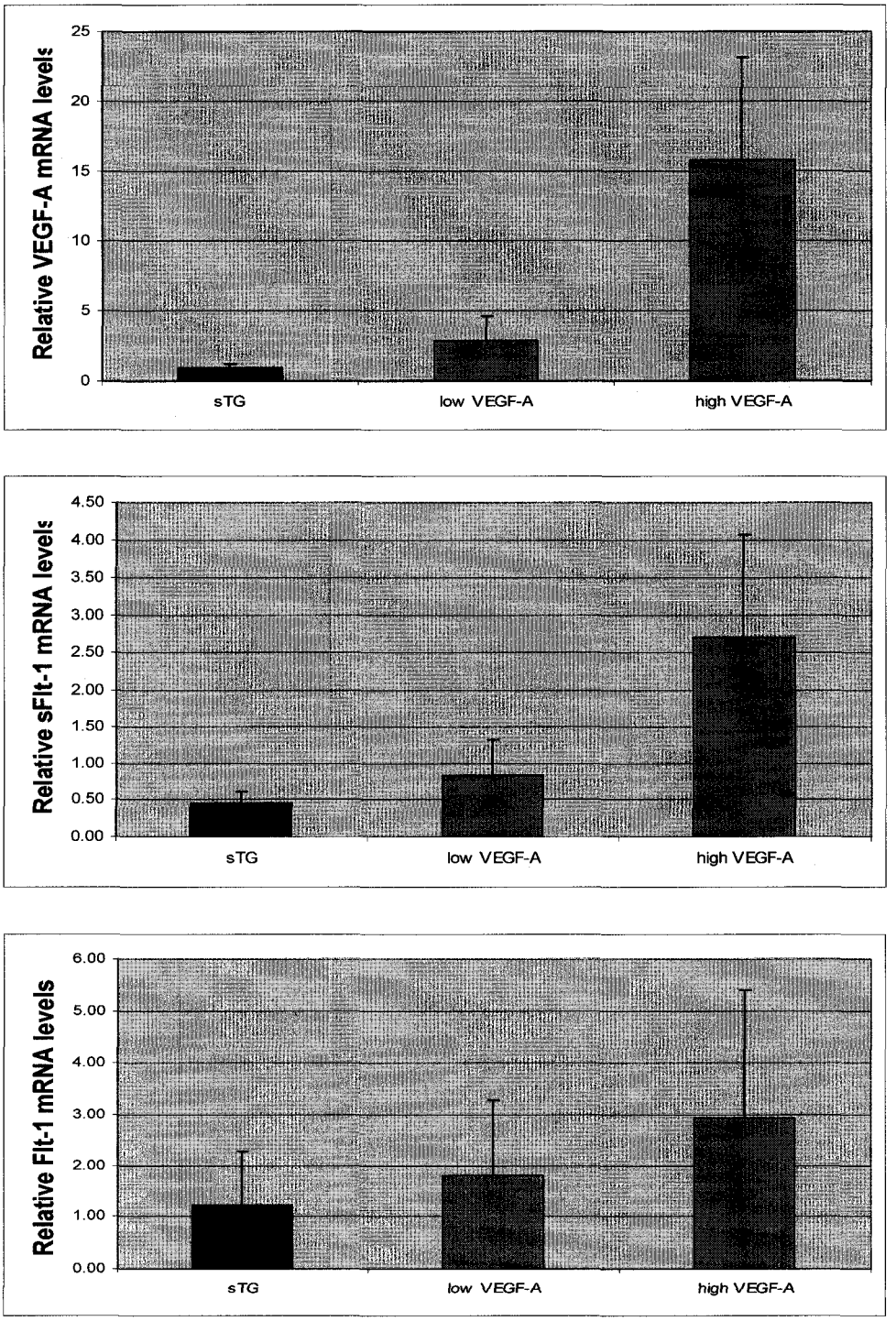


Figure 11. sFlt-1 and Flt-1 mRNA levels correlate to VEGF-A transgene expression. Real time RT-PCR was conducted using RNA from E10.5-16.5 VEGF-A induced mice, as described for

RT-PCR analysis. VEGF-A followed by sFlt-1 and Flt-1 mRNA levels were assessed to determine if mRNA levels for bitransgenic (bTG) animals correlated to VEGF-A transgene expression. Single transgenics (sTG) animals represent endogenous VEGF-A mRNA levels. Upregulation differences were considered significant at $P < 0.05$.

Table of VEGF-A values

Doxycycline treated animals
E10.5-16.5

Litter	Genotype	VEGF mRNA	Group
D37.1	sTG	1.322	endogenous
D37.2	sTG	0.421	endogenous
D37.3	sTG	0.818	endogenous
D37.4	bTG	1.325	hemizygous
D37.5	bTG	5.276	homozygous
D36.6	bTG	6.535	homozygous
D92.1	bTG	33.545	homozygous
D92.2	sTG	0.955	endogenous
D92.3	bTG	21.989	homozygous
D92.4	bTG	20.545	homozygous
D92.5	bTG	19.958	homozygous
D92.6	sTG	0.994	endogenous
D73.1	sTG	1.265	endogenous
D73.2	bTG	4.63	hemizygous
D73.3	bTG	15.195	homozygous
D73.4	bTG	9.458	homozygous
D73.5	sTG	1.108	endogenous
D73.6	bTG	9.277	homozygous
D73.7	sTG	*	*

Doxycycline treated animals
E12.5-14.5

Litter	Genotype	VEGF mRNA	Group
D82.1	sTG	0.225	endogenous
D82.2	bTG	0.784	hemizygous
D82.3	bTG	1.82	homozygous
D82.4	sTG	0.281	endogenous
D82.5	sTG	0.334	endogenous
D82.6	bTG	2.308	homozygous
D82.7	bTG	7.528	homozygous
D82.8	bTG	7.639	homozygous
D83.1	sTG	0.82	endogenous
D83.2	bTG	5.332	homozygous
D83.3	sTG	0.581	endogenous
D83.4	bTG	5.412	homozygous
D83.5	sTG	0.43	endogenous
D83.6	sTG	0.344	endogenous
D83.7	bTG	*	*
D83.8	sTG	0.385	endogenous
D83.9	sTG	0.372	endogenous

Table 1. Relative VEGF-A mRNA levels for individual animals. Bitransgenic (bTG) and single transgenic (sTG) animals were grouped based on VEGF-A mRNA levels obtained. Animals with lower VEGF-A mRNA levels were assumed as animals hemizygous for the VEGF-A transgene, whereas animals with higher VEGF-A levels were assumed homozygous for the VEGF-A transgene in response to VEGF-A gene dosage. Single transgenic animals were grouped as endogenous levels of VEGF-A.
* represents no value obtained for that animal.