

Development of the Pulmonary Endothelium in Development of the Pulmonary Circulation: Vasculogenesis and Angiogenesis

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INTRODUCTION

Role of the Pulmonary Vasculature

The cardiovascular system, comprised of the heart and blood vessels, is the first functional organ formed during embryogenesis in higher vertebrates. In the mouse, the heart and first vessels become functional as early as 8 days following fertilization, while in humans the cardiovascular system forms after approximately 3 weeks of development. Cardiovascular function is essential to the survival of higher organisms, because every cell requires nutrition, gas exchange, and elimination of wastes via blood vessels. The primary site of gas exchange is the vascular/alveolar interface, located deep within the lung. Once blood is oxygenated in the lung, pumping of the blood by the heart disperses oxygen-rich blood throughout the body, where exchange of gas within tissues occurs via capillary beds. Then, oxygen-depleted, carbon dioxide-rich blood is returned to the lungs via the vena cava, for the respiratory/circulatory cycle to begin anew. Despite decades of research into the biology of this vascular/pulmonary interface, little is known about how the pulmonary vasculature ensures its proper coordinated growth and intimate development along the tree-like epithelium of the developing lung.

Vascular Development Overview

Morphogenesis of the embryonic vascular system begins with the emergence of angioblasts, or endothelial progenitor cells, which are initially scattered within the mesoderm prior to their incorporation into patent vessels [1]. Angioblasts are fibroblast-like, mesodermal cells capable of migrating, recognizing other angioblasts, adhering, and organizing into vascular structures. Once an angioblast is recruited into forming a vascular “tube,” or vessel, it differentiates into a bona fide differentiated endothelial cell (EC). The defining cell type of the established cardiovascular system is thus the EC, which forms the seamless lining of the entire circulatory system. As the vasculature develops, the initial circulatory system is composed of a rather homogeneous system of primitive vessels, or “plexus.” However, as the embryo develops, this plexus reshapes and remodels into a hierarchical network of large and small vessels. In large vessels, such as the major arteries and veins, the endothelial inner lining becomes insulated by thick layers of extracellular matrix (ECM) components and smooth muscle. In capillary beds, where vessels taper to very narrow diameters, and gases and nutrients are actively exchanged, the endothelium is relatively more “naked” and in immediate contact with surrounding tissues. Thus, development of the vas-

cular system is a step-wise series of dynamic cellular activities, which together shape individual blood vessels, thereby ensuring proper distribution of oxygen-rich blood throughout the body. Interestingly, most key steps in specification and differentiation of vascular cell types are driven by the molecular interaction of vascular endothelial growth factor (VEGF) with its receptor vascular endothelial growth factor receptor VEGFR-2, which is expressed in vascular ECs. In this chapter, we will review the basic steps during systemic and pulmonary vessel development, since they are driven by many analogous mechanisms, and we will present new ideas regarding the molecular basis of their coordinated growth.

ONTOGENY OF VASCULAR CELLS

Endothelial Origin

To fully understand vascular development, it is essential to know where exactly endothelial precursors come from. Although their exact cell of origin has long remained elusive, angioblasts are known to differentiate exclusively from the mesoderm [2, 3]. In addition, it has been demonstrated that angioblasts arise in both extra- and intra-embryonic mesoderm, with their extra-embryonic emergence in the yolk sac preceding their differentiation in embryonic tissues. In mouse, the first extra-embryonic angioblasts can be detected as early as embryonic day (E) 6.5, while those in the embryo proper can be identified later, around E7.0 [4–6]. The first angioblasts identified in the yolk sac can be found within local proliferative foci of extra-embryonic mesoderm. These aggregations of angioblasts progressively take a more definitive shape, either as angioblast “cords” (linear aggregates) or blood islands (see following section) [5, 6]. In all vertebrates examined, these primitive vascular structures precede the formation of a functional and continuous vasculature.

Blood Islands and Hemangioblasts

As mentioned in the previous section, some of the earliest angioblasts identified in vertebrates are those in or near structures called “blood islands” [5, 7]. In mouse, blood islands are scattered in a ring around the distal yolk sac mesoderm [8–10]. In frog and fish, on the other hand, a single blood island is found on the ventral aspect of the gut. Blood islands have been described as “mesodermal cell aggregates,” where inner cells consist of blood or hematopoietic stem cells and outer cells comprise a mantle of angioblasts [5]. Thought to represent transitional structures, blood islands have been shown to grow and fuse, creating a continuous network of blood filled vessels [6, 11, 12]. However recent work calls into question this “blood island fusion” mechanism of vascular

development, and suggests instead that embryonic vessels are more likely to derive from ECs migrating and enveloping, or “capturing,” hematopoietic precursors, as they generate a continuous vasculature [5]. Regardless of the exact dynamics, blood islands have been observed for over a century and are a hallmark of the primitive vertebrate yolk sac vasculature.

The close spatial and temporal association of hematopoietic and EC development in the yolk sac blood islands led to the idea that both lineages originated from common precursor called the “hemangioblast” [1, 13–16]. This possibility is supported by the observation that vessel and blood progenitors express many common markers and mutation of a number of genes affects both lineages [11, 17]. For decades, evidence has accumulated that supports the existence of a hemangioblast [18–20]. However, the isolation of a truly bipotential cell in the embryo, with the capacity to give rise exclusively to both EC and hematopoietic cell types, has yet to be conclusively shown. Recent experiments demonstrate that most intra-embryonic ECs do not emerge from blood islands, and in addition, few blood and ECs actually arise from common progenitors [21–23]. Therefore, the question remains open as to the true nature of the hemangioblast, the breadth of its potential to give rise to different cell types, and its actual frequency within the early vertebrate embryo.

The Endothelial Cell

The fundamental building unit of the blood vessel is the EC. Together, blood vessels of an adult human consist of approximately 1×10^{13} ECs, which stitch together to form the hierarchical network of vessels that carry blood throughout the body [24]. One interesting question that arises is exactly how does one define the EC? Only two shared characteristics have been identified that can be applied to all ECs [25]. The first is anatomical, in that ECs adhere to one another and form the seamless inner lining of all blood vessels. The second is functional, in that ECs create a selectively permeable and active interface, between blood and tissues, which controls the passage of nutrients, gases, and immune cells. Surprisingly, beyond these two traits, no single definition can be applied globally to all ECs. Blood vessels are strikingly different from one tissue to the next. It has been said that there are as many different types of ECs as there are tissues [26]. In the last decade, ECs have been shown to be extremely heterogeneous in their transcriptional profile, structural features, and regionalized functions [27–29]. Therefore, perhaps a more apt definition of ECs is that they can generally be defined as the cells that line the lumen of blood vessels, but display a variable nature that is strikingly heterogeneous, dynamic, and plastic.

ONTOGENY OF THE VASCULATURE

Cellular Mechanisms of Blood Vessel Formation

Blood vessel development occurs via two principal and distinct cellular mechanisms, referred to as vasculogenesis and angiogenesis (Figure 1.1) [15, 30, 31–34]. The initial primitive vascular plexus emerges via vasculogenesis, which describes the *de novo* formation of blood vessels from individual angioblasts. Angiogenesis, in contrast, describes the growth and remodeling of the existing primitive vasculature, and occurs during normal growth of embryonic organs and tissues. Both vasculogenesis and angiogenesis strictly refer to “the genesis of blood vessels”; however, they have been used to describe very different cellular mechanisms of blood vessel formation.

Vasculogenesis

Vasculogenesis refers to the formation of blood vessels via the clustering and organization of individual angioblasts into linear aggregates, or “cords,” followed by

the formation of a patent lumen (Figure 1.1a) [15, 30, 35, 36]. In addition, the term has also been used to describe the fusion of blood islands into blood-filled tubes within the yolk sac. Vasculogenesis is known to be the primary mechanism by which the first embryonic vessels form [2, 36]. This includes the primordia of most primitive blood vessels, including the dorsal aortae and the endocardium, as well as the relatively homogeneous capillary network found in tissues such as the yolk sac. Vasculogenesis is therefore a term that describes a step-wise developmental process, which includes angioblast migration, proliferation, adhesion, morphogenesis, differentiation, and maturation into ECs. Coalescence of these individual vascular progenitors ultimately leads to the formation of a continuous network of vessels, which circulation depends on. “Vasculogenesis” and “neovascularization” are both terms that refer to this *de novo* formation of blood vessels, and are often used interchangeably. Two types of vasculogenesis have been described, type 1 and type 2, with the distinction being based on the location of angioblast emergence relative to the location of vessel formation. In type 1, angioblasts aggregate into cords, at

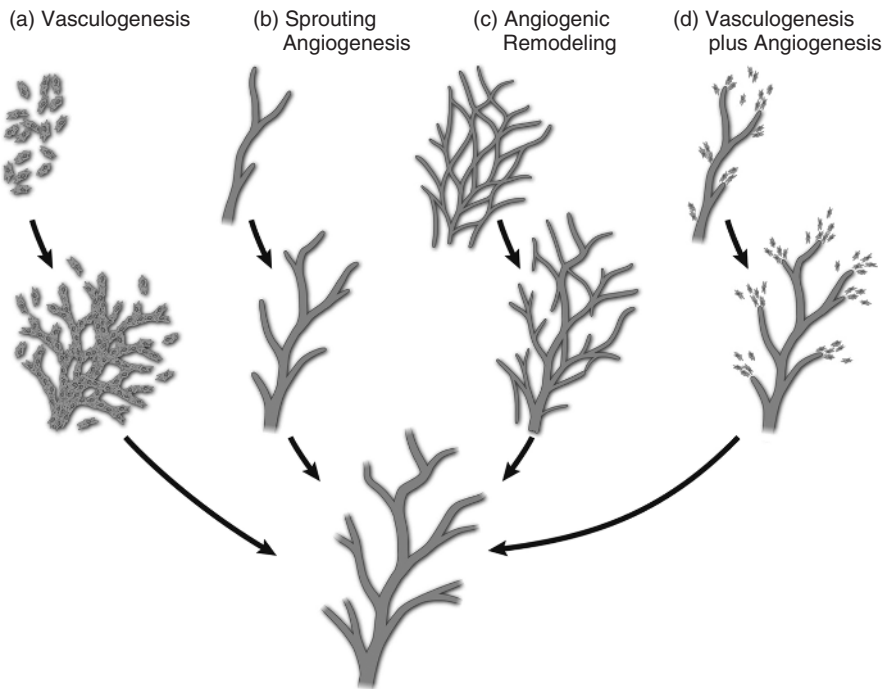


Figure 1.1 Schematic illustrating the different mechanisms of blood vessel formation. (a) Vasculogenesis is the *de novo* formation of vessels via aggregation of angioblasts within the mesoderm. (b) Sprouting angiogenesis is the formation and extension of new sprouts from pre-existing vessels. (c) Angiogenic remodeling is the reorganization and shape change of vessels within an existing vascular plexus. (d) In many tissues, including lung, vasculogenesis and angiogenesis are coordinated to create vascular beds within developing organs and tissues.

the same location where they emerge in the mesoderm. In type 2, angioblasts appear in the mesoderm, but then actively migrate to a different location, where they then coalesce into vessels. During embryonic vascular development, dorsal aortae formation in mouse occurs by vasculogenesis type 1 [37], while the formation of a single dorsal aorta in frog entails vasculogenesis type 2 [38, 39].

Tubulogenesis

Central to the concept of vasculogenesis is the concept of endothelial tubulogenesis. Morphogenesis of a vascular “tube,” from a “cord” of angioblasts or within a growing angiogenic sprout, occurs via tubulogenesis. Tubulogenesis has been described as occurring by two distinct mechanisms. In the first mechanism, the vascular lumen forms by the alignment and fusion of “intracellular spaces,” such as large vacuoles [40, 41]. Classical observations in the avian embryo suggest this first mechanism, where a lumen can be shown to form from the fusion and expansion of intracellular vacuoles into a long continuous space across many cells, at the center of a cord [40–45]. Alternatively, the lumen can be generated by the enlargement of an “extracellular space” located between adjacent angioblasts [46]. The latter mechanism for vascular “tube” formation primarily involves cellular rearrangements that drive the transformation of a solid cord of cells, into a patent cylinder. Based on zebrafish observations [46], it might be predicted that vacuole fusion-based tubulogenesis is likely to be predominantly used in angiogenic sprouting as discussed below, whereas rearrangement-based tubulogenesis is likely to occur primarily during vasculogenesis.

Angiogenesis

Following the formation of the initial primitive vascular plexus via vasculogenesis, the simple circulatory system is then elaborated and extended via angiogenesis. Two fundamentally distinct angiogenic mechanisms have been identified: “sprouting angiogenesis” and “angiogenic remodeling.” Sprouting angiogenesis is defined as the sprouting and extension of new vessels from pre-existing vessels. Quiescent cells within the walls of vessels proliferate, branch, and extend new sprouts into avascular tissues. Angiogenic remodeling encompasses the multiple gross changes that pre-existing vessels can undergo in their basic size or pattern, including the splitting or fusion of the vessel and the enlargement or shrinking of vessel diameter [47–49]. Often these changes in vessel size or shape occur in response to hemodynamic forces. Here, we describe the general features distinguishing each type of angiogenesis.

Sprouting Angiogenesis

Sprouting angiogenesis involves sprouting of new capillaries from the walls of pre-existing blood vessels (Figure 1.1b). Quiescent cells at a specific point along the vessel wall initiate a cascade of targeted cellular activities, all aimed at building an entirely new vessel branch from a pre-existing parent vessel. To create a new sprout, proteolytic degradation of the ECM surrounding the parent vessel is coordinated with proliferation of the sprouting ECs. Together these cellular activities generate a new growing vascular branch, which will eventually fuse with the wall of an adjacent vessel.

Cells at the distal tip of extending angiogenic sprouts, termed “tip” cells, have attracted recent attention. New capillary sprouts grow into the interstitium by the amoeboid migration of distal tip ECs. These invade surrounding avascular tissue, migrate as the sprout extends, fuse with the endothelium of an adjacent vessel, and open up a new connecting lumen [14]. Interestingly, the growth of new sprouts is not believed to occur by proliferation of the tip cells. As the angiogenic sprouts extend, it is within the growing stalk that new cells are added by mitotic proliferation of pre-existing ECs [50]. Classical observations of neural angiogenesis demonstrated that ECs located at the tip of sprouts exhibited a number of distinctive “filiform” processes, hypothesized to function in seeking out and fusing with other growing vessels [51]. More recent studies on endothelial tip cell filopodia in growing retinal vessels have shown that filopodia are the primary target of VEGF signaling and function to drive vessel growth and extension [52, 53].

Remodeling Angiogenesis

Another angiogenic process that generates basic morphogenetic changes in the vascular network architecture is “remodeling angiogenesis,” or “angiogenic remodeling.” In this angiogenic process, pre-existing vessels change in shape, size, and fundamental organization (Figure 1.1c). Generally, these changes involve a wide range of cellular modifications that dynamically alter blood vessel size or architecture. During remodeling, vessels of an initial embryonic plexus either enlarge or regress during development, accommodating the coordinated growth and differentiation of other tissues. Once the vascular system is mature, the vascular network becomes relatively stable and undergoes angiogenic remodeling only in select tissues, such as in female reproductive organs, wound healing, or during pathological processes (e.g., tumor growth).

A dramatic example of angiogenic remodeling involves the primary capillary plexus of the early murine yolk sac. Initially, this plexus presents as a relatively

homogeneous network of vessels, resembling a fisherman's net, with most vessels being of equal size, length, and similar appearance. However, this primitive plexus is rapidly remodeled and modified into the familiar hierarchical, tree-like array of larger and smaller blood vessels. These transformations occur via "angiogenic remodeling" [31, 54]. Angiogenic remodeling remains poorly understood, despite the fact many mouse mutants display clear failure of vascular remodeling.

A wide variety of cellular mechanisms underlie angiogenic remodeling, causing either an increase or decrease in vessel density. Here, we describe intussusception, regression, and pruning. Intussusception is the process of splitting and reorganizing pre-existing vessels, resulting in the expansion of a capillary network [55, 56]. During intussusception, proliferation of ECs within a vessel results in the formation of a large lumen that is subsequently split by intervening endothelial walls (thus resulting in the splitting of one vessel into two). Another mechanism of vascular remodeling, which in contrast decreases capillary density, involves endothelial regression [57]. Key steps in vessel regression include changes in EC shape, lumen narrowing, increased vacuolation, cessation of blood flow, detachment from the basement membrane, and cell death. Regression of vessels often occurs as a result of either a reduction of blood flow, cessation of VEGF-mediated maintenance, or other genetically determined processes, such as changes in expression of angiogenic cues in surrounding tissues. Yet another type of vascular remodeling, which also decreases vessel density and does not involve cell death, has been termed "pruning," as it resembles the process of thinning out excess branches on a tree [31]. Pruning was first observed in the embryonic retinal vasculature and involves the regression of redundant, parallel channels [58]. In these vessels, blood flow ceases, their lumens collapse and ECs retract out of the regressing vessel. In all cases of angiogenic remodeling described above, the principal goal is to fine tune the vasculature so that it perfuses tissues at the required density, satisfying local oxygen demands, by trimming excessive, unneeded vessels or reorganizing vessels to meet physiological demands.

Vasculogenesis and Angiogenesis within Organs

Vascularization of most developing embryonic organs has long been thought to occur primarily via angiogenic invasion of vessels. This was a sensible supposition, given that growing organs appeared to be vascularized by ingrowth of vessels that originated and sprouted from the pre-existing primary vascular plexus. However, improved technology for visualization of the vasculature and its precursors, using newly identified molecular markers and

new vascular reporters, has revealed that most organs develop at least part of their vasculature via *in situ* aggregation of local mesenchymal angioblasts or vasculogenesis [34]. This holds true for the growing vasculature of the lung, liver, stomach, spleen, pancreas, intestine, and kidney [32, 59–63]. During embryonic development of these organs, it is known that angiogenic sprouting from existing vessels also contributes to maintenance and extension of the primitive organ vasculature [34]. New observations have demonstrated that peripheral vasculogenic vessels often fuse with invading angiogenic vessels [64]. Thus, it seems likely that building a continuous vasculature within most organs is a coordinated joining of both vasculogenic beds with angiogenic ingrowth of sprouting vessels.

ARTERIAL VERSUS VENOUS DIFFERENTIATION

Once blood flow begins within the circulatory system, the immature vascular plexus becomes segregated into recognizable arteries and veins (Figure 1.2). Vessels can be categorized as either veins or arteries by a number of parameters, including the direction of blood flow within their lumens, anatomical and functional differences, as well as by the expression of several markers. For instance, the expression of ephrin B2 (Efnb2) ligand is enriched in arteries, while expression of the B4 ephrin receptor (EphB4) is enriched in veins. In addition, a variety of other markers are specific for arteries, including Dll4 [65, 66], Jag1 [67], Notch1 [68], Hey1 and Hey2 [69], activin receptor-like kinase 1 [70], and EPAS1/hypoxia-inducible factor (HIF) [71].

The mechanisms underlying the specification of arterial and venous cell fate are largely unknown. Previously, circulatory dynamics were thought to be the driving cause of arteries and veins developing into structurally and functionally different vessels. However, growing evidence points to a genetic program underlying this fundamental distinction. Indeed, labeling experiments in zebrafish suggest that arterial and venous EC fate may be determined before the formation of blood vessels [72]. Similarly, work in chicks has demonstrated that segregation of arterial and venous markers has already occurred in subpopulations of blood islands long before vessel formation [73]. Therefore, growing evidence points to hard-wired genetic cues specifying arteriovenous cell fate extremely early during vascular development.

Interestingly though, it also seems likely that different vascular beds experience artery/vein specification at different times. For instance, arteriovenous markers in certain organs, such as myocardium [74] and pancreas (Cleaver, unpublished), appear to acquire their identities much later during development. In addition, it is

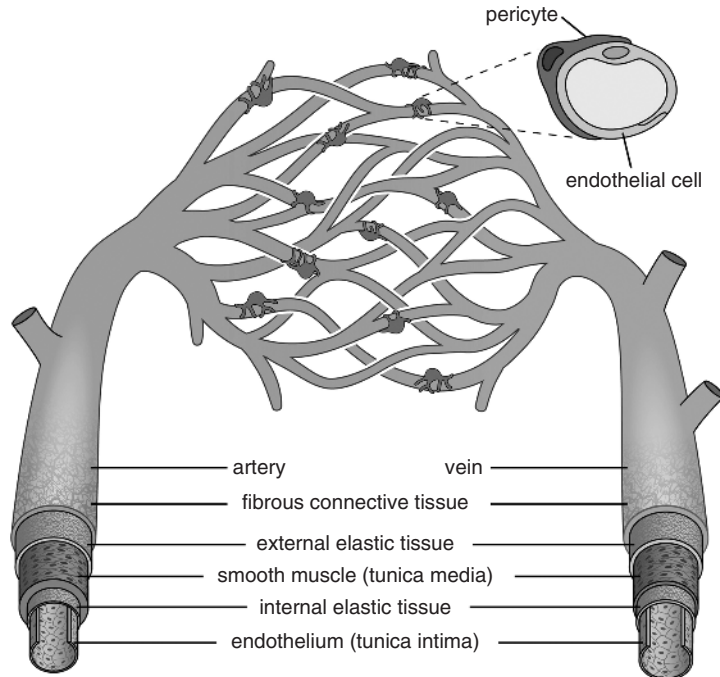


Figure 1.2 Fundamental architecture of blood vessels. Capillary beds perfuse tissues. Capillaries are small caliber vessels, the lumen often forming from single ECs. Capillaries are largely devoid of supportive cells, except for sparse coverage by pericytes. Capillaries are connected in a hierarchical fashion to larger arterioles and venules, which in turn connect to arteries and veins. Arteries and veins are insulated by thick layers of elastic, smooth muscle and fibrous tissues. A color version of this figure appears in the plate section of this volume.

known that arteriovenous cell fate is highly plastic and reversible. In grafting experiments in chicks, vascular ECs were shown to be plastic with respect to their arteriovenous fate [75]. In these experiments, fragments of arteries were heterotopically transplanted to different embryonic sites. Strikingly, cells from the grafted arteries would quickly colonize either host arteries or veins. When they colonized veins, arterial ECs turned off arterial markers and upregulated venous markers. Thus, EC fate remains plastic with respect to arteriovenous differentiation, at least for a period of time during early development.

KEY MOLECULES IN VASCULAR DEVELOPMENT

VEGF [76, 77], and its receptors VEGFR-1 (also called Flt-1) and VEGFR-2 (also called KDR or Flk-1) [78] have long been known to be critical regulators of endothelial differentiation, as well as blood vessel formation and morphogenesis [79]. VEGF-A is essential for proper vessel formation and selective expression of VEGF-A isoforms (murine 120, 164, 188; human 121, 145, 165,

189, 206) drives different aspects of vessel formation in many different organs, including the lung [80]. Here, we introduce the principal vascular developmental factors and outline their roles in vessel formation.

VEGF-A and its Isoforms

The VEGF family of growth factors consists of VEGF-A, B, C, D, and E, and placental growth factor (PlGF). All family members regulate at least some aspect of EC proliferation, migration, and/or survival [79, 81]. Gene targeting demonstrates that VEGF-A plays an essential role in early vessel development. VEGF-A expression is dynamic throughout embryonic development and is often expressed in tissues immediately adjacent to developing blood vessels [38, 77, 82, 83]. VEGF-mediated signaling drives both vessel formation by vasculogenesis, as well as angiogenic invasion of developing tissues. Mice lacking a single VEGF allele die early during embryogenesis (around E10.5). These VEGF-null embryos show a range of vascular defects, including severe abnormalities in EC differentiation, sprouting angiogenesis, vessel lumen

formation, and in the overall patterning of the vasculature [84, 85]. The profound vascular phenotype that results from the loss of a single allele of VEGF demonstrates that tight regulation of VEGF levels is critical for proper vascular development. However, given that angioblasts are present in the VEGF knockout embryos, it can be inferred that VEGF signaling is not required for initial specification of angioblasts [86], but is critical for their proper differentiation and morphogenesis.

VEGF-A presents a number of alternate isoforms, which are generated by alternative splicing of the VEGF-A mRNA. Resulting isoforms differ in their biological activities, as a direct result of differences in their receptor binding affinities and in their ability to diffuse within the extracellular environment. The larger forms of VEGF (VEGF₁₆₄, 188, and 205 in mouse) possess a motif that tethers them to various ECM components and thus decreases their diffusibility. The smallest isoform of VEGF lacks this domain and can freely diffuse. This form has been shown to drive chemotaxis of migrating angioblasts [39]. Gene targeting of these different isoforms results in a range of vascular defects [87]. Therefore, it seems likely the coordination of different isoforms is critical for the generation of a continuous and functional embryonic vasculature.

VEGFRs

The principal receptor for VEGF is the receptor tyrosine kinase VEGFR-2. VEGFR-2 has been shown to be critical for both vasculogenesis and angiogenesis, and is one of the most reliable markers of angioblasts and differentiated ECs. Expression of VEGFR-2 has been shown to be high during embryonic blood vessel formation and in tumor vessels [38, 77, 78, 88]. Mice lacking VEGFR-2 function die early during development, between E8.5 and E10.5, from almost total failure of vascular development [17]. Mutant animals lack almost all angioblast differentiation and either cord or vessel formation. In addition, these mice lack all hematopoietic cells. Thus, VEGFR-2 is a key regulator of both angioblast specification and differentiation. In this chapter, we will review its role during pulmonary vascular development in detail (see “Vascular Growth Factors in Lung Morphogenesis”).

VEGFR-1 displays structural and expression similarities to VEGFR-2, but appears to play a distinct role during vessel formation. VEGFR-1 is a high-affinity receptor for VEGF and PlGF, much like VEGFR-2 [89]. In contrast to VEGFR-2-null mutants however, loss of VEGFR-1 function does not affect early angioblast development, but it does affect their ability to assemble and organize into vessels [90]. In addition, VEGFR-1-deficient embryos actually show an increase, rather than a decrease, in the number of EC precursors throughout the

embryo [91]. While VEGFR-1, like VEGFR-2, possesses an intracellular tyrosine kinase domain, mutation of this domain does not impede normal vessel formation. This suggests that the intracellular portion of the receptor may not transduce active intracellular signaling. Instead, it has been proposed that VEGFR-1 normally functions to sequester excess VEGF ligand, which may regulate the number of differentiated angioblasts and subsequent EC proliferation.

FORMATION OF PULMONARY VASCULATURE

Once the embryo has established a rudimentary circulatory system capable of providing oxygen and nutrients to growing tissues, organ development begins, driven by genetic cues. Coordinately, organ vascular beds also begin to emerge and grow. Although a significant amount is known regarding the forces that drive embryonic vessel formation and lung branching morphogenesis, the angiogenic and vasculogenic mechanisms that establish the pulmonary circulation remain poorly understood. This is in part a result of the complexity of distal pulmonary development, where intimate association of alveolar and vascular tissues must be coordinated to create a functional interface that allows proper oxygen exchange in the mature lung. Given this interdependent relationship between alveolar and vascular development, it has proven difficult to distinguish the mechanisms underlying vascular emergence from those driving distal epithelial morphogenesis. In the second half of this chapter, we review the stages of pulmonary branching morphogenesis and place these in context with what is known regarding pulmonary vascular development. In addition, we also introduce new ideas regarding the molecular basis of their close association and coordinated growth.

ORIGIN OF THE LUNG

Lung morphogenesis initiates on the ventral aspect of the foregut. The first signs of lung formation are a thickening of the foregut epithelium and the subsequent evagination of the laryngotracheal groove. The groove then separates from the esophagus posteriorly, giving rise to the laryngotracheal tube. This parallel tube then grows distally into the underlying splanchnopleuric mesoderm. Morphogenetic changes of the endodermal epithelium result in the formation of two small lung buds, composed of inner epithelial pouches surrounded by a thick layer of mesoderm. This mesodermal layer consists of undifferentiated mesenchyme, vascular, and neuronal cells, surrounded by a thin layer of mesothelium. Following initial embryonic lung budding, early lung morphogenesis then involves a

stereotypic pattern of reproducible budding and branching events, that generate a complex, tree-like system of epithelial branches, which maintain medial–lateral and left–right axes and form the mature lung organ [92–96].

STAGES OF LUNG DEVELOPMENT

Lung development, including pulmonary neovascularization, can be divided into five classic chronological stages based on the growth and differentiation of specific pulmonary epithelial structures (Figure 1.3) [97–99].

- (i) **Embryonic stage**, when the evaginating foregut endodermal epithelium invades the adjacent primitive mesoderm (murine: 9.5–11.5 days; human: 3.5–7 weeks).
- (ii) **Pseudoglandular stage**, during which epithelial-lined airways (pre-acinar bronchi) undergo repeated dichotomous branching (murine: 11.5–16 days; human: 7–17 weeks).
- (iii) **Canalicular (or vascular) stage**, is marked by proliferation of the vasculature, emergence of capillaries, epithelial thinning, and differentiation of the alveolar type 1 and 2 cells (murine: 16.5–17.4 days; human: 17–27 weeks).

- (iv) **Saccular stage**, when vascularization and the number of terminal sacs increases, concurrent with formation of crests and cup-shaped alveoli (murine: 17.4–5 + days; human: 28–36 weeks).
- (v) **Alveolar stage**, during which the alveolar ducts and alveoli develop, mature, and proliferate two to threefold before reaching their adult number (murine: 5+ days; human: 36 weeks gestation onwards).

Progression of lung development through these five distinct stages is consistent across mammalian species.

ORIGIN OF LUNG VASCULATURE

Similar to vessel formation within the developing embryo [100], lung neovascularization is governed by complex interactions between ECs, endodermal and mesodermal cells, mural cells, the ECM, and the cellular microenvironment, as well as by epigenetics [28, 101]. Consistent with vessel formation in other tissues, angiogenesis and vasculogenesis are considered to work in concert to form the pulmonary vascular system [64, 99, 102–104]. Identifying the mechanisms underlying formation of the pulmonary circulation poses many challenges. Initial

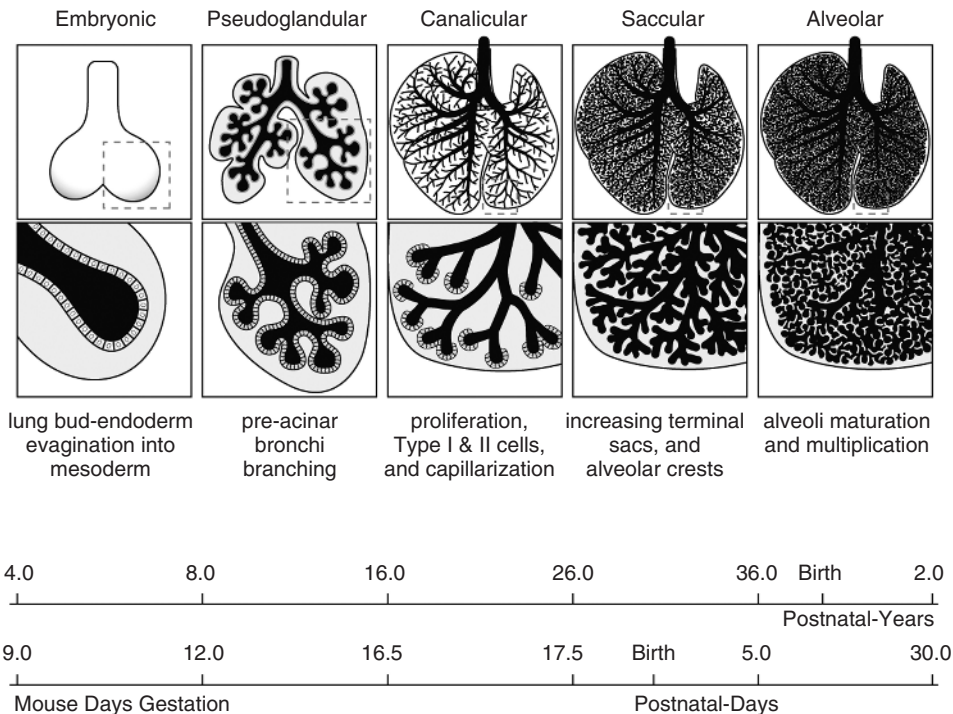


Figure 1.3 Diagram illustrating the stages of lung development that are consistent across mammalian species.

observations using staining for von Willebrand factor suggested that vessel formation in the emerging lung was predominately limited to the canalicular stage [105]. However, more recent observations using *in situ* hybridization and transgenic mouse studies that examined VEGFR-2 expression, generally considered to be a marker of primitive angioblasts and developing vessels, indicate that vessel formation occurs throughout all stages of lung development [106]. Thus, the evolution of available tools and reagents has resulted in an improved anatomical understanding of lung vessel location.

ANGIOGENESIS AND VASCULOGENESIS IN THE DEVELOPING LUNG

Serial histological reconstruction of human embryonic fetal lungs has provided significant insight into the developing lung vasculature. These histological studies indicate that during the embryonic stage of lung development, cells expressing the CD34 antigen (hematopoietic progenitor cell marker) coalesce and form the pulmonary arteries via vasculogenesis within the mesoderm [98, 107, 108]. As lung morphogenesis proceeds to the pseudoglandular stage, pulmonary arteries are believed to continue to be formed via vasculogenesis, while later, during the canalicular and alveolar stages, extension of these vessels occurs via angiogenic mechanisms [98, 107, 108]. Thus, based on these histological studies, it would appear that the development of pulmonary circulation employs sequentially the distinct mechanisms of vasculogenesis and angiogenesis.

In contrast to these histological findings, electron microscopy and methacrylate vessel-casting studies suggests that two independent vascular networks, one angiogenic and one vasculogenic, actually form in parallel and only later connect with each other to generate a continuous circulatory network within the lung [61]. Indeed, these studies suggest that these two networks, which arise simultaneously but independently from each other, have only rare anatomical communication between them during early lung development. Electronic microscopy studies identified vasculogenic pools of clustered angioblasts throughout the embryonic stage, as separate and peripherally located within the lung mesenchyme. To characterize angiogenic vessel formation, vessel casting was performed. The earliest point at which vessel casting could be accomplished, E12 – at the beginning of the pseudoglandular stage – indicated that arterial and venous vessels sprout at this stage from central pulmonary trunk vessels. Communication between the two networks was found to then gradually increase, until a complete vascular circuit is established by E17 just before term in the mouse embryo (term = E18.5) [61]. One complication is that the vessel casting technique is limited, as the location

of growing vessels in relationship to the mesenchyme and bronchi is not effectively revealed. Emerging angiogenic vessels are fragile making identification difficult, and casting at earlier stages prior to E12 of fetal development is limited by embryo size. However despite these limitations, casting studies were the first to identify the simultaneous development of the two parallel pulmonary vascular networks.

Analysis of the expression of EC-specific reporter genes has further expanded our understanding of vasculogenesis and angiogenesis during lung vascular development. Utilizing transgenic reporter mouse lines, both vasculogenic and angiogenic derived emergence of vessels has been observed. Distribution of Tie2 receptor expression in Tie2–lacZ transgenic mice suggests that vessels do not originate *de novo* in the lung bud mesenchyme, but are instead attracted to the lung bud and grow into the lung mesenchyme by angiogenic sprouting [109]. Indeed, vessels expressing Tie2 are observed extending from the medial gut tube toward the distal tip of the lung buds. Vessel emergence via vasculogenesis within the lung mesenchyme is supported by observations of VEGFR-2 reporter expression. VEGFR-2–lacZ transgenic mice, in contrast to the Tie2–lacZ pattern, reveal the presence of an intact vascular plexus within the mesenchyme in E10.5 mouse lungs [106]. Therefore vascular identification studies carried out with different markers reveal endothelial heterogeneity, indicating that different types of ECs are found in the proximal versus the distal lung bud mesenchyme. Alternatively, as VEGFR-2 is a more primitive EC marker Tie2/platelet-endothelial cell adhesion molecule (PECAM)-1 (CD31) [110], it is possible that observed differences may be based on the distinct stages of EC commitment in different regions of the bud. Nonetheless, these studies indicate that vessels are present within the distal mesoderm early, but do little to delineate the exact origin of the different vessel populations. Although initial studies suggested sequential vasculogenesis and angiogenesis, recent evidence continues to accumulate supporting the notion that separate parallel angiogenic and vasculogenic processes work coordinately to form the pulmonary vasculature throughout lung development.

In addition to the alveolar endothelial interface that supports oxygen exchange, central vessels are also found in close proximity to the central bronchi of the lung. Interestingly, bronchial circulation and the interface between the central bronchi and vasculature are poorly understood. To date, observations suggest that although arteries are adjacent to the bronchi extending into the peripheral airways in the mature lung, during early pulmonary development there is little contact between the vasculature and the central or peripheral airways [98, 107, 108]. However, there is histological evidence demonstrating that

by the canalicular stage bronchi and vessels are in close proximity and that an intact vascular network is found by casting at the saccular stage [61]. The contrast between these studies highlight a persistent void in our knowledge of the mechanisms that mediate formation of the bronchi/bronchial circulation interface.

Pulmonary Arterial and Venous Differentiation

The pulmonary circulation is composed of arterial and venous vessels that coordinate vascular flow to and from the distal oxygen exchanging alveolar cells. As mentioned in “Arterial versus Venous Differentiation,” recent studies have identified the endothelial marker EphB4 tyrosine kinase receptor and its membrane-bound ligand EfnB2 as specific venous and arterial vessels markers, respectively [111]. Interestingly, in contrast to other regions throughout the body, the pulmonary arteries carry un-oxygenated blood to the distal capillaries where the EC/alveolar interface facilitates oxygen exchange. Pulmonary veins then return oxygen-rich blood to the left side of the heart. Histological analysis of human fetal lungs (84–98 days gestation) suggests that while a subset of the vascular population expresses EfnB2, all pulmonary EC populations, venous and arterial, express EphB4 [98, 107, 108]. Furthermore, at this stage (E13.5) ECs lack fate specificity as they express both surface markers. It is only at E15.5 that EC arteriovenous cell fate specificity begins to emerge [112]. What is unclear is the stimulus that dictates pulmonary EC specification to either an arterial or venous fate. As oxygen levels *in utero* are relatively low in the developing fetus and the fetal lung is protected from high arterial flow pressures, it is not readily evident that a mechanical or oxidative stress mechanism is involved. An alternative possibility is signaling from smooth muscle cells (SMCs) that are known to line arterial but not the venous system [98, 107, 108]. The paucity of studies that examine arterial and venous EC fate specification highlight our lack of understanding of the mechanisms that regulate the emerging pulmonary vasculature and remain a challenge to pulmonary vascular biologist.

Extension of Primary Pulmonary Vascular Plexus to the Epithelial/Mesenchymal Interface

In light of previous studies on lung vascularization and our recent identification of blood flow in the early lung bud (before E10.5) [112], we set forth a novel proposal for the etiology of lung vascular network formation. We propose that a functional, blood-filled primitive vascular network is present in the mesoderm prior to the evagination of the endodermal lung epithelium (Figure 1.4a). Initially, the relatively homogenous web-like plexus lies within the gut tube mesodermal layer, and runs along the

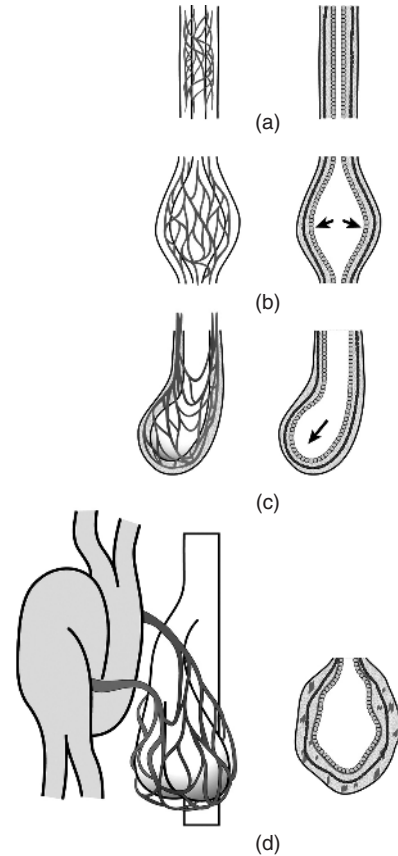


Figure 1.4 Proposal for the sequential progression of lung vascular development. A primitive blood filled vascular network, present within the mesoderm (a), is pushed outward by the invading endodermal bud epithelium (b). Progression of the endodermal epithelial invasion and distal lung bud expansion results in vascular plexus forming a purse-like pouch that narrows at the proximal neck (c). The growing vasculature of the lung bud always maintains a vascular connection with the central circulation system, and the proximal vessels remodel into fewer and larger vessels (d). As the bud grows and the lung vasculature extends and remodels, vasculogenic pools are also present in the distal mesoderm (d). Vascular remodeling of this plexus and the establishment of communication with the vasculogenic clusters completes a multilayered pulmonary vascular network. A color version of this figure appears in the plate section of this volume.

entire length of the foregut and beyond. As the endoderm buds into the mesoderm, the vascular plexus and mesodermal layers are pushed out with it, forming a vascular network that surrounds the budding epithelium like a fish net (Figure 1.4b). This can be seen in a number of studies

that describe early lung vasculature [109]. However, importantly, the vasculature at these early stages remains sandwiched within the middle of the mesodermal layer and is not in immediate contact with the underlying endodermal epithelium.

As budding continues, we propose that the lung bud extends distally with minimal proximal lung growth. This causes the distal vascular plexus to extend, while the proximal vascular plexus remains in relative close proximity to its origin within the foregut. As the bud tips grow out, proximal vessels remodel into fewer and larger vessels and both the arterial (anterior) and returning venous (posterior) systems take shape. Since there is minimal proximal growth relative to distal proliferation, the vascular plexus comes to form a purse-like pouch, with constriction of the proximal plexus around the thinning neck of the lung bud (Figure 1.4c). Simultaneously, in the distal mesenchyme of the lung bud, vasculogenic pools of angioblasts are also emerging (Figure 1.4d).

Around E12 in the mouse, vessels extend centripetally from their position within the mesenchyme toward the epithelial/mesenchymal interface by angiogenic sprouting. In addition, this same plexus also extends in the opposite direction, centrifugally outwards, and establishes communication with the vasculogenic clusters. Overall, remodeling of this plexus completes a multilayered pulmonary vascular network, within the lung bud, by embryonic day 17. This proposed mechanism is consistent with observed vessel formation in other organs where the vasculature is initially confined to a single layered plexus within the mesoderm, while adjacent endoderm and ectoderm layers are initially avascular. Similarly, lymphatic vessels in skin develop from a simple flat array of vessels, to a multilayered array [113]. In both cases, an initial plexus must grow out of a two-dimensional net-like network, and create a more three-dimensional array. Still to be determined is whether type 1 and/or 2 vasculogenic mechanisms are used in lung vascularization, and the timing and mechanisms underlying pulmonary vascular tubulogenesis and angiogenic remodeling during lung development.

Further complicating our understanding of pulmonary neovascularization has been the difficulty in pinpointing the stage at which the lung vasculature comes in contact with the epithelium. Early studies indicate that cells expressing VEGFR-2 mRNA are present in conjunction with pulmonary epithelium during much of lung development [59, 106]. Although adult murine and human lungs have vessels adjacent to the epithelium of bronchi, branching epithelium, and distal alveoli cells, this does not appear to be the case in embryonic lungs. Serial reconstruction of human embryonic fetal lungs [107] and identification of perfused vessels in the mesenchyme [112] indicates that primitive vessels are present in the mesenchyme but are not immediately adjacent to

the evaginating epithelium. Lack of consensus surrounding the stage during lung development where vessel emergence is observed and how vessels develop their interface with the alveolar cell elucidates the difficulty presented in dissecting out the pulmonary circulatory system.

VASCULAR GROWTH FACTORS IN LUNG MORPHOGENESIS

VEGF-A and its Isoforms

Similar to their roles in embryonic vasculogenesis, VEGF-A and its receptors, VEGFR-1, and VEGFR-2, are also essential for pulmonary vessel formation. Indirectly regulated by both fibroblast growth factor (FGF)-9 and “sonic hedgehog” signaling in the mesenchyme, VEGF-A expression mediates distal capillary density and plexus formation [114]. This is supported by the correlation of VEGF-A isoform-specific expression patterns with regional pulmonary vessel formation at different developmental timepoints. VEGF-A isoform distribution and timing suggests that different VEGF-A isoforms facilitate specific aspects of vessel formation. During the early pseudoglandular stages, when vessel formation is confined to the middle mesenchymal cell layer, initial expression of the 120 and 164 VEGF-A isoforms is distributed throughout the mesenchyme [115–118]. At this stage, primitive vessel building and recruitment occurs, and the vascular plexus surrounds the emerging lung bud. This is consistent with the fact that VEGF-A 120 is highly diffusible, allowing it to chemotactically recruit vessels from the plexus or from surrounding vasculogenic pools, while doing little to increase the vascular density within the region [80]. In contrast to VEGF-A 120’s highly diffusible properties, VEGF-A 164 exhibits only moderate diffusion capacity, and is therefore capable of both vessel recruitment and increases in vascular density. The presence of both VEGF-A isoforms 120 and 164 would suggest that during early stages of lung development, vessel formation in the mesenchyme occurs by both vessel recruitment (angiogenesis) and *de novo* differentiation (vasculogenesis). Taken together these findings suggest that in the mesenchyme VEGF120 expression is stimulating angiogenesis while VEGF164 facilitates simultaneous angiogenesis and vasculogenesis.

During the later part of the pseudoglandular stage, the VEGF-A 188 isoform that is notable for developing vascular density is found to be tightly associated with ECM and is found at the epithelial tips of the lung buds. Its expression initiates midway during lung development and gives rise to high local concentrations at the distal tips of the lung buds, which increase distal capillary network density [80, 116–118]. However, it is unclear whether the

increase in vascular network density results from vasculogenic or angiogenic mechanisms. It is worth noting that at E14, the overall expression of VEGF-A is markedly increased in epithelial cells at the tips of the expanding airways, which coincides strikingly with a dramatic increase in vessel density and vascular ingression into the epithelial/mesenchymal interface [112, 115–118]. The corresponding timing of increased VEGF expression in focal epithelial tip cells and the proximity of the epithelium to the extending vasculature are consistent with the facilitation of distal vessel formation. We propose that the burst in epithelial expression of VEGF-A in the lung is likely to attract the filopodia of the angiogenic tip cells toward the epithelial/mesenchymal interface (Figure 1.5). Differential VEGF-A isoform distribution and focal epithelial expression suggests that VEGF-A regulation is critical to vascular growth in pulmonary development.

The potent effect of VEGF on both the formation of pulmonary vessels and on developing airway epithelium can be demonstrated experimentally. Overexpression of the VEGF-A 164 isoform under the control of the human SP-C promoter results in increased vascularization, as expected, but also in marked lung abnormalities characterized by large dilated tubules, disrupted branching morphogenesis, and inhibition of type 1 epithelial cell differentiation [119]. Selective expression of the VEGF-A 120 isoform (which lacks heparin-binding capacity and

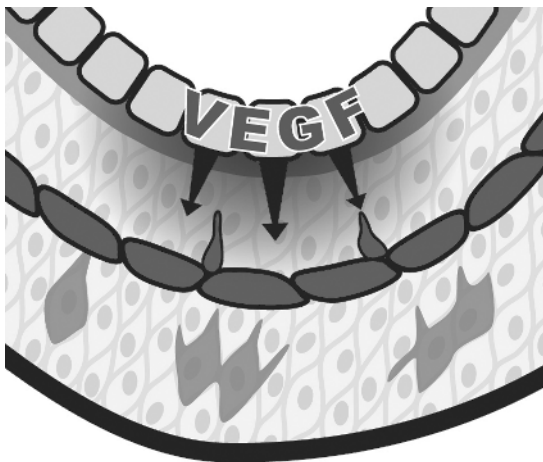


Figure 1.5 Vascular remodeling and establishment of intervascular connections is in part due to the interactions between epithelial VEGF gradients, the vasculogenic pools, and angiogenic extensions from the growing lung plexus. These forces work in concert to develop a functional gas-exchanging vascular/alveolar cell interface. A color version of this figure appears in the plate section of this volume.

therefore lacks ECM interaction domains in mice) resulted in impaired vascular development. Expression of only the VEGF-A 120 isoform resulted in the lack of directed extension of endothelial filopodia and a decrease in vascular branching [120]. Importantly, in addition to defects in lung microvasculature, these mutant mice also displayed a marked delay of airspace maturation [121]. The lack of branching and diminished EC filopodia was attributed to the disruption of the proper VEGF-A concentration gradient. Despite whether expression of one or all VEGF isoforms was altered during development, distal alveolar formation was altered. Together, these experiments suggest that all of the VEGF-A isoforms are necessary for normal alveolar/vascular air–blood barrier formation and confirms that the different VEGF-A isoforms have specific roles in lung morphogenesis [121].

VEGFRs

The influence of VEGF-A on neovascularization is not only regulated by local control of expression levels, but also by the selective expression of its receptors. VEGF-A binds multiple receptors including VEGFR-1, -2, and -3 (also known as Flt-4), and neuropilins 1 and 2. While it has been shown that each member of this family of closely related tyrosine kinases performs very different functions during blood vessel development, little is known about their different roles during development of the pulmonary vasculature. What has been demonstrated is that VEGFR-1 and VEGFR-2 both regulate EC proliferation and differentiation and are therefore essential for development of the pulmonary vasculature [122]. As during initial embryonic vessel formation, VEGFR-2 is more likely to mediate EC proliferation and differentiation, while VEGFR-1 plays a greater role in vessel branching and remodeling [122]. VEGFR-2 mRNA-expressing cells during lung development have been correlated with regions in which endothelial precursors are emerging within the mesenchyme via vasculogenesis [106].

Although all precursor EC express VEGFR-2, recent studies indicate that VEGFR-2 is also expressed on precursors to SMCs or pericytes. Presentation of either a VEGF or platelet-derived growth factor ligand to the precursor cell dictates the cell fate to either an EC or pericyte/SMC, respectively [123]. This observation thus limits the usefulness of engineered VEGFR-2 mRNA and VEGFR-2 reporter mice as a sole means to identify emerging vessels. However, by taking advantage of colocalization using antibodies against phosphorylated VEGFR2 and its “endothelial-differentiating” ligand VEGF, one can deduce that a cell positive for both would represent a cell committed to an endothelial fate. Studies that examine colocalization of phosphorylated VEGFR-2 in association with VEGF confirmed that the vasculature

is confined to the mesenchymal cells prior to E14.5–15.5 [112]. While most studies have associated VEGFR-2 expression with vascular and perivascular cells, a study by Ahlbrecht *et al.* determined that epithelial cells in later stages of development also initiate VEGFR-2 expression and simultaneously secrete VEGF [124]. In contrast, neuronal cells lack VEGFR and only express VEGF and neuropilin receptors [125]. Clearly these studies point to the growing need to examine the role of the different tyrosine kinases in response to the VEGF ligand in different cell types.

Environmental Influences on VEGF Expression

Although cell-autonomous factors, like receptor availability and composition of intracellular signaling mediators, are strong determinants of VEGF signaling, tissue interactions, ECM, and environmental factors also play an important role in VEGF regulation. Explant experiments demonstrate that epithelial/mesenchymal interactions are required for induction or maintenance of vascular precursors [59]. Specifically, fetal lung mesenchyme isolated and grown in culture in the absence of lung epithelium maintains few VEGFR-2 cells. In contrast, lung mesenchyme recombined with lung epithelium develops abundant VEGFR-2-positive cells. The necessity of both lung rudimentary tissues suggests that during early pulmonary development epithelial/mesenchymal signaling is essential for the proper emergence of vascular precursors and subsequent development of lung vasculature [59].

Oxygen tension, a mediator of the transcription factor HIF, has been shown to regulate VEGF expression levels. Signaling through the HIF–VEGF–VEGFR system in fact actively participates in lung alveolarization and maturation [126]. Genetic ablation of HIF-2 α resulted in the development of fatal respiratory distress syndrome in neonatal mice [127]. Associated with the reduction in HIF-2 α were lowered alveolar VEGF levels. This resulted in alveolar capillaries that failed to remodel properly and a concomitant insufficient surfactant production by alveolar type 2 cells. However, this could be rescued by either intra-uterine or postnatal intratracheal instillation of VEGF [127]. Further demonstrating the profound impact of HIF on VEGF protein expression, hyperoxia exposure (>95% O₂ days 4–14) resulted in depressed HIF-2 α and VEGF mRNA levels [128, 129] resulting in not only a reduction in vessel density, but also arrested lung alveolarization [130]. Mediation of environmental oxygen tension is observed in the premature newborn where fetal lungs are exposed to relatively high oxygen levels compared to what they would experience *in utero*. This premature oxygenation results in the onset of pathologic lung hypoplasia or bronchopulmonary dysplasia (BPD). Studies examining lung development in premature infants

using a baboon model of BPD determined that there was a marked and selective downregulation of HIFs [131]. Inhibition of HIF degradation augmented distal alveolar angiogenesis and ameliorated the pathological alveolar dysplasia and physiological consequences of BPD [132, 133]. These studies suggest that environmental influences on VEGF expression play a significant role in the evolution of neonatal lung disease.

ENDOTHELIAL-SPECIFIC FACTORS

In addition to the VEGF and VEGFR family, ECs themselves generate factors that contribute to the regulation of their behaviors during vessel formation. For example, angiopoietin-1 protein is likely to be required for pulmonary vessel integrity and quiescence. High angiopoietin-1 levels in nitrofen-induced hypoplastic lungs were associated with a significant reduction in peripheral capillaries [134]. Further supporting a role for endothelial-selective growth factors in pulmonary vascular development, transgenic mice with an endothelial nitric oxide synthase mutation exhibit capillary hypoperfusion, misaligned pulmonary veins and also display a paucity of distal arteriolar branches [135]. These endothelial-specific factors, while not characterized as endothelial growth factors, directly impact vessel formation during development and warrant further studies to better understand their contribution to lung pulmonary vascular development.

NON-ENDOTHELIAL-SPECIFIC GROWTH FACTORS

In contrast to factors that have endothelial-specific receptors, growth factors secreted from other cell types also contribute to vessel formation. For example, secreted factors such as FGFs influence vessel formation by altering vascular integrity [136] and distal alveolar formation [137]. However, the effects of FGFs are not limited to vessel formation, as lung branching and distal alveolar cell differentiation are directly impacted by FGF levels. Although these studies are beyond the scope of this chapter, review articles by Cardoso and Maeda nicely elaborate in greater detail on the interactions between transcriptional factors and lung morphogenesis [93, 138]. Further examination of nonendothelial-specific growth factors and their contribution to overall lung growth, including vessel formation, is important in broadening our understanding of pulmonary vascular development.

ECM

The ECM has also been shown to be critical in modulating embryonic organ and tissue development, including

blood vessel formation. Interactions between adhesion molecules mediate cell–cell cohesion and facilitate establishment of epithelial cell polarity [139]. Despite our growing understanding of secreted growth factors in lung vascularization, the role of the ECM in this process is poorly understood.

One abundant pulmonary ECM component is laminin. In the developing lung, laminin is the predominant ECM molecule found at the epithelial/mesenchymal interface [140–144]. Owing to its proximity to the developing vasculature, laminin is ideally positioned to influence lung vessel formation. Recent experiments have shown that laminin regulates vessel lumen diameter, but overall has little impact on vessel emergence [145]. In these studies, deletion of laminin from embryoid bodies due to a laminin $\gamma 1$ deletion results in minimal impact on vessel emergence and organization, but does increase the frequency of vessels with wide lumens [145].

In contrast to the relatively minor role of laminin on vessel construction, recent studies suggest that the ECM protein tenascin-C is required for pulmonary vessel network formation. Tenascin-C is known to be downstream of the paired-related homeobox gene (*Prx1*) and *Prx1*-null mice die soon after birth from respiratory failure. Histological analysis of *Prx1*-null mice reveals hypoplastic lungs with a marked reduction in both vessel number and tenascin-C expression as compared to control littermates. Ihida-Stansbury *et al.* suggest that not only is *Prx1* required for tenascin-C expression, but that tenascin-C is required for *Prx1*-dependent differentiation of fetal pulmonary EC precursors and vascular network formation [146, 147]. Together, these studies demonstrate that ECM molecules are important mediators of vessel formation in the developing lung.

ANTIANGIOGENIC FACTORS

In contrast to the positive role of many growth factors on vascular development, negative/inhibitory vascular factors provide a counterbalance to vessel formation during lung development. The antiangiogenic protein endothelial-monocyte activating polypeptide (EMAP) II, which is activated by its cleavage from p43 [148–150], plays a significant role during lung vascular development.

EMAP II temporal/spatial expression during lung development is consistent with a role in maintaining specific avascular regions during lung development. During the early, pseudoglandular stage (E14.5–15.5), prior to vascularization of the epithelial/mesenchymal interface, EMAP II was found to be highly expressed. Strikingly, its expression is downregulated coincident with the canalicular stage (E16.5), as this region becomes vascularized. EMAP II expression is limited to the perivascular expression into adulthood [151]. Exogenous delivery of

the endogenous antiangiogenic protein EMAP II in a fetal lung allograft model [150] markedly decreased lung vasculature, it induced lung dysplasia and it inhibited distal epithelial cell differentiation. Conversely, and as predicted, delivery of an EMAP II-blocking antibody significantly enhanced vasculature and accelerated differentiation of the distal lung [150].

Early postnatal lung development is profoundly influenced by experimental vascular inhibition, demonstrating the requirement for tight regulation of pulmonary angiogenic factors. Thalidomide, fumagillin, the VEGFR-2 inhibitor SU5416, or PECAM-1-blocking antibodies delivered in the early postnatal period result not only in vascular interruption, but in coincident gross abnormalities in lung development [152, 153]. For example, delivery of the VEGFR-2 inhibitor in the early postnatal period initiates an attenuation of lung development noted by a concomitant decrease vessel formation and alveolarization [152, 153]. Similar results are noted when PECAM-1 is inhibited resulting in the disruption of alveolar septation and reduced endothelium [152, 153]. These studies provide support a role for tight regulation of vascular regulators during lung morphogenesis.

Cross-Talk between Pulmonary Vasculature and Epithelium

As pulmonary morphogenesis progresses, the distal alveoli and ECs have a greater influence on each other's development. This is evident as disruption of either the emerging distal air sacs composed of alveolar clefts or vasculature results in an alteration in the normal morphogenesis of the other. In contrast to embryonic and early pseudoglandular stages, where the vasculature and branching airways are separated by several cell layers, the later pseudoglandular and canalicular stages are characterized by thinning of the mesenchyme and increasing proximity of the lung epithelial and ECs. The close proximity of the two cell types is critical for the facilitation of oxygen exchange across the epithelial/endothelial interface during later development.

The mutual dependence of vasculature and the organs they perfuse is exemplified when vessels are experimentally disrupted. For instance, in lung, vessel inhibition is associated with alterations in epithelial cell morphogenesis. Inhibition of VEGF using a soluble receptor in lung renal capsule grafts [154] inhibited vascular development and epithelial development supporting a role for VEGF in the coordination of epithelial and vascular development [155]. Whereas blockade of vessel growth using the antiangiogenic protein EMAP II in a lung allograft model [150] inhibits epithelial morphogenesis [148]. Furthermore, studies indicate that endogenous VEGF induces fetal epithelial proliferation *in vitro* fetal human

lung explants [156], while conversely VEGF blockade interrupts alveolar structural integrity [157]. In addition, transgenic studies where pulmonary blood vessel formation is altered by overexpression of VEGF164 isoform using the SP-C promoter results in concomitant disruption of branching morphogenesis and inhibition of alveolar type 1 cell differentiation [119]. It is important to note that VEGFRs are not found on the epithelium, suggesting that the vasculature is the target, and that the epithelium responds secondarily. On the other hand, inhibition of lung structural maturation by inhibition of transforming growth factor- β 1, thyroid transcriptional factor-1, or Wnt7b resulted in vascular malformations in conjunction with severe alterations in distal lung alveolar morphogenesis [96, 158–160]. Taken together, these studies indicate that there is a direct and mutually dependent relationship between vessel formation and epithelial morphogenesis.

It has become increasingly apparent that an intimate and reciprocal relationship between epithelial and ECs is fostered throughout distal lung development, likely via cell–cell signaling mediated by VEGF-A. This theory is supported by several key observations: (i) VEGF-A distribution in development, (ii) EC facilitation of distal epithelial cell differentiation, and (iii) the strikingly evident reciprocal influence that alveolar and vascular development have on each other. First, during lung development the epithelial cells generate VEGF that is deposited in the subepithelial matrix within the lung branches. This results in a clear proximal-to-distal VEGF gradient, with VEGF epithelial expression being highest at the tips of the branching distal airways at E13.5 and lowest at the proximal epithelium [117]. Corresponding to the epithelial VEGF gradient, phosphorylated VEGFR-2 signal can be found on the tips of the pulmonary ECs that are extending toward the epithelial/mesenchymal interface during the pseudoglandular stage [112]. Taken together, this suggests that the epithelial basilar VEGF gradient serves as a guidance and endothelial differentiation signal [123].

The basilar epithelial location of VEGF also suggests a morphologic role where a cross-talk interaction between the VEGF expressing basilar epithelial surface and the ECs initiate distal epithelial differentiation. Previous studies have shown that ECs contribute important paracrine signals that influence the development of surrounding organs. For example, during pancreatic development, key events of endocrine differentiation occur only in close association with ECs [161, 162]. In liver, hepatocyte migration and differentiation require similar signals from blood vessel ECs [60]. Similarly, in lung development, VEGF also patterns and coordinates epithelial/vascular morphogenesis [155, 163]. These studies

indicate that without VEGF-A tightly coordinating distal epithelial differentiation and vascular development, progression of epithelial proliferation and sacculation are altered. Interestingly, distal lung differentiation does progress, but the epithelial cell numbers and structure are limited. This suggests that VEGF-A has a broad influence on distal lung formation. Importantly, these studies reinforce the fundamental concept that vascular and epithelial cell cross-talk are essential in the formation of the alveolar/vascular interface that is essential for oxygen exchange.

CONCLUSIONS AND PERSPECTIVES

Lung vascular development is clearly a complex process. Guided by both pro- and antiangiogenic factors, the ECM, epithelial/mesenchymal interactions, and angiogenic and vasculogenic mechanisms work together to establish a functional site of gas exchange at the alveolar/endothelial interface. Mediated by a wide array of vascular growth factors, receptors, and arterial/venous guidance cues, vessel formation is derived by vasculogenic and angiogenic forces. Furthermore, during development vascular growth factors mediate not only endothelial morphogenesis, but also influence directly and indirectly affect a broader cellular community. This results in the close association and coordination of vascular formation and epithelial differentiation, where alteration in either system inevitably and dramatically influences the formation of the other. The intimate relationship between these two interconnected processes makes it exceedingly difficult to identify the individual contributions to either component. Thus, designing methods to distinguish the contribution and regulation of vascularization from epithelial morphogenesis, development of an in-depth understanding of the angiogenic and vasculogenic progression during the early stages of lung formation, and identification of the arterial and venous contributions all remain exciting challenges for future studies.

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