Vascular Endothelial Growth Factor Gene Therapy Increases Survival, Promotes Lung Angiogenesis, and Prevents Alveolar Damage in Hyperoxia-Induced Lung Injury
Evidence That Angiogenesis Participates in Alveolarization

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**Background**—Bronchopulmonary dysplasia (BPD) and pulmonary emphysema, both significant global health problems, are characterized by a loss of alveoli. Vascular endothelial growth factor (VEGF) is a trophic factor required for endothelial cell survival and is abundantly expressed in the lung.

**Methods and Results**—We report that VEGF blockade decreases lung VEGF and VEGF receptor 2 (VEGFR-2) expression in newborn rats and impairs alveolar development, leading to alveolar simplification and loss of lung capillaries, mimicking BPD. In hyperoxia-induced BPD in newborn rats, air space enlargement and loss of lung capillaries are associated with decreased lung VEGF and VEGFR-2 expression. Postnatal intratracheal adenovirus-mediated VEGF gene therapy improves survival, promotes lung capillary formation, and preserves alveolar development in this model of irreversible lung injury. Combined VEGF and angiopoietin-1 gene transfer matures the new vasculature, reducing the vascular leakage seen in VEGF-induced capillaries.

**Conclusions**—These findings underscore the importance of the vasculature in what is traditionally thought of as an airway disease and open new therapeutic avenues for lung diseases characterized by irreversible loss of alveoli through the modulation of angiogenic growth factors. (Circulation. 2005;112:2477-2486.)

**Key Words:** angiogenesis ▪ lung ▪ gene therapy ▪ pediatrics ▪ oxygen

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States.⁹ BPD and emphysema are characterized by interrupted development and loss of alveolar structures, and therapy is palliative. Perinatal lung injury in neonates born during the late canalicular stage of lung development disrupts the normal sequence of lung growth, resulting in a histological pattern of “alveolar simplification” (fewer and larger alveoli with fewer septae), loss of small pulmonary arteries, and decreased capillary density. Structural abnormalities closely mimicking human BPD have been demonstrated in a newborn rat model of BPD caused by exposure to hyperoxia.¹⁰–¹² The mechanisms and signal-transduction pathways that regulate the normal alveolar development remain poorly understood, and even less is known about how these pathways are altered in disease. Interactions between airways and blood vessels are critical for normal lung development, suggesting that a
coordinated and timely release of vascular-specific growth factors from respiratory epithelial cells promotes alveolar development.

Vascular endothelial growth factor (VEGF) is crucial for blood vessel formation. In the lung, VEGF mRNA and protein are localized to distal airway epithelial cells and the basement membrane subjacent to the airway epithelial cells. This suggests that translocation of VEGF protein occurs after its synthesis in the epithelium. VEGF is present in alveolar type II cells in the developing mouse lung, and its expression peaks during the canalicular stage, when most of the vessel growth occurs in the lung, then decreases until day 10 postnatal (P10), when it plateaus at adult levels. VEGF receptor 1 (VEGFR-1) and VEGFR-2 mRNA expression also increases during normal mouse lung development, and these receptors are localized on pulmonary endothelial cells, closely apposed to the developing epithelium. This spatial relationship suggests that VEGF plays a role in the development of the alveolar capillary bed. Consistent with this, infants who die of BPD have little or no VEGF in their lung epithelium, and their pulmonary vasculature lacks VEGF receptors. On the basis of the normal interactions between airways and blood vessels in lung development and the observed abnormalities in both the vasculature and airways in BPD, we hypothesized that (1) VEGF-driven angiogenesis is crucial for normal alveolarization; (2) oxygen-induced experimental BPD impairs angiogenesis and decreases alveolarization by decreasing lung VEGF expression; and (3) overexpression of VEGF enhances lung angiogenesis, thereby promoting alveolarization and reversing established BPD.

**Methods**

**Pharmacological VEGF Inhibition**

VEGF-TrapR1/R2 (VEGF-Trap) (25 mg/kg; Regeneron Pharmaceuticals), a soluble combined truncated form of VEGFR-1 and VEGFR-2 fused to the Fc portion of immunoglobulin G, was administered subcutaneously to rat pups at P4, P7, and P10.

**Endothelial Network Formation Assay**

The formation of cord-like structures by human pulmonary artery endothelial cells (HPAECs) was assessed on Matrigel-coated wells. HPAECs (80,000 cells/well) were seeded into 24-well plates coated with Matrigel (BD Biosciences). The media (200 μL, Cascade Biologics) were supplemented with the agents (VEGF-Trap, hFc, or adenoviral constructs) and incubated at 37°C for 8 to 12 hours in normoxia (21%) or hyperoxia (95%). Cord-like structures were observed with an inverted phase contrast microscope (Olympus) and quantified by measuring the number of intersects and the length of structures in random fields from each well using OpenLab (Quorum Technologies Inc).

**Oxygen-Induced Lung Injury**

All procedures were approved by the Animal Health Care Committee of the University of Alberta. Rat pups were exposed to normoxia (21%, control group) or hyperoxia (95% O2, BP0 group) from birth to P14 in sealed Plexiglas chambers (BioSpherix) with continuous O2 monitoring. Dams were switched every 48 hours between the hyperoxic and normoxic chambers to prevent damage to their lungs and to provide equal nutrition to each litter. Litter size was adjusted to 11 pups to control for effects of litter size on nutrition and growth. Rat pups were euthanized at various ages with intraperitoneal pentobarbital, and lungs and heart were processed according to the experiments performed.

**Lung Morphometry**

Lungs were fixed with a 4% glutaraldehyde solution through the trachea under a constant pressure of 20 cm H2O. The trachea was then ligated, and the lungs were immersed in fixative overnight at 4°C. Lung volume was measured by water displacement. Lungs were processed and embedded in paraffin. Serial step sections, 4 μm in thickness, were taken along the longitudinal axis of the lobe. The fixed distance between the sections was calculated so as to allow systematic sampling of 10 sections across the whole lobe. Lungs were stained with hematoxylin and eosin (H&E). Alveolar structures were quantified on a motorized microscope stage using the mean linear intercept method.

**Scanning Electron Microscopy on Lung Vascular Casts**

A freshly prepared mixture of Mercosol catalyst and resin (Ladd Research Industries) at a 50:1 ratio was injected into the main pulmonary artery through a 30-gauge needle until the left cardiac atrium was filled with Mercosol. The catalyst was placed in a wholemount solution of 20% KOH for 2 days, with daily changes of solution. The vascular casts were rinsed in distilled water, air-dried in an oven at 40°C for 1 hour, and mounted on a stub. After having been sputter-coated with gold (Edwards S150B; Edwards), the samples were examined by a Hitachi SEM S-2500.

**Lectin Staining of Lung Endothelium**

FITC Lycopersicon esculentum lectin (100 μg/20 g in 0.5 mL, Vector Laboratories) was injected into the pulmonary artery, followed by a perfusion with 1% PFA in PBS, pH 7.4, at 30 mm Hg for 2 minutes. Lungs were then inflated through a tracheotomy with warmed 1% low-melt agarose, cooled with iced PBS, and embedded in warmed 3% agarose for sectioning. Thick sections (80 to 120 μm) were mounted in Vectashield and visualized under a confocal microscope.

**Barium-Gelatin Angiograms and Arterial Density Counts**

A barium-gelatin mixture (60°C) was infused at 70 mm Hg pressure into the main pulmonary artery catheter for 3 to 4 minutes until surface filling of vessels with barium was seen uniformly over the surface of the lung. At this pressure, the barium evenly filled arteries and capillaries. The main pulmonary artery was tied off under pressure, and the lungs were inflation-fixed with formalin. The right upper lobe was embedded in paraffin, and sections were cut and stained with H&E. Barium-filled pulmonary capillaries were counted per high-power field ×100 magnification. Four to 5 lungs per animal, 5 sections per lung, and 10 high-power fields per section were counted.

**Immunoblotting**

Protein expression in whole lungs was measured with immunoblotting using available antibodies, as previously described. The intensity of the bands was normalized to the intensity of a reporter protein (actin) using the Kodak Gel-doc system.

**Laser Capture Microdissection**

To quantify mRNA in various lung compartments, laser capture microdissection (LCM) was performed on frozen sections by use of the microdissector PixCell II (Arcturus Engineering). LCM specimens were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) using specific primers. Primers for each gene were designed by use of Primer Express software. Total RNA was extracted using an RNAeasy Mini Kit (Qiagen). The TaqMan One-Step RT-PCR Master Mix reagent kit (Applied Biosystems) was used to quantify the copy number of cDNA targets. Levels of mRNA were normalized to a housekeeping gene (18S rRNA) and expressed as \(2^{-\Delta\Delta C_T}\), as described previously.
Preparation of Recombinant Adenoviral Vector
We used a recombinant adenovirus (serotype 5) vector that was rendered replication-deficient by virtue of deletions of the E1 and E3 genes. Ad5-encoding genes, each under a cytomegalovirus promoter, were generously provided by GenVec, Gaithersburg, MA (for green fluorescent protein [GFP] and for VEGF145-GFP) and by Regeneron Pharmaceuticals (for angiopoietin-1 [Ang*1]).

In Vivo Ad5 Intratracheal Administration
Gene delivery into the airways of newborn rats was performed through intratracheal puncture at P4 or P21. After halothane anesthesia, the trachea was exposed through a neck incision. The gene of interest (VEGFR2) was delivered through a tracheal puncture with a short, 30-gauge needle (Becton-Dickinson). After closure of the incision with biological glue (Vetbond, 3 mol/L), rats were allowed to recover.

Statistics
Values are expressed as the mean±SEM. Intergroup differences were assessed by Student’s paired t test or a factorial ANOVA, as appropriate. Post hoc analysis used a Fisher’s probable least significant difference test (Statview 5.1, Abacus Concepts). Survival curves were derived by the Kaplan-Meier method, and differences evaluated by log-rank tests. A value of P<0.05 was considered statistically significant.

Results
VEGF Inhibition Decreases Lung Angiogenesis and Impairs Alveolarization
In an in vitro endothelial network formation assay, inhibition of VEGF signaling by VEGF-Traps decreased cord-like structure formation by HPAECs (Figure 1A). In vivo, subcutaneous injection of VEGF-Traps in rat pups between postpartum days 4 and 14 (P4–14, during the alveolar period of lung development) reduced overall body weight and decreased the ratio of lung weight to body weight (LW/BW) compared with control and hFc-treated animals. VEGF-Traps decreased lung VEGF and VEGFR-2 expression. Scanning electron microscopy of Mercox casts of the pulmonary vasculature showed that VEGF-Trap-treated lungs have a significantly less dense vascular network compared with control and hFc-treated lungs. Decreased capillary density in VEGF-inhibited lungs is confirmed by the quantification of barium angiograms. Representative H&E-stained lung sections showing larger and fewer alveoli in VEGF-Trap–treated lungs. Decreased alveolarization is quantified by use of the mean linear intercept (Lm), which was significantly increased in VEGF-Trap versus control and hFc.
Impaired Alveolarization in Oxygen-Induced Lung Injury Is Associated With Decreased Lung Angiogenesis and VEGF Expression

Exposure of newborn rats to hyperoxia (95%) from birth to P14 impaired alveolar growth, as assessed by the mean linear intercept (Lm) (Figure 2A). Lung capillaries were visualized by labeling the pulmonary endothelium with FITC-L. esculentum lectin injected into the pulmonary artery at P14. Pups housed in room air showed a dense capillary network, whereas those exposed to hyperoxia had a rarified capillary network (Figure 2B). The quantification of arterial density with barium angiograms confirmed decreased numbers of pulmonary vessels in hyperoxia (Figure 2B). In this model, enlarged air spaces and decreased lung angiogenesis were associated with decreased lung VEGF and VEGFR-2 expression (Figure 2C).

In Vivo Intratracheal VEGF145 Gene Therapy Enhances Survival and Promotes Alveolarization in Irreversible Oxygen-Induced BPD

First, we showed the effect of VEGF gene therapy in vitro in the endothelial network formation assay. Hyperoxia decreased endothelial cord-like structure formation by HPAECs; VEGF gene transfer significantly counteracted the effect of oxygen and promoted endothelial network formation (Figure 3A). Because VEGF inhibition decreases alveolarization and because oxygen-induced hypoaerialization is associated with decreased VEGF expression, we investigated the therapeutic potential of VEGF gene therapy to restore normal alveolarization in the rat BPD model. Exposure of newborn rats from birth to P14 to hyperoxia (95%) results in impaired alveolar growth and vascular abnormalities that persist into “adult” life.10–12 Animals were randomized into 4 groups: (1) control, room air; (2) hyperoxia (O2); (3) O2+intratracheal administration of a replication-deficient adenovirus carrying genes for the human VEGF145 isoform and the GFP reporter (O2+Ad5-VEGF145) administered at P4; and (4) O2+Ad5 carrying GFP only (O2+Ad5-GFP). Intratracheal gene transfer resulted in efficient GFP expression in the distal lung (Figure 3B). VEGF145 gene therapy significantly increased lung VEGF protein expression (Figure 3B). VEGF145 gene expression was highest 4 days after infection and declined gradually over a period of 21 days (Figure 3B). Laser capture microdissection showed compartmentalized VEGF145 gene expression, with maximal transgene expression in bronchi and lower expression in pulmonary arteries and alveoli (Figure 3C). No VEGF145 mRNA was detected in the other groups. VEGF gene transfer was also associated with increased alveolar endothelial nitric oxide synthase (eNOS) expression (Figure 3C).

In vivo, VEGF gene transfer at P4 improved survival (Figure 4A) compared with O2 and O2+Ad5-GFP–exposed animals. Ad5-VEGF145–treated animals had increased lung capillary density (Figure 4B) and preserved alveolarization (Figure 4C), compared with O2- and O2+Ad5-GFP–exposed animals. Because this model is thought to produce an irreversible lung injury,10–12 we also tested the ability of VEGF gene transfer to reverse established lung hypoaerialization by overexpressing VEGF after the insult. In this rescue experiment, VEGF gene therapy performed at P21 significantly improved the lung architecture (Lm=39±2 μm) compared with O2-exposed (Lm=44±4, P<0.01) and O2+Ad5-GFP–exposed (Lm=43±1, P<0.01) animals 2 weeks after treatment and was not different from control (Lm=36±2, P=0.08) (Figure 4 D).

Scanning electron microscopy revealed that VEGF-induced lung capillaries, although more numerous than in BPD lungs, are fenestrated and appear immature (Figure 5). Because the angiogenic growth factor Ang-1 is crucial for...
subsequent vessel stabilization and maturation, we hypothesized that combined VEGF and Ang-1 gene transfer would alleviate the enhanced permeability of VEGF-induced angiogenesis. Combined Ad5-VEGF145 + Ang*1 gene therapy given at P4 improved survival (88%), increased lung capillary formation (Figure 6A), and improved lung architecture (Figure 6B) compared with hyperoxia. Pulmonary vessels were less fenestrated compared with Ad5-VEGF145–treated animals (Figure 6A). Combined Ad5-VEGF145 + Ang*1 gene therapy was also associated with a decreased wet/dry lung weight compared with O2-, O2/H11001 Ad5-GFP–, and O2/H11001 Ad5-VEGF145–exposed animals (Figure 6C), suggesting decreased vessel permeability.

**Discussion**

Here, we show that VEGF-driven angiogenesis is critical for normal lung alveolar development and demonstrate the therapeutic potential of postnatal, intratracheal gene transfer for lung diseases that are currently viewed as having irreversible loss of alveoli. As proof of concept, we showed that inhibition of VEGF with a very potent high-affinity blocker impaired alveolarization, mimicking BPD/emphysema. Likewise, irreversible oxygen-induced hypoalveolarization was associated with decreased vascular growth and VEGF expression. Furthermore, VEGF overexpression through intratracheal VEGF145 gene therapy improved survival and was able to preserve and restore normal alveolarization in this model, even when given late in the disease.

In 1959, Liebow observed that the alveolar septa in centrilobular emphysema were remarkably thin and almost avascular. He postulated that a reduction in the blood supply of the small precapillary blood vessels might induce the disappearance of alveolar septa. Despite this early observation, pulmonary vessels were thought for many years to be passive bystanders in lung development, following the branching pattern of the airways. Histological observations showed that endothelial tubes line up around the terminal buds of the airways, suggesting an inductive influence on the part of the epithelium. Recent data suggest that angiogenesis is actively involved in distal lung development. Inacti-
vation studies of VEGF alleles\textsuperscript{13,16} and knockouts of VEGFR-1\textsuperscript{114} and -2\textsuperscript{15} result in lethal embryonic phenotypes characterized by deficient organization of endothelial cells. Mice with a targeted deletion of the VEGF heparin-binding isoforms VEGF\textsubscript{164} and VEGF\textsubscript{188}, however, are viable and display a variety of vascular defects, including a significant reduction in the formation of air spaces and capillaries, resulting in delayed airway maturation.\textsuperscript{31} Conversely, excessive expression of VEGF in the normal developing lung disrupts lung architecture and causes pulmonary hemorrhage and hemosiderosis.\textsuperscript{32} VEGF-induced angiogenesis is in part mediated by NO. Lungs of eNOS-deficient mice exhibit a paucity of distal arteriolar branches and misalignment of pulmonary veins, characteristic features of alveolar dysplasia.\textsuperscript{33} In this study, we used VEGF-Trapp, a potent and specific VEGF blocker,\textsuperscript{22} to study the role of VEGF-driven angiogenesis on normal alveolar development. Rat pups treated during the alveolar period of lung development (P4–14) with VEGF-Trapp displayed decreased vascular growth associated with enlarged airspaces reminiscent of BPD/emphysema (Figure 1B and 1C). In our study, chronic VEGF inhibition was also accompanied by a strong downregulation of VEGFR-2 protein. Our interpretation is that VEGF has a feedback to induce VEGFR-2. Prolonged lack of VEGFR-2 stimulation by VEGF (14 days) may have resulted in a downregulation of VEGFR-2. Our results are consistent with previous reports showing that a VEGFR-2 aptamer or antiangiogenic agents (such as fumagillin and thalidomide) given to rat pups impair alveolar and lung vascular development.\textsuperscript{34} In adult rats, disruption of VEGF signaling (using the VEGFR-1 and -2 blocker SU5416) leads to enlargement of the air spaces, indicative of emphysema,\textsuperscript{35} suggesting that VEGF is required for the maintenance of both the pulmonary vasculature and alveolar structures throughout adulthood. We show that the reverse is also true: in this model of established hypoalveolarization, ie, the hypoxic BPD model, vascular growth is arrested and VEGF signaling is impaired (Figure 2). This is consistent with experimental BPD in other species\textsuperscript{36,37} and with findings in humans.\textsuperscript{21}
Whether inhibition of vascular growth may be a cause rather than simply a consequence of impaired alveolarization remains controversial. Nonetheless, these data suggest a therapeutic potential for angiogenic growth factor modulation in lung diseases characterized by alveolar damage.

To explore this possibility, we used the hyperoxic BPD model of lung injury, hypothesizing that Ad5-mediated overexpression of VEGF would promote lung angiogenesis and thereby stimulate alveolarization. Traditionally, the transgene expression induced by Ad5-mediated gene transfer lasts 2 weeks. The transient nature of transgene expression is ideal in our setting because it covers exactly the target period of alveolarization in rats. Gene delivery into the airways of newborn rats was performed intratracheally at P4. Intratracheal injection of the virus has the advantage of locally administering the gene of interest to the target site, ie, the distal airways and small pulmonary arteries, while avoiding dissemination and induction of angiogenesis in extrapulmonary sites, as we showed previously. Another strength of this approach of postnatal treatment is that it mimics the clinical setting in which exogenous surfactant is routinely administered to premature infants with hyaline membrane disease. Hence, concomitant administration of lung protective/regenerative agents with surfactant through the endotracheal tube in premature infants likely to develop BPD is appealing and clinically relevant. The LCM technique allowed quantification of mRNA expression of the gene of interest in each compartment of the lung, being maximal in the bronchi but also expressed to a lesser extent, but equally, in the small pulmonary arteries and alveoli (Figure 3C). VEGF gene transfer also increased alveolar eNOS expression, suggesting that part of the beneficial effect of VEGF might be NO-mediated. Recent data suggest that inhaled NO treatment preserves alveolar architecture in experimental BPD and decreases the incidence of BPD in premature infants with respiratory failure. VEGF gene therapy given at P4 improved survival and increased lung capillary density and promoted alveolarization (Figure 4). These lung capillaries seen in scanning electron microscopy were dramatically fenestrated, consistent with a leaky phenotype of immature vessels lacking pericyte coating (Figure 5). Similar features have been observed in the lung and in other organs in which VEGF was overexpressed. Normally, VEGF and Ang function together during vascular development, with VEGF acting early during vessel formation and Ang-1 acting later during vessel remodeling, maturation, and stabilization. In the skin, concomitant overexpression of VEGF and Ang-1 alleviates VEGF-induced vascular permeability. In the present study, lung coexpression of Ang-1 and VEGF also enhanced angiogenesis, and scanning electron microscopic images provide evidence that the combined gene therapy approach resulted in more mature capillaries (Figure 6A) that were less permeable (Figure 6C).

Hypoxia is a major stimulator of VEGF expression. Premature exposure of the developing lung to a hyperoxic environment is expected to downregulate VEGF expression. Even ambient O2 levels (21%), ie, premature birth per se, may interfere with normal lung vascular development. Hypoxia upregulates VEGF gene transcription by activating the hypoxia-inducible transcription factors HIF-1 and HIF-2, which
bind the hypoxia-response element in the \textit{VEGF} promoter.\textsuperscript{46,47} HIF-2 is expressed in fetal type 2 pneumocytes.\textsuperscript{46} Interestingly, newborn mice lacking HIF-2 have deficient lung surfactant and die of respiratory failure.\textsuperscript{48} The possibility that this pathology may be the result of diminished VEGF expression is suggested by experiments in which administration of VEGF protein resulted in thinning of the alveolar walls, restoration of surfactant production, and reduction of respiratory distress in wild-type mice.\textsuperscript{48} The study suggests that the pneumotrophic effect of VEGF might have a therapeutic potential for lung maturation in preterm infants at risk for respiratory distress syndrome.

With the use of antenatal steroids and the introduction of exogenous surfactant administration, few premature infants die as a result of acute respiratory distress caused by biochemical lung immaturity (ie, surfactant deficiency); conversely, management of the structural lung immaturity remains problematic, and with improved perinatal care, a new form of lung disease has emerged that lacks specific treatment, resulting in a chronic form of respiratory distress, ie, BPD. The remaining challenge in perinatal medicine is to decrease the incidence/severity of BPD. In this respect, we show that VEGF treatment induces formation of new lung blood vessels, and this treatment improves lung development in an experimental model of chronic lung disease after premature birth.\textsuperscript{49} Our data provide strong evidence that angiogenesis in general, and VEGF in particular, is necessary for alveolarization during normal lung development and that inhibition of VEGF during a critical period of lung growth contributes to the late sequelae of BPD. Furthermore, our data suggest that modulation of vascular growth factors may have therapeutic potential for lung diseases characterized by irreversible loss of alveolar structures.

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Discourse

Dr. Gavin Thurston is an employee of and has stock options in Regeneron Pharmaceuticals, which has a proprietary interest in VEGF trap.

References

Lung diseases characterized by impaired development/loss of alveoli, such as bronchopulmonary dysplasia (BPD) in premature infants or emphysema in adults, are significant global health problems. Currently, there is no specific treatment for these debilitating and life-threatening diseases. Understanding the mechanisms that regulate alveolar development is crucial for developing new treatment strategies. Here, we report that the vascular endothelial growth factor, VEGF, crucial for blood vessel development, regulates normal alveolar development, prevents alveolar damage, and regenerates alveoli after lung injury. We show that VEGF blockade during the critical period of alveolar development in newborn rats leads to air space enlargement and loss of lung capillaries, mimicking BPD. In experimental BPD in newborn rats induced by hyperoxia, air space enlargement and loss of lung capillaries are associated with decreased lung VEGF and VEGFR-2 expression. Intratracheal adenovirus-mediated VEGF gene therapy improves survival, promotes lung capillary formation, and preserves/restores alveolar development in this model of irreversible lung injury. Combined VEGF and angiopoietin-1 (another angiogenic growth factor crucial for blood vessel maturation) gene transfer matures the new vasculature, reducing the vascular leakage seen in VEGF-induced capillaries. Our findings underscore the importance of the vasculature in what is traditionally thought of as an airway disease and open new therapeutic avenues for lung diseases characterized by irreversible loss of alveoli through the modulation of angiogenic growth factors.

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