

# Unique aspects of the developing lung circulation: structural development and regulation of vasomotor tone

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**Abstract:** This review summarizes our current knowledge on lung vasculogenesis and angiogenesis during normal lung development and the regulation of fetal and postnatal pulmonary vascular tone. In comparison to that of the adult, the pulmonary circulation of the fetus and newborn displays many unique characteristics. Moreover, altered development of pulmonary vasculature plays a more prominent role in compromised pulmonary vasoreactivity than in the adult. Clinically, a better understanding of the developmental changes in pulmonary vasculature and vasomotor tone and the mechanisms that are disrupted in disease states can lead to the development of new therapies for lung diseases characterized by impaired alveolar structure and pulmonary hypertension.

**Keywords:** pulmonary circulation, fetal, newborn, endothelial, smooth muscle.

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Lung development has been classically divided into five overlapping stages in humans and rodents, on the basis of gross histological features. They are termed the embryonic (human: weeks 4–7 of pregnancy; mice: days E9.5–E12), pseudoglandular (humans: weeks 5–17; mice: E12–E16.5), canalicular (humans: weeks 16–26; mice: E16.5–E17.5), saccular (humans: weeks 24–38; mice: E17.5–P4), and alveolarization stages (humans: week 36 to infancy; mice: P4–P14).<sup>1</sup> The development of the lung starts with the formation the primary left and right lung buds in the ventral foregut endoderm, at about week 4 of pregnancy in the human and at day E9.5 in the mouse. During the pseudoglandular stage, the major airway architecture, including the conducting airways and respiratory bronchiole of the lung, is established, resulting from recursive branching morphogenesis.<sup>2,3</sup> This process is likely to be achieved by three geometric forms, termed domain branching, planar bifurcation, and orthogonal bifurcation.<sup>4</sup> Continuity between the heart and the capillary plexus of the lung occurs as early as day 34 of pregnancy in the human, with the artery extending from the outflow tract of the heart and the vein connecting to the prospective left atrium. With the development of the circulation, a mesenchymal capillary plexus forms between the arteries and the veins.<sup>5,6</sup> A similar phenomenon has been found at day E10.5 in the mouse.<sup>7</sup> At the end of the pseudoglandular stage, all precinar pulmonary and bronchial arteries are in place and correspond to the bronchial branching pattern in human lung.<sup>2,3</sup>

During the canalicular stage, a great increase in the number of lung capillaries occurs. These capillaries are arranged in close ap-

position to the epithelium to form the first air-blood barrier. The subsequent saccular stage is characterized by the formation of the last generation of airways, which end with clusters of thin-walled primitive alveoli (sacculi). At this stage capillaries form a bilayer within the cellular intersacculi septa. Alveolarization is the final stage of lung development. During this period, alveoli are formed by the outgrowth of secondary septa that subdivide terminal sacculi into anatomic alveoli. The interalveolar septa are thinned, the double capillary layer matures into a single-layer adult form, and the microvasculature undergoes marked growth and development.<sup>2,3</sup> Studies by Abman and colleagues<sup>8</sup> suggest that the disturbed alveolar growth and vasculogenesis may constitute an important factor for bronchopulmonary dysplasia (BPD).

The normal development of pulmonary vasculature is an essential part of lung development. The impairment of this process is involved in the pathogenesis of various pediatric pulmonary vascular diseases (PVDs), such as pulmonary artery hypertension and BPD. In comparison with adult PVDs, disruption of lung vascular growth and development plays an especially prominent role in the pathobiology of pediatric PVDs.<sup>3,8</sup> In this review, we summarize our current knowledge on lung vasculogenesis and angiogenesis during normal lung development and the regulation of fetal and postnatal pulmonary vascular tone. Clearly, a better understanding of the mechanisms underlying the developing changes in pulmonary vasculature and pulmonary vasoreactivity would be of help to combat pediatric PVDs more effectively.

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## VASCULOGENESIS AND ANGIOGENESIS

Formation of the pulmonary circulation is dependent on two basic processes: vasculogenesis and angiogenesis. Vasculogenesis is the de novo formation of blood vessels from angioblasts or endothelial precursor cells that migrate and differentiate in response to local cues (growth factors, extracellular matrix) to form vascular tubes. Angiogenesis is the formation of new blood vessels from preexisting ones.<sup>9</sup> There are currently three different postulations regarding pulmonary vascular development: one proposes that central angiogenesis occurs concurrently with peripheral vasculogenesis,<sup>10-12</sup> a second considers vasculogenesis as the primary process in the developing lung,<sup>7,13,14</sup> and the third suggests that distal angiogenesis may act as the mechanism for lung vascular morphogenesis.<sup>5,15</sup> These discrepant models may result, in part, from the lack of genetic tools for sophisticated cell lineage tracing as well as the lack of a time-based simultaneous detection of related signaling profiles.<sup>9</sup>

The development of pulmonary vasculature is closely correlated with and interacts with the airway growth. Studies demonstrate that lung vascularization initially originates in the mesenchyme, distal to the epithelium. In response to epithelial-derived vascular endothelial growth factor (VEGF), the endothelial cells move toward the epithelium, where they eventually form epithelium-capillary bilayers, which are essential for gas exchange.<sup>16</sup> In mice, epithelial deletion of Wntless (Wls), a mediator of WNT ligand secretion, results in decreased distal pulmonary microvascular development. Epithelial Wls-mutant mice die at birth from respiratory failure due to lung hypoplasia and pulmonary hemorrhage.<sup>17,18</sup> Conversely, impairment of pulmonary vasculature development by VEGF inhibitors in both fetal and newborn rats is accompanied by diminished alveolar development, histologically similar to pathologic findings in clinical BPD.<sup>19,20</sup>

A critical step of microvascular maturation occurs during the alveolar stage of lung development.<sup>21</sup> Capillaries, which are initially organized as double capillary layers in the immature gas-exchange region, later fuse or remodel to form a single capillary layer. This process is completed in the rat by about the third postnatal week but can persist postnatally in infants with developmental lung disorders and Down syndrome (Thurlbeck). The alveolar wall thins, and its cellular composition changes. In the rat, the thickness of the alveolar wall and the air-gas barrier (the distance between alveolar gas and capillary blood) decreases by 20%–25%. Between birth and adulthood, the alveolar and capillary surface areas expand nearly 20-fold and the capillary volume by 35-fold. Further expansion of the capillary network subsequently occurs via two angiogenic mechanisms: sprouting angiogenesis from preexisting vessels and intussusceptive growth. Intussusceptive microvascular growth occurs by internal division of the preexisting capillary plexus (insertion of transcapillary tissue pillars) without sprouting, which may underlie alveolar growth and remodeling throughout adult life and thus be amenable to therapeutic modulation for lung regeneration.<sup>22,23</sup> Much more must be learned about the anatomic events underlying lung vascular development and time-specific mechanisms that regulate growth and function at each stage. Nonetheless, current knowledge, gained mostly from developmental biology and cancer literature about key

angiogenic growth factors, has been exploited to investigate the role of lung angiogenesis during normal alveolar development.

## ROLE OF ANGIOGENIC GROWTH FACTORS DURING LUNG VASCULAR DEVELOPMENT

### VEGF is pivotal for proper blood vessel formation

In mammals, 5 VEGF molecules have been identified: VEGF-A, placenta growth factor, VEGF-B, VEGF-C, and VEGF-D. Among them, VEGF-A (hereafter referred to as VEGF) is the one essential molecule for all aspects of vascular development, from the endothelial lineage specification to vessel maintenance. VEGF exerts its effect by binding to transmembrane tyrosine kinase receptors, VEGF receptors 1 (VEGFR-1, flt-1) and 2 (VEGFR-2, flk-1/KDR), and neuropilin 1, which are expressed on vascular endothelium. VEGF signaling is transduced via VEGFR-2, whereas VEGFR-1 modulates this process in a negative manner. Upon binding of VEGF, activated VEGFR-2 undergoes dimerization and autophosphorylation of specific intracellular tyrosine residues of the receptors. VEGFR-2 activation stimulates several signaling pathways, including the rapidly accelerated fibrosarcoma kinase (Raf)–mitogen-activated protein kinase kinase (MEK)–extracellular signal-regulated protein kinase 1 and 2 (ERK 1/2) pathway, which enhances cell proliferation; the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)–protein kinase B (PKB) pathway, which promotes cell survival; the cell division cycle 42 (Cdc42)–p38 mitogen-activated protein kinase (p38 MAPK) pathway; and tyrosine-protein kinase CSK (Src), which induces cell migration. Src activation and PI3K-PKB-mediated increase in nitric oxide (NO) production can increase cell permeability, which may facilitate angiogenesis by providing exposure to the proangiogenic extracellular environment (Fig. 1).<sup>24-26</sup>

The absolute requirement of VEGF for development of the embryonic vasculature in mice has been demonstrated by inactivation studies of VEGF alleles<sup>27,28</sup> and knockouts of VEGFR-1<sup>29</sup> and VEGFR-2.<sup>30</sup> In each of these studies, VEGF-related gene inactivation causes lethal phenotypes that are characterized by deficient organization of endothelial cells. Inducible Cre-*loxP*-mediated gene targeting or administration of a soluble VEGF receptor chimeric protein (mFlt (1–3)-IgG) to inactivate VEGF in early postnatal life increased mortality, stunted body growth, and impaired organ development. The impact of VEGF inhibition lessened with postnatal maturation in this model.<sup>31</sup>

### VEGF promotes normal lung growth

The spatial relationship between receptor and ligand suggests that VEGF plays a role in the development of the alveolar capillary bed. In addition, pharmacological and genetic inactivation of VEGF arrests alveolar development. VEGF messenger RNA (mRNA) and protein are localized to distal airway epithelial cells and the basement membrane subjacent to the airway epithelial cells.<sup>32</sup> VEGFR-1 and VEGFR-2 mRNA expression increases during normal mouse lung development<sup>33,34</sup> and is localized to the pulmonary endothelial cells closely apposed to the developing epithelium.<sup>35</sup> VEGF<sub>120</sub>, VEGF<sub>164</sub>, and VEGF<sub>188</sub> are present in alveolar type II cells in the developing

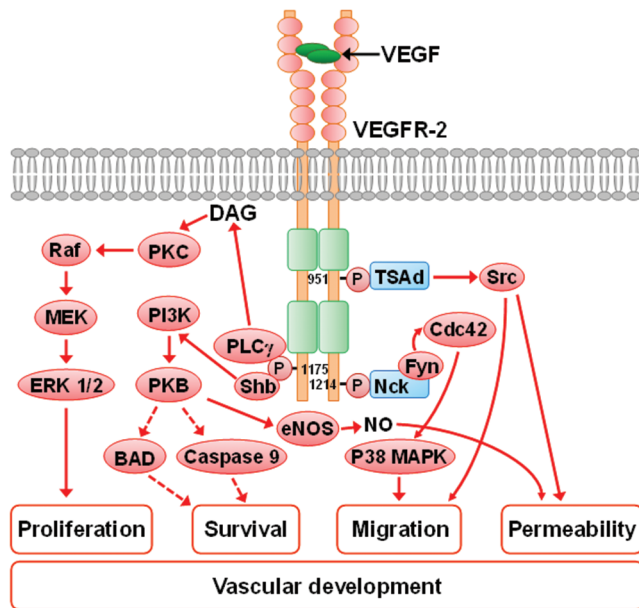


Figure 1. Scheme depicting the possible mechanism of VEGFR-2-mediated vascular effects (cell proliferation, survival, migration, and enhanced permeability) related to vascular formation. VEGFR-2 is composed of an extracellular domain containing seven immunoglobulin-like domains, a transmembrane domain, and an intracellular domain of two kinase domains separated by a kinase-insert domain. The binding of the VEGF dimer to the receptor leads to the dimerization and autophosphorylation of specific intracellular tyrosine residues of the receptors. The phosphorylation of Y1175 recruits PLC $\gamma$ , resulting in DAG release and activation of PKC and the Raf-MEK-ERK 1/2 signaling pathway and thus in stimulated cell proliferation. The phosphorylated Y1175 may also recruit adaptor protein Shb, which leads to activation of PI3K-PKB signaling; consequently, BAD and caspase are suppressed, which results in increased cell survival. Increased PI3K-PKB activity increases eNOS-dependent NO production, resulting in increased cell permeability, which facilitates angiogenesis through providing a proangiogenic extracellular environment. The phosphorylation of Y951 provides a binding site for TSA1, which causes the activation of Src, leading to increased cell migration and permeability. Increased cell migration also results from activation of Cdc42-p38 MAPK as a result of the phosphorylation of Y1214 and the formation of the Nck-Fyn complex. Solid arrows indicate activation and dashed arrows inhibition. BAD: B-cell lymphoma 2-associated death promoter; Cdc42: cell division cycle 42; DAG: diacylglycerol; eNOS: endothelial nitric oxide synthase; ERK 1/2: extracellular signal-regulated protein kinase 1 and 2; Fyn: proto-oncogene tyrosine-protein kinase Fyn; MEK: mitogen-activated protein kinase; Nck: Nck adaptor protein 1; NO, nitric oxide; p38 MAPK: p38 mitogen-activated protein kinase; PI3K: phosphatidylinositol-4,5bisphosphate 3-kinase; PKB: protein kinase B, also known as Akt; PKC: protein kinase C; PLC $\gamma$ : phosphoinositide phospholipase C- $\gamma$ ; Raf: rapidly accelerated fibrosarcoma kinases; Shb: SH2-domain-containing adaptor protein B; Src: tyrosine-protein kinase CSK; TSA1: T cell-specific adaptor molecule; VEGF: vascular endothelial growth factor; VEGFR-2: VEGF receptor 2.

mouse lung, and their expression peaks during the canalicular stage, when most of the vessel growth occurs in the lung, then decreases until postnatal day 10 (P10), when it increases to levels that are maintained through adulthood.<sup>35</sup>

Targeted exon deletion of the VEGF gene reveals that mice that lack the heparin-binding isoforms VEGF<sub>164</sub> and VEGF<sub>188</sub> display a variety of vascular defects, including a significant reduction in the formation of air spaces and capillaries, resulting in distended and underdeveloped alveoli.<sup>36</sup> Likewise, pharmacological and genetic VEGF inhibition during alveolar development decreases alveolarization and pulmonary arterial density, features encountered in clinical BPD.<sup>19,20,36-39</sup> Chronic treatment of adult rats with the VEGFR-1 and VEGFR-2 blocker SU5416 leads to enlargement of the air spaces, indicative of emphysema,<sup>40</sup> suggesting that VEGF is required not only for the formation but also for the maintenance of the pulmonary vasculature and alveolar structures throughout adulthood. Conversely, lung overexpression of VEGF during normal lung development disrupts the lung architecture, suggesting a tight regulation of VEGF-driven angiogenesis to insure proper lung development.<sup>37,41</sup>

In a series of elegant experiments, Vu and colleagues used pharmacological VEGF inhibition in lung renal capsule grafts<sup>39</sup> and genetic VEGF inactivation<sup>42</sup> to show that selective inactivation of VEGF in respiratory epithelium results in an almost complete absence of pulmonary capillaries in the fetal mouse, demonstrating the dependence of pulmonary capillary development on epithelium-derived VEGF. Deficient capillary formation in VEGF-deficient lungs was associated with a defect in primary septum formation, coupled with suppression of epithelial cell proliferation and decreased hepatocyte growth factor (HGF) expression. Lung endothelial cells express HGF, and selective deletion of the HGF receptor gene in respiratory epithelium phenocopies the malformation of septa, confirming the requirement for epithelial HGF signaling in normal septum formation and suggesting that HGF serves as an endothelium-derived factor that signals to the epithelium. These observations suggest that inhibition of vascular growth in the fetus directly impairs distal airspace growth.<sup>42</sup> Selective VEGF inactivation during the early postnatal period causes similar disruption of distal lung development, resulting in sustained reductions of alveolar and vascular growth in the infant mouse.<sup>26</sup>

### Perturbation of NO is associated with arrested lung vascular growth

The role of the endothelium-derived relaxing factor NO in the regulation of the pulmonary vascular tone in the perinatal period is well established; however, little was known about its potential role in the structural development of the pulmonary vasculature.<sup>3</sup> Lungs of late-fetal and neonatal endothelial NO synthase (eNOS)-deficient mice have a paucity of distal arteries and reduced alveolarization<sup>43</sup> and are more susceptible to failed vascular and alveolar growth after exposure to mild hypoxia and hyperoxia.<sup>44</sup> Recent studies suggest that VEGF-induced lung angiogenesis is in part mediated by NO. SU5416 (a VEGF inhibitor)-induced arrested alveolar and vascular growth in

newborn rats is associated with decreased lung eNOS protein expression and NO production; treatment with inhaled NO improves vascular and alveolar growth in this model.<sup>45</sup> Soluble guanylyl cyclase (sGC) is the primary effector enzyme for signaling by NO.<sup>3</sup> In sGC- $\alpha$ 1 knockout mouse pups, the number of small airway structures and the lung volume are decreased, compared with those in wild-type mice, indicating the involvement of NO-cGMP (cyclic guanosine monophosphate) signaling in lung development.<sup>46</sup> However, recombinant human VEGF protein treatment restores lung structure after exposure to mild hyperoxia in eNOS<sup>-/-</sup> postnatal mice, suggesting that VEGF can enhance vascular and alveolar growth through non-eNOS-related mechanisms, such as HGF signaling or enhanced vitamin A production.<sup>26,47</sup>

Counterintuitively, NF- $\kappa$ B, a transcription factor traditionally associated with inflammation, plays a unique protective role in the neonatal lung. Constitutive NF- $\kappa$ B expression is higher in the neonatal lung than in the adult lung, and inhibition of constitutive NF- $\kappa$ B activity decreases pulmonary endothelial cell survival and proliferation and impairs in vitro angiogenesis. NF- $\kappa$ B inhibition during the alveolar stage of lung development in mice reduces pulmonary capillary density and leads to alveolar simplification reminiscent of BPD.<sup>48</sup>

The Forkhead Box (Fox) family of transcription factors plays important roles in regulating expression of genes involved in cellular proliferation and differentiation. Newborn *foxf1*<sup>+/-</sup> mice with low Foxf1 levels die with defects in alveolarization and lung capillaries, whereas those with increased pulmonary Foxf1 levels survive and display normal adult lung morphology.<sup>34</sup> Endothelial-specific deletion of Foxf1 is embryonically lethal and results in fewer blood vessels in the lung.<sup>49</sup> In humans, FOXF1 haploinsufficiency is associated with alveolar capillary dysplasia with misalignment of pulmonary veins.<sup>50</sup>

EphrinB2 and its receptor EphB4 belong to the Eph/ephrin family, which is critically involved in cardiovascular development.<sup>51,52</sup> Expression of ephrinB2 and its receptor EphB4 peaks during the stage of intense lung vascular growth in the developing rat lung. EphrinB2 and EphB4 are expressed in cells that ultimately differentiate into arteries and veins, respectively, preceding the formation of morphologically distinct arteries and veins.<sup>53-55</sup> Expression of ephrinB2 and EphB4 is not restricted to arterial versus venous vessels until the canalicular stage.<sup>16,52</sup> In vivo ephrinB2 knockdown using intranasal small interfering RNA during the postnatal stage of alveolar development and vascular maturation arrests alveolar and vascular growth.<sup>56</sup> Mice homozygous for the hypomorphic knock-in allele *ephrinB2* <sup>$\Delta$ V/ $\Delta$ V</sup> die by 2 weeks of age with enlarged airspaces.<sup>57</sup>

### Axonal guidance cues (AGCs) contribute to lung angiogenesis

AGCs are molecules that guide the outgrowth of axons to their targets. Among these AGCs, members of the ephrin family also promote angiogenesis, cell migration, and organogenesis outside the nervous system. Thus, AGCs are also appealing candidates in guiding the outgrowth of secondary crests during alveolar development and repair.<sup>58</sup> Semaphorin 3, another molecule of the AGC

family, may have an important role in the orchestration of pulmonary vascular development during alveolarization. The congenital loss of semaphorin 3–neuropilin 1 signaling in transgenic mice resulted in disrupted alveolar-capillary interface formation and high neonatal mortality.<sup>59-62</sup>

### ROLE OF LUNG ENDOTHELIAL PROGENITOR CELLS DURING LUNG VASCULAR DEVELOPMENT

#### Endothelial cell function is important for lung growth

Administration of an anti-PECAM-1 antibody that inhibits endothelial cell migration, but not proliferation or survival in vitro, also impairs alveolarization in neonatal rats, without reducing endothelial cell content.<sup>63</sup> In the endothelium, platelet endothelial cell adhesion molecule (PECAM-1), in addition to being an adhesion molecule, affects the expression and activity of eNOS. Endoglin is an essential molecule for angiogenesis. Its expression and activity are compromised in the absence of PECAM-1.<sup>64</sup> Pulmonary capillary endothelial cells may also initiate and sustain alveologenesi by increasing MMP14 (matrix metalloproteinase 14)-dependent bioavailability of epidermal growth factor receptor ligands on the epithelial cells.<sup>65,66</sup>

#### Lung endothelial progenitor cells (EPCs)

An observation by Schwartz and Benditt<sup>67</sup> suggested that the presence of fast-growing endothelial niches within the aortic intima that were enriched with replication-competent cells was required to repopulate injured or senesced cells. More recently, large differences in proliferative potential have been documented between pulmonary macro- and microvascular endothelial cells,<sup>68</sup> with rat pulmonary microvascular endothelial cells proliferating twice as fast as rat pulmonary artery endothelial cells (PAECs). Thus, the pulmonary microcirculation seems to be enriched with EPCs that display vasculogenic competence while maintaining functional endothelial microvascular specificity.<sup>69</sup> These so-called resident microvascular endothelial progenitor cells (RMEPCs) are highly proliferative and express endothelial cell markers (CD31, CD144, eNOS, and von Willebrand factor) and progenitor cell antigens (CD34 and CD309) but not the leukocyte marker CD45. RMEPCs interact with *Griffonia simplicifolia*, display restrictive barrier properties, and possess vasculogenic capacity, as demonstrated in vitro and in vivo, forming ultrastructurally normal de novo vessels. Nucleosome assembly protein 1, incriminated in the control of cell growth, is preferentially expressed by RMEPCs and functions as an important regulator of the proliferative and vasculogenic endothelial cell phenotype.<sup>70</sup> These RMEPCs share features of human cord blood-derived endothelial colony-forming cells (ECFCs). Developing human fetal and neonatal rat lungs contain ECFCs with robust proliferative potential, secondary colony formation, and de novo blood vessel formation in vivo when transplanted into a matrigel plug under the skin of immunodeficient mice.<sup>71</sup> Furthermore, human fetal lung ECFCs exposed to hyperoxia in vitro and neonatal rat ECFCs isolated from hyperoxic alveolar growth-arrested lungs mimicking BPD proliferate less, show decreased clonogenic capacity, and form fewer capillary-like networks. These findings indicate

that impaired ECFC function contributes to the lack of lung repair and the persistence of arrested alveolar growth in BPD. Consequently, exogenous administration of human cord blood-derived ECFCs restores alveolar and lung vascular growth in hyperoxic rodents.<sup>71</sup> However, lung engraftment was low, suggesting that the effect of these exogenous ECFCs was through a paracrine effect. Accordingly, administration of cell-free ECFC-derived conditioned media exerted similar therapeutic benefits in the hyperoxia- and bleomycin-induced models of BPD in rodents.<sup>71,72</sup> Overall, these findings further support the crucial role of angiocrine-derived factors in lung repair and offers promising new therapeutic strategies to prevent/restore lung damage.

### DIVERSITY OF THE SMOOTH MUSCLE CELL (SMC) PHENOTYPE IN DEVELOPMENT

During development, SMCs undergo rapid proliferation, during which time the vessel wall acquires its complement of SMCs. Replication rates decrease as the animal matures, with quiescence of SMCs in the adult animal.<sup>73-75</sup> However, after injury to an adult artery, SMC replication rates increase, at least transiently, to levels similar to those exhibited during embryonic life.<sup>76</sup> Similarly, adult SMCs do not exhibit serum-independent growth and have lower growth responses to mitogen stimulation than fetal SMCs. In an observation that is consistent with high in vivo growth rates, several investigators have reported the isolation of unique fetal aortic SMCs that differ substantially from adult cells. These SMCs, isolated from rat pups, demonstrate a characteristic epitheloid morphology, secrete platelet-derived growth factor (PDGF), and express high levels of cytochrome P450 IA1, PDGF-b chain, and elastin mRNAs.<sup>77,78</sup>

An even more interesting cell has been isolated from earlier embryonic periods that exhibits an intrinsically activated growth phenotype, dependent on Akt and mTOR (protein kinase B and mechanistic target of rapamycin, respectively) signaling, apparently independent of autocrine secretion of growth factors. This cell is ultimately growth inhibited by the timed acquisition of the endogenously produced growth factor PTEN (phosphatase and tensin homolog).<sup>78</sup> In contrast, SMCs isolated in culture from uninjured adult aortas or carotid arteries show a spindle-shaped morphology, low to undetectable PDGF secretion, and greatly reduced cytochrome P450 IA1 and elastin mRNA expression. During normal development, SMCs demonstrate compositional changes in cytoskeletal and contractile protein expression.<sup>79</sup> Fetal and neonatal aortic SMCs express primarily nonmuscle  $\beta$ -actin, whereas adult SMCs express predominantly SMC-specific  $\alpha$ -actin isoforms.<sup>80</sup> A gradual increase in the number of desmin- and tropomyosin-positive aortic SMCs also occurs with increasing age.<sup>81</sup> A reversion from the adult to the fetal or neonatal phenotype is often observed for each of these parameters in SMCs in experimental intimal thickening, atherosclerotic plaques, and pulmonary hypertension.<sup>75,82,83</sup> These observations strongly suggest that SMCs undergo maturation from a fetal to an adult phenotype during normal development and that reexpression of “fetal-like” markers of cell phenotype occurs upon arterial injury or entry of SMCs into a growth phase. It is also clear that expression of fetal or less differentiated cell phenotypic charac-

teristics may have a significant impact on functional responses of the injured vessel wall.

An obvious question raised by these observations is, what is the response of the fetal and neonatal circulation (still undifferentiated) to injury? With regard to the pulmonary circulation, infants dying with persistent pulmonary hypertension often exhibit a striking distal extension of SMCs, thickening of the media and adventitia, and excessive accumulation of the matrix proteins in vessel walls that appear to be acquired over very short periods of time. The SMC and adventitial changes appear particularly malignant when compared to adult forms of pulmonary hypertension. In fact, numerous observations demonstrate that the proliferative and matrix-producing potential of the neonatal pulmonary circulation in response to injury exceeds that of the adult. For example, when exposed to hypoxic, hyperoxic, or toxic stimuli, neonatal animals develop more severe, more rapidly progressive, and potentially less reversible pulmonary hypertension and right ventricular hypertrophy than adult animals. Further, structural changes, including cell proliferation and matrix-protein accumulation, are more dramatic than those observed in adults.<sup>84-86</sup>

The observation that neonatal cells normally exist in a “synthetic” or “primed” state provides support for the notion that in neonates the potential for vascular cell growth is heightened.<sup>75</sup> Alterations in receptor expression, signal transduction (including protein kinase C [PKC]), proto-oncogene expression (including *c-fos* and *c-myc*), and growth factor production have been demonstrated during periods of rapid cell growth and differentiation and are consistent with the rapid proliferative response of the neonate to growth-promoting stimuli.<sup>77,87,88</sup> Matrix protein-producing genes for elastin and collagen demonstrate their greatest activity in late fetal and early neonatal life.<sup>89,90</sup> Tropoelastin mRNA expression, for example, is nearly completely suppressed in the adult.<sup>91</sup> Thus, the hypothesis has been raised that the capacity of the SMCs to respond to matrix-producing stimuli depends not only on local hormonal and cell-cell interactions but also on the developmental state of the cell. The possibility that the normal fetal-to-adult transition in vascular cell phenotype may fail to occur under certain conditions in the neonatal period has also been raised. The persistence of fetal-like characteristics of vascular wall cells could therefore contribute to the excessive growth and matrix-protein production observed under conditions of severe neonatal pulmonary hypertension.

### SITE-SPECIFIC HETEROGENEITY OF SMC PHENOTYPE IN VASCULAR WALL

In addition to the developmentally regulated differences in SMC phenotype, there is a growing body of literature documenting the existence of phenotypic heterogeneity within the resident SMC population of a given tissue at a specified developmental stage. It is becoming clear that SMCs in the pulmonary artery (and aorta) form a phenotypically heterogeneous, yet highly organized, cell population.<sup>92,93</sup> Distinct phenotypic differences have been documented in SMCs at specific locations and along the longitudinal axis of the pulmonary artery and aorta.<sup>93</sup> Marked differences in the production of elastin and collagen have been demonstrated in aor-

tic SMCs, with elastin production being greatest near the heart.<sup>94</sup> An inverse gradient for the expression of desmin has also been demonstrated.<sup>95</sup> These differences in the biosynthetic phenotype are stable in culture, suggesting the existence of multiple medial SMC phenotypes.

#### REGULATION OF SMC PROLIFERATION DURING DEVELOPMENT

Much of the available literature suggests that the bulk of vascular SMC replication during development occurs before birth. With regard to the SMCs, the active replicative state slows considerably in the perinatal period and then virtually ceases under normal conditions by adulthood. Therefore, an understanding of the factors and mechanisms responsible for cell proliferation in fetal and neonatal life and the mechanisms that suppress cell replication once morphogenesis is complete is crucial if we are to understand the proliferative response of the vascular SMC to injury.

Cook et al.<sup>74</sup> defined the distinct phases of SMC replication that occur during early vascular development. At the embryonic stage, the rat aorta is characterized by a very high *in vivo* replication rate, significant autonomous capabilities *in vitro*, and a generalized lack of responsiveness to exogenously added SMC mitogens. The capacity for autocrine growth *in vitro* appears to be lost between gestational days 18–20 in the fetal rat and appears to be correlated with a drop in *in vivo* replication rates just after the embryonic-fetal transition. Autocrine growth was due not to hyperresponsiveness of the cells to growth stimuli but rather to intrinsically controlled developmental timing mechanisms operating independently of exogenous growth factors.

In contrast, mechanisms involving growth factors are likely involved in the regulation of vascular cell growth in the neonatal period. While the autonomous growth capability observed in fetal cells has been lost, neonatal cells demonstrate enhanced growth capacity, compared to adult cells. Neonatal bovine pulmonary artery SMCs are more responsive than adult cells to several growth-promoting stimuli. Specifically, SMCs harvested from the lobar pulmonary artery of neonatal calves grow faster in serum-containing media than matched SMCs obtained from an adult. Although the neonatal cells lack autocrine growth capacity, they are more resistant to the induction of a quiescent state than their adult counterparts. This has been demonstrated by measuring basal [<sup>3</sup>H]-thymidine incorporation after 72 hours of serum deprivation.<sup>96</sup> In addition, these neonatal vascular cells also have enhanced mitogenic responses to growth factors such as insulin-like growth factor 1 (IGF-1) and phorbol myristate acetate, factors that activate different but potentially synergistic growth-promoting pathways.<sup>97,98</sup> The mechanisms responsible for these enhanced growth responses remain unknown but may involve expression of increased numbers of cell surface receptors specific for a particular ligand or increased ability to generate second messengers in response to growth factor binding. For instance, IGF-1 binding levels have been found to be much higher in near-term fetal and neonatal aortic SMCs than in the adult. IGF-2 binding activity is increased over IGF-1 levels in fetal cells but then falls below them in the postnatal period.<sup>99</sup> Thus, receptor expression for each rele-

vant growth factor may be uniquely regulated during development and could contribute to enhance neonatal responsiveness to growth stimuli.

Increased responsiveness of neonatal cells to phorbol esters, cell-permeable activators of PKC, is also intriguing, because in this case the cell surface receptors have been bypassed altogether. This signal transduction pathway has been shown to be important in the pulmonary artery SMC (PASMC) proliferative response to hypoxia, as well as in the hypertrophic response to mechanical loading, and is susceptible to development change.<sup>97,98,100,101</sup> In addition, increased expression of certain isoforms of PKC have been shown to cause increased responsiveness to growth factors and to promote cell differentiation.<sup>102</sup> A modest increase in whole-cellular PKC catalytic activity in neonatal versus adult bovine PASMCs has been shown.<sup>103</sup> More work on intrinsic differences in signaling pathways leading to enhanced growth responses in neonatal versus adult cells may lead to explanations of exaggerated growth responses to pathologic stimuli.

There may also be more subtle developmental differences in the growth responses of vascular cells to mitogenic signals. For instance, it has been demonstrated that neonatal rat aortic SMCs exhibit altered responses to transforming growth factor beta (TGF- $\beta$ ), a family of peptides capable of stimulating or inhibiting replication in adult SMCs. In neonatal SMCs, TGF- $\beta_1$  acts exclusively as a growth inhibitor. In addition, TGF- $\beta_1$  acts to significantly increase the size of these cultured neonatal rat cells. Adult cells do not demonstrate this increase in size in response to TGF- $\beta_1$ . Here, then, is an instance where the type, rather than the magnitude, of the response is uniquely altered in neonatal vascular cells.<sup>104,105</sup>

Differences in growth properties of neonatal bovine vascular cells, as compared to those of adult cells, can be further enhanced by application of hypoxic stress to experimental animals before harvesting. The vascular-wall SMCs subsequently obtained from hypoxia-exposed animals grow even faster in culture than their normoxia-exposed counterparts. The mechanisms of these “acquired” growth properties are unknown. They could represent a regression to the autocrine (exogenous growth factor-independent) growth properties of fetal vascular cells or a further increase in the as-yet-unidentified mechanisms that underlie enhanced neonatal cell responsiveness to mitogens. Here there is great interest in the possibility of epigenetic changes in these cells leading to this growth phenotype.

#### AGE-SPECIFIC REGULATION OF EXTRACELLULAR MATRIX EXPRESSION BY SMC

There is little question that a major function of the SMCs in the developing vessel is the production of connective tissue, particularly elastin and collagen. In large arteries, such as the aorta, elastin and collagen make up more than 70% of the dry weight of the vessel wall.<sup>106</sup> Thus, study of the mechanisms controlling elastin synthesis during normal vascular development, as well as the elastogenic response to injury in both the neonate and the adult, is crucial for a complete understanding of vascular growth in health and disease. In most mammalian tissues, the bulk of elastin production occurs during fetal and neonatal periods and is essentially complete by the first decade of life. For instance, the levels of tropoelastin mRNA parallel

the deposition of elastin in the developing bovine nuchal ligament.<sup>107</sup> In addition, the steady-state levels of tropoelastin mRNA correlate with the functional levels of the mRNA as determined by cell-free translation and immunoprecipitation, indicating pretranslational regulation. The temporal sequence of elastogenesis is unique. Elastic fibers are deposited during a defined developmental window and persist for the life of the organism. In contrast, many other extracellular matrix proteins are continually made and turned over.

Although factors that influence the initiation, maintenance, and cessation of elastogenesis *in vivo* are unknown, results obtained from *in vitro* models have demonstrated that various mediators affect tropoelastin expression, and these may have regulatory functions *in vivo*. Several mediators, including TGF- $\beta$ , IGF-1, and glucocorticoids, coordinately increase elastin synthesis and tropoelastin mRNA. In general, the stimulation effected by these mediators is about 2–4-fold above control levels.<sup>89,108</sup> Repressors of tropoelastin synthesis include epidermal growth factor,<sup>109</sup> recombinant interleukin 1,<sup>110</sup> 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>),<sup>111</sup> and phorbol esters.<sup>112</sup> Unlike mediators that stimulate production, factors and conditions that downregulate tropoelastin synthesis profoundly influence production and, with sufficient exposure, can completely repress tropoelastin expression. Because elastogenesis proceeds through defined stages, diverse regulatory mechanisms may be active during different periods of expression. Indeed, tropoelastin pre-mRNA undergoes age-specific alternative splicing, resulting in the translation of multiple distinct protein isoforms.<sup>113,114</sup> This may have particular importance in the early lung, since tropoelastin expression is also noted in alveolar tip cells and abrogated expression is associated with decreased alveolarization and vascular growth.<sup>115</sup>

The SMC response in lobar pulmonary arteries to hypertension reveals yet another example that elastin production is controlled by different means at various stages of development. Increased deposition of elastin is characteristic of the vascular remodeling seen in pulmonary hypertension in both newborns and adults, but the site of this increased production differs between the two groups. In neonates, when elastogenesis is ongoing, increased deposition is detected in the medial wall and is apparently a consequence of sustained fetal expression.<sup>116</sup> By maturity, tropoelastin expression in medial SMCs has ceased, but renewed production is evident in neointimal cells.<sup>117</sup> It is not clear why some SMCs in the adult arteries do not contribute to matrix remodeling associated with pulmonary hypertension, but this age-specific response suggests that not all adult SMCs have the capacity to reexpress tropoelastin.

### REGULATION OF VASOMOTOR TONE

During fetal life, the lung is fluid filled, oxygen tension is low, and pulmonary blood flow is limited, while pulmonary vascular resistance (PVR) exceeds systemic vascular resistance. Blood is shunted away from the lungs at the level of the ductus arteriosus (DA).<sup>3</sup> At birth, pulmonary blood flow increases 8–10-fold,<sup>118</sup> and pulmonary arterial pressure decreases to 50% of systemic levels within 24 hours after birth,<sup>119</sup> concomitant with an increase in oxygen tension. The critical physiologic stimuli that account for perinatal pulmonary vasodilatation include rhythmic distention of the lung<sup>120</sup> and an in-

crease in oxygen tension<sup>121–124</sup> and shear stress.<sup>125</sup> Data accumulated over the past several decades implicate distinct and complementary roles for both PAECs and PASMCs in promoting fetal lung growth and development and in mediating the postnatal adaptation of the pulmonary circulation.

In the human fetus at weeks 20 and 38 of gestation, blood flow to the lungs is 13% and 21% of biventricular output, respectively. Maternal hyperoxygenation has no effect on human fetal pulmonary blood flow between weeks 20 and 26 of gestation, but significant increases occur in the fetus from week 31 to week 36 of gestation, suggesting that the capacity of the pulmonary circulation to sense and respond to an increase in oxygen tension is developmentally regulated.<sup>122</sup> In fetal lambs at 0.4 and at 1.0 gestation, pulmonary blood flow is 3.7% and 7.0% of the combined cardiac output, respectively.<sup>123</sup> In near-term and term fetal lambs, pulmonary arteries and veins display vigorous responses to various vasoactive agents and changes in oxygen tension.<sup>121,124</sup> However, the acute pulmonary vascular dilator response to increased oxygen tension is not sustained during more prolonged exposure, suggesting the presence of developmental vasoregulatory mechanisms that oppose vasodilation in the fetus.<sup>121,125</sup>

With the onset of breathing in the human infant, pulmonary blood flow increases from 21% to 100% of cardiac output to enable exchange of gas in the lungs.<sup>122</sup> There are several vasoactive molecules that play a key role in modulating pulmonary vascular tone, including endothelium-derived nitric oxide (EDNO), prostaglandin I<sub>2</sub> (PGI<sub>2</sub>), endothelin 1 (ET-1), and platelet-activating factor (PAF). The role of each of these molecules in the perinatal pulmonary circulation is addressed below.<sup>3,126</sup>

### PULMONARY ARTERY ENDOTHELIAL CELL (PAEC)

The PAEC plays a critically important role in the developing pulmonary circulation, both during and after fetal life. Vasoactive products produced by the pulmonary endothelial cells simultaneously preserve and constrain fetal pulmonary blood flow. During fetal life, inhibition of ET-1, a vasoconstrictive protein produced by the endothelium, causes marked pulmonary vasodilation.<sup>127</sup> Conversely, pharmacologic inhibition of NO decreases pulmonary blood flow and increases pulmonary artery blood pressure.<sup>128</sup> These two observations suggest that carefully circumscribed pulmonary blood flow is of critical importance in normal pulmonary vascular development.

In response to key perinatal physiologic stimuli, the pulmonary endothelium elaborates vasoactive mediators such as NO, endothelin, and prostaglandins.<sup>127–130</sup> PAEC NO production is necessary<sup>131</sup> for the normal transition of the pulmonary circulation from a high- to a low-resistance circuit. Among the most important properties of the pulmonary circulation is the ability to sense and respond to alterations in oxygen tension. During air-breathing life, the pulmonary circulation adapts to respond to decreases in oxygen tension in such a manner as to match ventilation and perfusion and thereby mitigate hypoxemia. At birth, it is biologically imperative for the pulmonary circulation to respond to an acute increase in oxygen tension. Interestingly, the capacity of the pulmonary circulation to sense and respond to an acute increase in oxygen tension is develop-

mentally regulated. Not until gestation is approximately 85% complete is the fetal pulmonary circulation able to respond to an increase in oxygen tension.<sup>124</sup> Vasodilator agents that act through endothelial cell-mediated mechanisms produce only transient pulmonary vasodilation.<sup>121,132</sup> The relatively narrow developmental window wherein the fetal pulmonary circulation is able to respond to an increase in oxygen tension may serve a protective purpose, as either too much or too little pulmonary blood flow compromises pulmonary vascular development.<sup>133,134</sup>

The capacity of PAECs to produce NO significantly enhances the ability of the pulmonary circulation to dilate in response to increasing oxygen tension with maturation.<sup>135</sup> By the end of gestation, the pulmonary circulation is exquisitely sensitive to alterations in oxygen tension, as an increase of only 8 Torr results in a 3-fold increase in pulmonary blood flow.<sup>136</sup> Several investigators have demonstrated that production of NO by the endothelial cells mediates oxygen-induced pulmonary vasodilatation.<sup>128,137</sup> In a confluent monolayer of fetal PAECs, an acute increase in oxygen tension directly increases cytosolic calcium. In the PAEC, an acute increase in oxygen tension results in membrane depolarization and an increased rate of calcium entry. The sustained and progressive increase in PAEC intracellular calcium concentration ( $[Ca^{2+}]_i$ ) entails calcium release from intracellular stores. The observation that acetylcholine causes a greater increase in PAEC cytosolic calcium under hypoxic than under normoxic conditions provides inferential evidence that the low-oxygen-tension environment of the normal fetus may ready the perinatal pulmonary circulation to respond to an acute increase in oxygen tension by augmented loading of intracellular stores. In PAECs and lung tissue derived from fetal lambs with chronic uterine pulmonary hypertension, the response of the pulmonary circulation to vasodilator stimuli is attenuated. Expression of eNOS and NO production are decreased, characterized by impaired pulmonary vasodilation at delivery.<sup>138</sup> PAECs derived from this ovine model of perinatal pulmonary hypertension of the newborn (PPHN) do not respond to an acute increase in oxygen tension with either an increase in  $[Ca^{2+}]_i$  or NO production, and they grow and form tubes poorly *in vitro*.<sup>139</sup> These findings suggest that intrauterine events, including sustained hemodynamic stress, cause sustained endothelial cell dysfunction in the early perinatal period.

The response of the PAEC to birth-related physiologic stimuli is critically important for postnatal adaptation of the pulmonary circulation. PAECs must contract and increase production of NO<sup>131,140</sup> and prostacyclin,<sup>141,142</sup> as well as decrease endothelin production,<sup>143</sup> in a remarkably short time frame to facilitate the transition of the neonatal pulmonary circulation from high to low tone and thereby enable effective gas exchange to occur in the lungs. Shear stress is an essential physiologic stimulus that prompts PAECs to increase NO production. In the chronically instrumented fetal lamb model, pharmacologic inhibition of NO production prevents shear-stress-induced pulmonary vasodilatation.<sup>128</sup> The increase in fluid shear stress after birth resulting from increased pulmonary blood flow also increases NO production by altered phosphorylation of eNOS and increased eNOS expression. In primary cultures of

ovine fetal PAECs, shear stress activates eNOS through reduction in phosphorylation of eNOS at Thr495 and increased phosphorylation at Ser1177.<sup>143,144</sup> A complex consisting of PECAM-1, vascular endothelial cadherin, and VEGFR-2 is localized at the gap junction of endothelial cells, serving as a mechanotransducer for shear stress. PECAM-1 is activated by shear stress, resulting in a Src family kinase-mediated, ligand-independent transactivation of VEGFR-2. The activation of VEGFR-2 may activate eNOS through Akt-dependent phosphorylation of Ser1177. Activation of VEGFR-2 can also cause Ser1177 phosphorylation via PKA.<sup>145</sup>

In fetal PAECs, acute shear stress initially upregulates eNOS expression that may be due to increased phosphorylation of c-Jun<sup>146</sup> and reduced binding of STAT3 to the eNOS promoter.<sup>147</sup> Heat shock protein 90 (Hsp90), a constitutively expressed molecular chaperone, provides an additional level of control for endothelial cell NO production. Hsp90 associates with eNOS to facilitate eNOS activation. Hsp90 also recruits activated Akt in the eNOS complex and maintains Akt activity, both by preventing proteosomal degradation of upstream PI3K and by inhibiting dephosphorylation of Akt by phosphatase 2A, thereby enhancing Akt-mediated activation of eNOS (Fig. 2).<sup>148</sup> In resistance pulmonary arteries of the piglet, the physical interactions between Hsp90 and eNOS mature over the first weeks of life.<sup>149</sup> In isolated perfused lungs of newborn piglets, Hsp90 inhibition reduces eNOS-Hsp90 interaction and NO production and thereby attenuates acetylcholine-induced pulmonary vasodilatation.<sup>150</sup> Moreover, decreased association of Hsp90 with eNOS occurs in pulmonary hypertension induced by prenatal ductal ligation in fetal lambs. As a result of the diminished interaction, NO release is impaired, as is the vasodilator response to adenosine triphosphate (ATP), an important mediator of perinatal pulmonary vasodilatation.<sup>151</sup>

Pulmonary endothelial cells produce the prostaglandin molecules PGI<sub>2</sub> and PGE<sub>2</sub>, both of which are potent vasodilators. Substantial evidence suggests that these molecules play a meaningful role in modulating fetal and neonatal pulmonary vascular tone. In isolated resistance pulmonary arteries and veins of term fetal lambs, the cyclooxygenase inhibitor indomethacin has no effect on arteries under low oxygen tension but causes contraction of arteries under higher oxygen tensions, suggesting that oxygen may regulate the synthesis of the dilator prostanoids.<sup>152</sup> PGI<sub>2</sub> production has been found to be more sensitive to the inhibitory effect of hypoxia in fetal ovine pulmonary arteries and veins than in those of adult sheep.<sup>153</sup> In fetal goats and lambs, ventilation-induced pulmonary vasodilatation is associated with increased production of PGI<sub>2</sub>. Despite pharmacologic inhibition of cyclooxygenase, PVR decreased with the onset of air-breathing life, indicating that while PGI<sub>2</sub> is involved in the decrease in PVR at birth, it is not absolutely required.<sup>129,130,141</sup> The decrease in PVR caused by PGI<sub>2</sub> at birth is modest in comparison to that induced by EDNO.<sup>154</sup>

Vasodilatation induced by PGI<sub>2</sub> and PGE<sub>2</sub> is mainly mediated by cyclic adenosine monophosphate (cAMP), which is elevated after the activation of adenylyl cyclase (AC). Forskolin, a direct AC stimulator, caused a greater relaxation of pulmonary veins in newborn than in fetal lambs, whereas 8-Br-cAMP induced similar



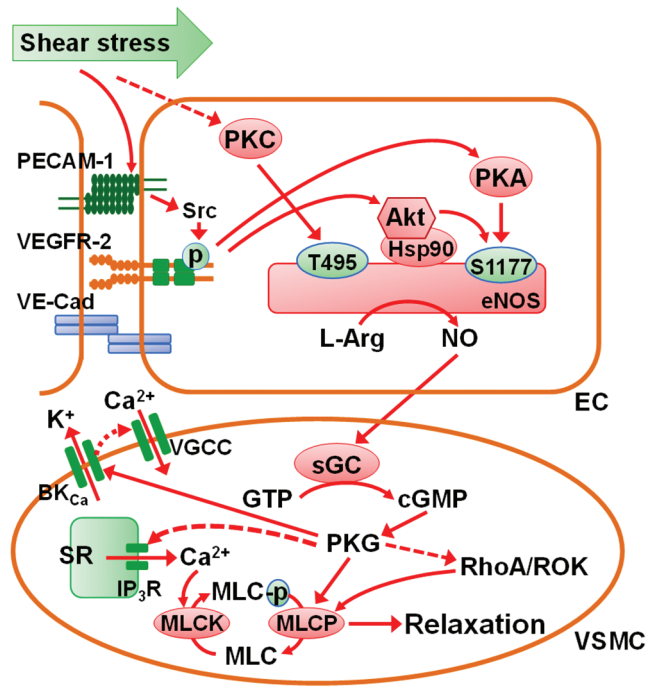


Figure 2. Mechanism for shear stress-induced activation of eNOS and NO-mediated relaxation of vascular smooth muscle cells (VSMCs). A complex consisting of PECAM-1, VE-cadherin, and VEGFR-2 localized at cell-cell junctions serves as a mechanotransducer for shear stress. The tension exerted by shear stress on PECAM-1 activates a Src family kinase, resulting in ligand-independent transactivation of VEGFR-2, followed by activation of Akt (also known as protein kinase B), phosphorylation of serine 1177 (S1177) of eNOS, and production of NO. Activation of VEGFR-2 can also cause S1177 phosphorylation via PKA. Heat shock protein 90 (Hsp90), when associated with eNOS, can recruit Akt into the eNOS complex and maintains Akt activity. In contrast to S1177, the phosphorylated threonine 495 (T495) of eNOS exerts inhibitory effect on the enzyme. T495 can also be phosphorylated by PKC. Shear stress may enhance eNOS activity by inhibiting PKC activity. NO released from the endothelial cells (ECs) can readily diffuse into the underlying VSMCs, where it activates sGC, resulting in the conversion of guanosine triphosphate (GTP) to cGMP (cyclic guanosine monophosphate). The elevated cGMP causes vasodilatation, primarily via PKG, by reducing the intracellular  $Ca^{2+}$  concentration and decreasing the sensitivity of myofilaments to  $Ca^{2+}$ . PKG stimulates  $Ca^{2+}$ -activated  $K^+$  ( $BK_{Ca}$ ) channels, resulting in membrane hyperpolarization and suppression of  $Ca^{2+}$  entry. PKG also reduces the release of  $Ca^{2+}$  from sarcoplasmic reticulum (SR) into cytosol by inhibiting  $IP_3$  receptor ( $IP_3R$ ) activity. The decreased cytosolic  $Ca^{2+}$  concentration leads to diminished activity of myosin light-chain kinase (MLCK), decreased phosphorylation of myosin light chain (MLC), and reduced contractility. PKG causes reduced  $Ca^{2+}$  sensitivity of myofilaments by directly stimulating the activity of myosin light-chain phosphatase (MLCP) as well as interfering with the inhibitory action of RhoA/Rho kinase (ROK) signaling on MLCP. These actions leads to increased dephosphorylation of MLC and thus relaxation of VSMCs. Solid arrows indicate activation and dashed arrows inhibition. eNOS: endothelial

responses in vessels of both ages. Furthermore, the stimulated AC activity is greater in newborn than in fetal veins. Hence, the greater response of pulmonary veins of the newborns may in part be attributable to AC activity greater than that in the fetal vessels.<sup>155</sup>

Recent data demonstrate that RhoA, a member of the Rho family of small GTPases,<sup>156</sup> plays a significant role in determining vascular tone,<sup>157</sup> cell contraction, migration, cell permeability,<sup>158</sup> gene expression, and differentiation.<sup>159,160</sup> RhoA activation occurs with GTP (guanosine triphosphate) association and translocation of the molecule to the plasma membrane where Rho kinase (ROK) is stimulated.<sup>161</sup> RhoA activation can result via a G-protein-coupled receptor or via tyrosine kinases. In the pulmonary vasculature, hypoxia activates RhoA.<sup>157,162</sup> RhoA activation increases  $Ca^{2+}$  sensitivity of the contractile myofilaments in vascular SMCs, in part, through inhibition of light-chain phosphatase. Inhibition of myosin light-chain phosphatase results from phosphorylation of the myosin-binding subunit of myosin light chain phosphatase (MYPT-1) or the myosin light chain phosphatase inhibitor protein CPI-17.<sup>163</sup> Phosphorylation of CPI-17 by ROK may be more significant in sustained vasoconstriction than is phosphorylation of MYPT-1.<sup>164,165</sup>

During fetal life, ROK activity maintains high PVR in the fetal lung.<sup>166</sup> Moreover, ROK inhibition causes NO-independent perinatal pulmonary vasodilatation. Because compromised NO production plays a role in PPHN, ROK inhibition might provide an additional therapeutic tool. ROK inhibition increases PAEC NO production and barrier function of endothelial cell monolayers and restores growth and tube formation of PAECs from experimental PPHN in vitro.<sup>139,167</sup> These findings suggest that, in addition to its role in SMCs, ROK activity further modulates endothelial cell growth and functions in the normal developing lung and PPHN.

The consequences of an attenuated response of endothelial cells to an acute increase in oxygen tension are significant as blood shunts away from the lungs, resulting in severe central hypoxemia that is unresponsive to high concentrations of inspired oxygen.<sup>168</sup> Recent data provide clear evidence that inadequate production of NO and relatively increased production of endothelin play an etiologic role in PPHN.<sup>169</sup> The observation that endothelial cells from an animal model of PPHN demonstrate no increase in either  $[Ca^{2+}]_i$  or only a minimal increase in NO production in response to an acute increase in oxygen tension provides the first direct evidence that chronic intrauterine pulmonary hypertension compromises endothelial cell oxygen sensing and NO production.<sup>138</sup> Moreover, the observation that acetylcholine caused a greater increase in cytosolic calcium in PAECs from hypoxic than in those from normoxic lambs suggests that the normally low oxygen tension environment of the developing pulmonary circulation prepares the lung for extrauterine,

nitric oxide synthase;  $IP_3R$ : inositol 1,4,5-trisphosphate receptor; L-Arg: L-arginine; NO: nitric oxide; PECAM-1: platelet endothelial cell adhesion molecule; PKA: protein kinase A; PKC: protein kinase C; PKG: cGMP-dependent protein kinase; sGC: soluble guanylyl cyclase; Src: tyrosine-protein kinase CSK; VGCC: voltage-gated  $Ca^{2+}$  channel; VE-cad: vascular endothelial cadherin; VEGFR-2: vascular endothelial growth factor receptor 2.

air-breathing life, providing a mechanistic link between endothelial cell cytosolic calcium and an acute increase in oxygen tension, one of the central perinatal physiologic stimuli at birth.<sup>118</sup>

The observation that fetal PAECs maintained in culture respond to an acute increase in oxygen tension with an increase in cytosolic calcium, in direct contrast to the response of PAECs derived from the adult pulmonary vasculature,<sup>170,171</sup> lends weight to the proposition that maturation-related differences in the pulmonary circulation extend not only to the molecular expression but also to the physiology of individual cellular constituents. Further, the observation that PAECs derived from fetal lambs with chronic intrauterine pulmonary hypertension do not respond to an acute increase in oxygen tension with either an increase in cytosolic calcium or increased NO production<sup>138</sup> provides still further evidence of a link, albeit an inferential one, between an acute increase in oxygen tension and NO production in fetal PAECs.

Thus, in the perinatal pulmonary circulation, PAECs are able to sense and respond to an acute increase in oxygen tension with an increase in cytosolic calcium. The acute increase in oxygen tension leads to membrane depolarization and an increase in the rate of extracellular calcium entry. Augmented entry of extracellular calcium leads to calcium release from inositol triphosphate (IP<sub>3</sub>)-sensitive intracellular calcium stores. The sustained elevation of PAEC calcium results, in part, from release of calcium from ryanodine-sensitive stores. From a teleologic perspective, the response of the PAECs to an acute increase in oxygen tension is a biologic imperative. Compromised PAEC oxygen sensing may underlie the attenuated response of the pulmonary circulation to perinatal vasodilator stimuli, thereby leading to PPHN.

### CYCLIC GMP SIGNALING

NO causes pulmonary vasodilatation, primarily via activation of soluble guanylate cyclase (sGC). NO binds to the heme moiety of sGC, thereby inducing a conformational change that increases cGMP production from GTP by approximately 200-fold.<sup>172,173</sup> In pulmonary arteries of piglets, the molecular expression of the sGC  $\beta_1$  subunit increases with age in a manner that parallels the increasing response to NO.<sup>174</sup> Moreover, pulmonary sGC activity stimulated by sodium nitroprusside, a NO donor, is approximately 7-fold greater in 1- and 8-day-old rats than in adult rats.<sup>175</sup>

PKG (cGMP-dependent protein kinase) is the primary enzyme responsible for vasodilatation caused by NO-cGMP activity. In vascular SMCs, relaxation is contingent on activation of the type I isoform (PKG-I).<sup>176,177</sup> Calcium-sensitive potassium (BK<sub>Ca</sub>) channels are an important phosphorylation target of PKG,<sup>178,179</sup> and the activation of PKG by NO-cGMP signaling seems to be sufficient to activate BK<sub>Ca</sub> channels.<sup>180</sup> Reduced PKG-I expression may contribute to the development of nitrate tolerance, a condition wherein the vascular sensitivity to nitrovasodilators is diminished.<sup>181,182</sup> The capacity to induce cGMP-mediated relaxation in pulmonary arteries increases with maturation.<sup>183,184</sup>

In response to sustained exposure to normoxia, the expression and activity of PKG that favor diminished tone in PSMCs normally increase.<sup>185</sup> Experimental evidence indicates that PKG has three distinct effects that mediate diminished SMC tone. First, receptor phos-

phorylation by PKG enables ryanodine-sensitive calcium stores to produce a local and quantal release of calcium, resulting in BK<sub>Ca</sub> channel opening, membrane hyperpolarization, and subsequent closure of calcium channels to decrease cytosolic calcium.<sup>178,186</sup> Second, PKG directly activates the BK<sub>Ca</sub> channel through phosphorylation at serine 1072 of the alpha chain.<sup>187,188</sup> Third, PKG may inhibit the voltage-operated calcium channel (VOCC) by direct phosphorylation of the channel or by PKG-induced activation of a phosphatase.<sup>189</sup> In addition to the effects on PKG-I expression, sustained normoxia decreases expression of PASM C BK<sub>Ca</sub> and VOCCs.<sup>190</sup>

Evidence from studies in fetal and newborn ovine pulmonary vessels indicates that cGMP-mediated relaxation is primarily mediated by PKG in the perinatal period.<sup>191-193</sup> Furthermore, PKG activity is regulated by oxygen tension. Relaxation of pulmonary arteries and veins of term fetal lambs to 8-Br-cGMP, a cell-permeable cGMP analog, is greater after exposure for 4 hours to normoxia (partial pressure of oxygen [P<sub>O<sub>2</sub>]: 140 mmHg) than after exposure to hypoxia (P<sub>O<sub>2</sub>: 30 mmHg). The decreased relaxation of pulmonary veins to cGMP in hypoxia may result from reduced expression of PKG protein and mRNA as well as posttranscriptional modification of PKG by peroxynitrite and other reactive oxygen species, specifically in veins.<sup>193,194</sup> Acute hypoxia also induces an accumulation of ubiquitinated PKG-I in ovine fetal and newborn PSMCs. Such a modification was not evident in ovine fetal systemic (cerebral) artery SMCs. Ubiquitinated PKG-I $\alpha$  was unable to bind the cGMP analog 8-(2-aminoethyl)thioguanosine-3',5' (AET)-cGMP, a ligand for the unmodified protein, and reoxygenation reversed the acute hypoxia-induced accumulation of ubiquitinated PKG-I. PKG-I ubiquitination induced by acute hypoxia appears to play a unique role in the regulation of the pulmonary vascular SMC vasoreactivity and relaxation mediated by the NO-cGMP-PKG-I pathway.<sup>195</sup></sub></sub>

### ROLE OF THE CALCIUM-SENSITIVE K<sup>+</sup> CHANNEL IN THE PERINATAL LUNG

While physical factors and vasoactive products elaborated by the pulmonary vascular endothelium are involved in the regulation of perinatal pulmonary vascular tone,<sup>3,131</sup> sustained and progressive perinatal pulmonary vasodilatation requires pulmonary vascular K<sup>+</sup> channel activation. PASM C K<sup>+</sup> activation causes membrane hyperpolarization, closure of VOCCs, a decrease in cytosolic calcium, and vasodilatation.<sup>196</sup> Data demonstrate that activation of BK<sub>Ca</sub> channels in the pulmonary vasculature plays a critical role in perinatal pulmonary vasodilatation. The first definitive evidence that BK<sub>Ca</sub> channel activation mediates O<sub>2</sub>-induced fetal pulmonary vasodilatation came from studies in acutely instrumented late-gestation ovine fetuses.<sup>197</sup> O<sub>2</sub>-induced fetal pulmonary vasodilatation was blocked by either tetraethylammonium (TEA), a K<sup>+</sup> channel blocker, or iberiotoxin, a specific BK<sub>Ca</sub> channel antagonist, but was unaffected by glibenclamide, a blocker of the K<sub>A,TP</sub> channel, suggesting that O<sub>2</sub> causes fetal pulmonary vasodilatation through BK<sub>Ca</sub> channel activation.<sup>196,197</sup> Inhibitors of either guanylate cyclase- or cyclic nucleotide-dependent kinases also attenuated O<sub>2</sub>-dependent fetal pulmonary vasodilatation, implying that elevated fetal O<sub>2</sub> acts to increase guanylate cyclase activity and cGMP concentration and to activate cyclic nucleotide-dependent kinases, causing BK<sub>Ca</sub> channel activation and

vasodilatation.<sup>197</sup> Vasodilatation of perinatal pulmonary arteries caused by ventilation was inhibited by TEA.<sup>198</sup> This observation implies that ventilation causes sustained and progressive perinatal pulmonary vasodilatation through activation of TEA-sensitive K<sup>+</sup> channels. NO causes perinatal pulmonary vasodilatation, in part, through activation of the BK<sub>Ca</sub> channel.<sup>199</sup> Even shear stress-induced fetal pulmonary vasodilatation, induced by compression of the DA during fetal life, requires BK<sub>Ca</sub> and voltage-dependent K<sup>+</sup> channel activation.<sup>125</sup>

### Ontogeny of the pulmonary vascular K<sup>+</sup> channel

While the BK<sub>Ca</sub> channel is crucially important in the perinatal pulmonary circulation, its importance seems to decrease with maturation. The observation that the K<sup>+</sup> channel setting resting membrane potential (RMP) in the pulmonary circulation changes after birth from a BK<sub>Ca</sub> to a voltage-gated K<sup>+</sup> (K<sub>v</sub>) channel suggests developmental regulation of K<sup>+</sup> channels in the pulmonary circulation.<sup>200</sup> In coordination with a maturation-related decrease in BK<sub>Ca</sub> channel expression and activation, the K<sub>v</sub> channel expression increases with maturation. There is relatively more K<sub>v</sub>2.1 channel protein and message in the adult than in the fetal and neonatal pulmonary circulation. The increase in the K<sub>v</sub>2.1 channel parallels the increasing capacity of PSMCs to sense and respond to acute decreases in oxygen tension as, in response to acute hypoxia, [Ca<sup>2+</sup>]<sub>i</sub> increases more rapidly and to a greater degree in adult than in fetal PSMCs.<sup>201</sup> The relatively greater abundance of the K<sub>v</sub>2.1 channel in the adult may represent an adaptation that allows the pulmonary circulation to respond to a specific physiologic signal that is relevant to a particular developmental stage. Reports that the O<sub>2</sub> sensor in the adult PSMC is a K<sub>v</sub> channel are consistent with this view,<sup>202</sup> since the K<sub>v</sub> channel is inactivated by acute hypoxia, causing PSMC depolarization, opening of VOCCs, an increase in [Ca<sup>2+</sup>]<sub>i</sub>, and vasoconstriction. Thus, the maturation-related increase in hypoxic pulmonary vasoconstriction that has been previously reported might derive from the parallel increases in K<sub>v</sub>2.1 channel activity, protein, and message with maturation.<sup>203,204</sup>

In contrast to pulmonary vascular K<sub>v</sub> channel expression and activity, pulmonary vascular BK<sub>Ca</sub> activity and expression decrease with maturation. Data from both pulmonary arteries and PSMCs in primary culture indicate that BK<sub>Ca</sub> channel expression is greatest in the fetal lung and decreases with maturation.<sup>205</sup> Indeed, the physiology of pulmonary vascular SMCs is consistent with the molecular biology. In fetal PSMCs, RMP is determined by the BK<sub>Ca</sub> channel, while in adult PSMCs, K<sub>v</sub> channel activity determines RMP.<sup>200</sup> In fetal PSMCs, an acute increase in oxygen tension results in membrane hyperpolarization and a decrease in PSMC [Ca<sup>2+</sup>]<sub>i</sub> and has no effect on adult pulmonary arterial membrane potential or cytosolic calcium.<sup>205</sup> Taken together, these observations indicate that the developmental regulation of BK<sub>Ca</sub> channel expression allows for the fetal PSMCs to be uniquely well adapted to respond to an acute increase in oxygen tension with a decrease in [Ca<sup>2+</sup>]<sub>i</sub> and vasorelaxation.

### Hypoxia-inducible factor-1 (HIF-1) modulates BK<sub>Ca</sub> channel expression

HIF-1, a key transcription factor that is central to oxygen homeostasis, enables the cell to respond to changes in oxygen availability,

an essential response in many developmental, physiological, and pathological processes. Semenza<sup>206</sup> and others have shown that HIF-1 regulates the transcription of many genes involved in the cellular and systemic response to oxygen availability, including genes involved in angiogenesis (i.e., VEGF), oxygen transport (i.e., erythropoietin), and energy metabolism (i.e., glycolytic enzymes). HIF-1 is also a key player in pathophysiological processes such as cancer, because hypoxic microenvironments in a tumor trigger expression of angiogenic genes that promote the growth of the newly vascularized tumor.<sup>206,207</sup>

From a developmental perspective, the transition from the relatively low-oxygen-tension intrauterine environment to air-breathing life entails an unprecedented and absolutely required response to an increase in oxygen availability. The physiologically low-O<sub>2</sub> environment of the fetus might be essential for fetal vascular growth and lung morphogenesis<sup>206</sup> as well as other early embryo developmental programs.<sup>208</sup> Because HIF-1 is a master regulator of the oxygen response, it follows that the molecular mechanisms responsible for fetal life and its transition to newborn life might involve it. Under normoxic conditions, the  $\alpha$  chain of HIF-1 (HIF-1 $\alpha$ ) is eliminated by degradation, rendering HIF-1 inactive. In low-O<sub>2</sub> environments, HIF-1 forms a dimer, is stabilized, and can translocate to the cell nucleus, thereby promoting transcription of hypoxia-sensitive genes.<sup>209</sup>

Deferoxamine mesylate (DFX) is an iron chelator that prevents HIF-1 $\alpha$  degradation. Normoxic fetal PSMCs treated with DFX increased expression of the  $\alpha$  subunit of the BK<sub>Ca</sub> channel, implying that hypoxia may upregulate BK<sub>Ca</sub> channel expression via HIF-1.<sup>210</sup> In human PSMCs, knockdown of HIF-1 $\alpha$ , a master transcriptional factor for the hypoxic response, using a short hairpin RNA plasmid blocked the hypoxic induction of BK<sub>Ca</sub>  $\beta$ 1-subunit expression.<sup>211</sup> Considering that HIF-1 $\alpha$  is more stable and less prone to destruction in fetal and neonatal PSMCs than in adult PSMCs,<sup>212</sup> it is possible that the greater activity of BK<sub>Ca</sub> channels in perinatal pulmonary vasculature could be partially ascribed to a high level of HIF-1 $\alpha$ . In contrast to BK<sub>Ca</sub> channels, the expression of K<sub>v</sub> channels is downregulated by HIF-1 $\alpha$  in PSMCs of the rat and mice. This effect is indirect, resulting from HIF-1-mediated upregulation of ET-1 and the subsequent ET-1-mediated suppression of its expression (Fig. 3).<sup>213</sup> Changes in oxygen tension at birth might signal an important transcriptional adaptation of the fetus to the air-breathing life of the newborn infant. Resnik et al.<sup>212</sup> investigated the role of HIF-1 in that transition and found that expression of HIF-1 $\alpha$  is developmentally regulated, as is the expression of the prolyl and asparagyl hydroxylases and other competing factors that control HIF-1 $\alpha$  expression and activity. The well-coordinated developmental regulation of the components of the HIF-1 pathway in the fetus suggests that the fetus represents a particular transcriptional paradigm that, in response to differential oxygen availability, produces a specific physiological and developmental response.

### PLATELET-ACTIVATING FACTOR (PAF)

PAF, a molecule that modulates vascular tone, was originally described as a soluble factor involved in leukocyte-dependent histamine and serotonin release from platelets.<sup>214</sup> In 1972, Benveniste et al.<sup>215</sup> demonstrated that this soluble factor was also released from

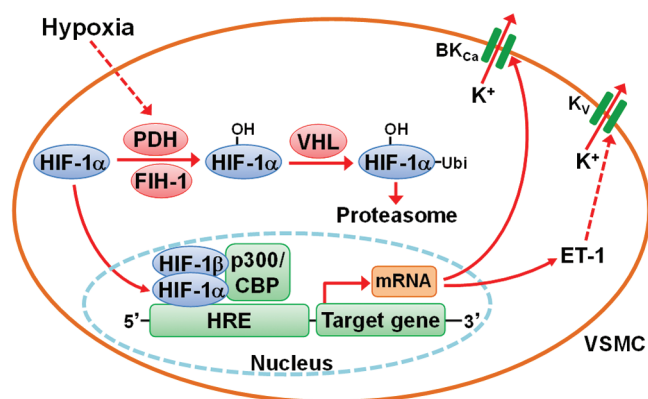


Figure 3. Role of hypoxia-inducible factor-1 (HIF-1) on the expression of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  ( $\text{BK}_{\text{Ca}}$ ) and voltage gated  $\text{K}^+$  ( $\text{K}_v$ ) channels in pulmonary arterial smooth muscle cells (PASMCs). In mammalian cells, including PASMCs, prolyl hydroxylase domain-containing enzyme (PHD) and factor inhibiting HIF-1 (FIH-1) act as cellular oxygen sensors and control the abundance of the  $\alpha$  isoform of HIF-1 ( $\text{HIF-1}\alpha$ ). Under normoxia,  $\text{HIF-1}\alpha$  is hydroxylated by PHD and FIH-1 and ubiquitinated by the von Hippel-Lindau protein (VHL), resulting in proteasomal degradation. The activities of PHD and FIH-1 depend on the presence of oxygen, and hence these enzymes are inhibited under hypoxic conditions, leading to reduced degradation of  $\text{HIF-1}\alpha$ . Consequently,  $\text{HIF-1}\alpha$  is translocated into the nucleus, dimerizes with the  $\beta$  isoform of HIF-1 ( $\text{HIF-1}\beta$ ), forms a complex with the transcriptional coactivator p300/CBP and binds to hypoxia-responsive elements (HRE) of HIF target genes to induce the expression of  $\text{BK}_{\text{Ca}}$ . HIF also promotes the expression of ET-1, while increased ET-1 suppresses the expression of  $\text{K}_v$  channels. Solid arrows indicate activation and dashed arrows inhibition. CBP: cAMP-response element-binding protein (CREB)-binding protein; ET-1: endothelin 1; Ubi: ubiquitin.

rabbit basophils after immunoglobulin E stimulation and coined the term “PAF.” Multiple cell types release PAF in response to inflammatory stimuli such as angiotensin II, thrombin, histamine, leukotrienes, or tumor necrosis factor  $\alpha$ .<sup>216</sup> Most cells that produce PAF also have PAF receptors and are, therefore, PAF targets.<sup>217</sup> PAF acts via specific receptors on responsive cells.<sup>218</sup> PAF receptor binding leads to activation of phospholipase C and consequently increased production of  $\text{IP}_3$  and diacylglycerol. The elevated  $\text{IP}_3$  stimulates  $\text{Ca}^{2+}$  release from the intracellular calcium stores while the increased diacylglycerol level causes PKC activation and enhanced sensitivity of myofilaments to  $\text{Ca}^{2+}$ . These events evoke or augment vasoconstriction.<sup>219</sup>

The hemodynamic effects of PAF are contingent on both anatomical site and developmental stage. In fetal lambs, PAF receptor blockade reduces PVR more than systemic vascular resistance, suggesting that PAF plays a role in the maintenance of the high-tone fetal pulmonary vasculature.<sup>220</sup> PAF levels are higher in fetal than in adult lung and plasma.<sup>221</sup> Moreover, hypoxia augments PAF receptor expression and PAF levels decrease after birth.<sup>222</sup> Hypoxia serves to potentiate the release of calcium from  $\text{IP}_3$ -sensitive stores.<sup>223</sup>

Within 90 minutes after birth, plasma PAF levels decrease in newborn lambs, perhaps because of upregulation of PAF-acetylhydrolase (PAF-Ah) activity and mRNA expression.<sup>221</sup> PAF-Ah inactivates

PAF and thus reduces its vasoconstrictive action. In PASMCs and in the whole lung, PAF-Ah activity is augmented by oxygenation.<sup>221</sup> Elevated cGMP and cAMP levels resulting from oxygen-induced release of EDNO and  $\text{PGI}_2$  may further decrease PAF receptor binding and PAF-stimulated  $\text{IP}_3$  production in a PKG-dependent manner.<sup>224</sup> These developmental changes likely facilitate the postnatal adaptation of the pulmonary circulation.

### ROLE OF VEINS IN REGULATION OF PULMONARY CIRCULATION

In the past, pulmonary veins have been viewed as conduit vessels similar to those in the systemic circulation; however, over the past 2 decades, a large amount of evidence has accumulated to indicate that pulmonary veins can exhibit substantial vasoactivity and contribute to the regulation of total pulmonary vasomotor tone and resistance.<sup>3,225</sup> In the fetus, pulmonary veins contribute a significant fraction to total PVR. Studies show that the veins may play a more important role in modulating the pulmonary circulation in the fetus and the newborn than in the adult.<sup>226-229</sup> It is not clear why, teleologically, there is a need for an increased venous resistance and venous reactivity in the lungs during the perinatal period; however, venous reactivity does appear to be at a maximum in the fetal lung. In perinatal sheep, not only acetylcholine (an EDNO-dependent vasodilator) but also exogenous NO causes a greater increase in the intracellular content of cGMP and relaxation in pulmonary veins than in arteries.<sup>3</sup> At birth, the veins, as well as the arteries, relax in response to EDNO and dilator prostaglandins, thereby assisting in the fall in PVR. These effects are oxygen dependent and modulated by PKG. In a number of species, including the human, pulmonary veins are also the primary sites of action of certain vasoconstrictors, such as endothelin<sup>230</sup> and thromboxane.<sup>231</sup> In various pathological conditions, there is an increased synthesis of these vasoactive agents that may lead to pulmonary venous constriction, increased microvascular pressures for fluid filtration, and formation of pulmonary edema. The significant role of veins in regulation of the pulmonary circulation must be appreciated to better prevent, diagnose, and treat lung disease.

### DUCTUS ARTERIOSUS (DA)

Closure of the DA at birth is a necessary step for the normal transition from fetal to air-breathing life.<sup>232</sup> Previous studies have demonstrated that an acute increase in  $P_{\text{O}_2}$  causes an increase in tension of DA rings. In rabbit DA SMCs, acute normoxia inhibits the  $\text{K}^+$  current, leading to membrane depolarization.<sup>233-235</sup> Pharmacologic blockade of the  $\text{K}_v$  channels mimics the effect of acute normoxia. Patent DA is a common complication of extreme prematurity. Preterm rabbit DAs display reduced  $\text{O}_2$  constriction. This is associated with decreased  $\text{O}_2$ -sensitive  $\text{K}^+$  current and decreased expression of  $\text{K}_v$  channels.<sup>236</sup> Accordingly, overexpression of the  $\text{O}_2$ -sensitive  $\text{K}^+$  channels  $\text{K}_v1.5$  or  $\text{K}_v2.1$  via gene transfer confers  $\text{O}_2$  responsiveness to preterm rabbit DAs. Consistent with such data, 4-aminopyridine mimics an acute increase in oxygen tension in DA SMCs.<sup>237</sup> Moreover, preventing extracellular  $\text{Ca}^{2+}$  entry accentuates the initial increase in  $[\text{Ca}^{2+}]_i$  in DA SMCs exposed to acute normoxia.

An acute increase in oxygen tension directly increases  $[Ca^{2+}]_i$  in a subconfluent monolayer of DA SMCs. Pharmacologic inactivation of voltage-sensitive, but not ATP- or calcium-sensitive,  $K^+$  channels mimics the effect of an acute increase in oxygen tension, providing support for the proposition that  $K_v$  channel inactivation mediates the  $O_2$ -induced increase in DA SMC  $[Ca^{2+}]_i$ .<sup>237</sup> Alternative reports demonstrate that an acute increase in oxygen tension initially decreases the rate of calcium entry into DA SMCs.<sup>238</sup> Data demonstrate that entry of extracellular calcium is not necessary for the initial increase in DA SMC  $[Ca^{2+}]_i$ . Rather, release of calcium from  $IP_3$ -sensitive stores accounts for the initial increase in DA SMC  $[Ca^{2+}]_i$ . The sustained and progressive increase in DA SMC  $[Ca^{2+}]_i$  entails subsequent extracellular calcium entry.<sup>237</sup> The initial release of  $Ca^{2+}$  from intracellular stores is necessary to trigger the second phase of extracellular  $Ca^{2+}$  entry, in a process termed calcium-induced calcium entry. The observation that blockade of calcium release from ryanodine-sensitive stores has no effect on the normoxia-induced increase in DA SMCs offers further support for the notion that release of calcium from an  $IP_3$ -sensitive store plays a pivotal role in the normoxia-induced increase in DA SMC  $[Ca^{2+}]_i$ .<sup>238</sup> Thus, pathological patency of the DA may result from defects in oxygen sensing.

### CONCLUDING REMARKS

This review provides information surrounding the complexity of the developmental regulation of pulmonary vascular growth, structure, and physiology. Both the vascular endothelium and the SMCs are adapted to modulate vascular growth and tone in the low-oxygen-tension environment of the developing lung. Cellular constituents of the pulmonary circulation possess developmentally regulated properties that ensure well-coordinated cell proliferation and growth in the fetal and neonatal pulmonary circulation, in conjunction with the distal airspace. Maturation of molecular mechanisms underlie the ability of the lung circulation to adapt to postnatal life with an exponential increase in pulmonary blood flow at birth. Should the pulmonary circulation be subject to stress or injury during fetal life, alterations in the production of critical growth factors, vasoactive molecules, or ion channel expression or increases in cell and matrix proliferation might ensue, resulting in the failure of transition at birth due to abnormal pulmonary vascular growth, structural remodeling, and vascular tone and vasoreactivity. Finally, we speculate that insights into mechanisms that preserve endothelial maturation and function may further enhance lung development and long-term outcomes in diverse forms of neonatal pulmonary hypertension associated with preterm birth, developmental lung diseases, and congenital heart disease.

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