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Molecular and Functional Insights Into the Pulmonary Vasculature

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Molecular and Functional Insights Into the Pulmonary Vasculature

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Chapter 1
Transcription Factors Regulating Embryonic Development of Pulmonary Vasculature

Craig Bolte, Jeffrey A. Whitsett, Tanya V. Kalin, and Vladimir V. Kalinichenko

Abstract Lung morphogenesis is a highly orchestrated process beginning with the appearance of lung buds on approximately embryonic day 9.5 in the mouse. Endodermally derived epithelial cells of the primitive lung buds undergo branching morphogenesis to generate the tree-like network of epithelial-lined tubules. The pulmonary vasculature develops in close proximity to epithelial progenitor cells in a process that is regulated by interactions between the developing epithelium and underlying mesenchyme. Studies in transgenic and knockout mouse models demonstrate that normal lung morphogenesis requires coordinated interactions between cells lining the tubules, which end in peripheral saccules, juxtaposed to an extensive network of capillaries. Multiple growth factors, microRNAs, transcription factors,

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and their associated signaling cascades regulate cellular proliferation, migration, survival, and differentiation during formation of the peripheral lung. Dysregulation of signaling events caused by gene mutations, teratogens, or premature birth causes severe congenital and acquired lung diseases in which normal alveolar architecture and the pulmonary capillary network are disrupted. Herein, we review scientific progress regarding signaling and transcriptional mechanisms regulating the development of pulmonary vasculature during lung morphogenesis.

1.1 Introduction

The mature lung is a complex organ consisting of a multiplicity of cell types organized in a precisely controlled pattern during morphogenesis. Perturbations of pulmonary developmental programs cause congenital malformations that are a common cause of morbidity and mortality in infancy. The pulmonary vasculature, which provides efficient gas exchange between air and blood, is composed of the bronchial and pulmonary vascular networks (Bridges and Weaver 2006). The bronchial circulation is directly supplied by the systemic circulation and is derived from the aorta. The bronchial circulation is formed in close proximity to the conducting airways and provides oxygen and nutrient-rich blood to pulmonary tissues. The pulmonary vasculature is distinct from the systemic circulation, delivering deoxygenated blood to the lung from the right ventricle of the heart via the pulmonary artery and returning oxygen-rich blood to the left atrium via the pulmonary vein. The pulmonary vascular network can be subdivided into proximal and peripheral circulations. The proximal circulation consists of pulmonary veins and arteries. The peripheral pulmonary circulation consists of a mesh of microvessels and capillaries which create the vascular bed within the alveolar region of the peripheral lung where gas exchange occurs. A complex lymphatic system is also present in the lung that serves to maintain tissue fluid clearance. The development of the pulmonary capillary network will be the primary focus of this chapter.

Morphogenesis of the embryonic pulmonary vasculature occurs via two main processes: vasculogenesis (de novo formation of blood vessels) and angiogenesis (branching of preexisting blood vessels). Vasculogenesis is highly active during early stages of embryogenesis, as endothelial progenitor cells (angioblasts) differentiate into endothelial cells. Angiogenesis is the major process by which the pulmonary vasculature develops. Both angiogenesis and vasculogenesis require precise transcriptional regulation and signaling mediated by Vascular Endothelial Growth Factor (VEGF). Formation of embryonic vasculature depends on signaling through ANGIOPOIETIN/TIE2, PDGF, PI3K/AKT, TGF-β, SHH, WNT, and NOTCH, as well as transcription factors FOXF1, FOXM1, FOXC1/2, ETV2, HAND1, MEF2C, PROX1, HEY1/2, COUP-TFII, TBX4, SNAIL, GATA, SOX, and KLF (reviewed in De Val and Black 2009; Arora and Papaioannou 2012; Tiozzo et al. 2012). Temporal and spatial coordination of these signaling and

transcriptional programs generates the extensive pulmonary vascular network required for lung function.

1.2 Lung Morphogenesis

1.2.1 Structural Development of the Respiratory Tree and Pulmonary Vasculature

The pulmonary vascular tree develops in close proximity to the epithelial lining of the lungs in a process mediated by crosstalk between respiratory epithelial cells and those of the pulmonary mesenchyme. Lung development in mice proceeds through distinct anatomic stages defined as: embryonic [prior to embryonic day 9.5 (E9.5)], pseudoglandular (E9.5–16.5), canalicular (E16.6–17.4), saccular (E17.5–postnatal day 5 (P5), and alveolar (P5–P20) (Fig. 1.1) (Costa et al. 2001; Cardoso and Lu 2006; Morrisey and Hogan 2010; Warburton et al. 2010; Herriges and Morrisey 2014; Kool et al. 2014). Epithelial tubules in the embryonic lung undergo branching morphogenesis during the pseudoglandular stage to generate a primitive tree-like network of tubules (Metzger et al. 2008; Morrisey and Hogan 2010; Warburton et al. 2010). Three modes of branching sequentially transform the lung buds into a more complex organ. Domain branching creates a series of new buds which arise from the bronchial stalk at specified distances, generating a series of smaller bronchi. Subsequently, the tip of the growing buds divides by either planar or orthogonal bifurcation based on a series of clues to prevent steric hindrance of the growing respiratory tree (Metzger et al. 2008; Morrisey and Hogan 2010; Warburton et al. 2010). During the canalicular stage, branching morphogenesis continues in the periphery of the lung thereby increasing the number of subdivisions of the tubular network. Terminal bronchioles give rise to respiratory bronchioles and alveolar ducts as airway epithelium differentiates (Warburton et al. 2010). Blood vessels grow with branching of the airways, increasing the complexity of the pulmonary vascular network (Morrisey and Hogan 2010; Warburton et al. 2010). The saccular stage involves thinning of the interstitium and differentiation of alveolar type I and type II epithelial cells in association with rapid capillary growth and increasingly close apposition of endothelial-lined capillaries and epithelial-lined alveolar saccules. The complexity of the pulmonary lymphatic network increases with advancing development (Warburton et al. 2010). While gas exchange can occur at the saccular stage, further septation of the saccules to form alveoli increases surface area and efficiency of gas exchange. Formation of secondary septae creates alveoli lined by type I and type II epithelial cells in close apposition to the capillary network allowing efficient diffusion of gases between the pulmonary circulation and the alveolar space (Morrisey and Hogan 2010; Warburton et al. 2010). Although there is considerable knowledge regarding the

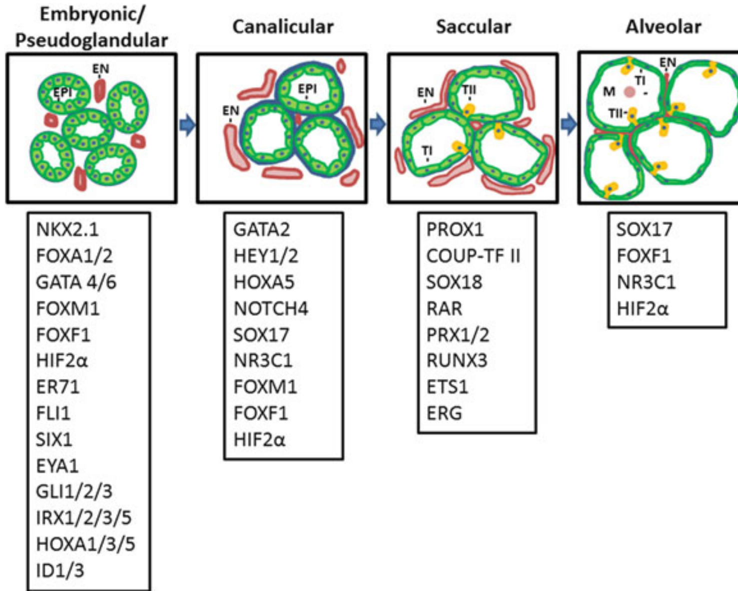

Fig. 1.1 Stages and transcriptional regulators of lung vascular development. The murine lung progresses through four developmental stages in utero and final fine-tuning occurs postnatally during the alveolar stage. Images represent the appearance of the epithelial tree as well as developing pulmonary vasculature during the pseudoglandular, canalicular, saccular, and alveolar stages of lung development. The major transcription factors regulating development of the pulmonary vasculature are listed for each stage. Abbreviations: *EN* endothelium, *EPI* epithelium, *TI* type I pneumocyte, *TII* type II pneumocyte, *M* macrophage

signaling pathways regulating development and differentiation of respiratory epithelium, development of pulmonary vasculature is not well understood.

1.2.2 Signaling Pathways Regulating Lung Morphogenesis

Multiple growth factors, transcription factors, and their associated signaling cascades are involved in formation of the pulmonary vasculature (Table 1.1). WNT and BMP signaling pathways are required for specification of the lung (Herriges and Morrisey 2014). Mice lacking WNT2/2b or β-CATENIN fail to generate a lung field, whereas over-activation of this pathway expands the specified lung field into the anterior foregut (Goss et al. 2009). Loss of BMP4 or BMP receptors 1A/B results in embryonic lethality as endoderm fails to form a trachea or, subsequently, the bronchial tree (Domyan et al. 2011). Formation of the gut tube also requires retinoic acid (RA) signaling through its receptors (RARs) (Cardoso and Lu 2006; Warburton et al. 2010). RA antagonism in mice from conception results in mid-gestation lethality and loss of lung field specification (Niederreither et al. 1999; Desai et al.

Table 1.1 Signaling molecules and transcription factors regulating pulmonary vascular development as determined by genetic mouse models

Gene	Mouse model	Vascular phenotype	Reference
Signaling molecules			
Vegf	*SPC-Vegf*	Abnormal pulmonary vascular development	Zeng et al. (1998)
Vegf	*Lyve-1-Cre Vegfr2*$^{-/-}$	Lymphatic hypoplasia, decreased blood vessel development in yolk sac, liver, and lung	Dellinger et al. (2013)
Tgf-β	*SPC-Tgf-β1 TG* (constitutively active)	Developmental arrest in pseudoglandular stage, increased vascular density	Zeng et al. (2001)
Shh	*Tie2-Cre Shh*$^{-/-}$	Simplified pulmonary vasculature	van Tuyl et al. (2007)
Wnt	*Wnt2*$^{-/-}$;*Wnt2b*$^{-/-}$	Lung agenesis, lack of lung specification	Goss et al. (2009)
Notch	*Tie2-tta Tre-Notch4*	Arterial-venous shunts	Miniati et al. (2010)
Bmp	*Shh-Cre Bmpr1a*$^{fl/+}$; *Bmpr1b*$^{-/-}$	Die at birth, tracheal agenesis and aberrant bronchi	Domyan et al. (2011)
Pten	*Dermo-Cre Pten*$^{-/-}$	Reduced pulmonary capillary network, ACD-like syndrome	Tiozzo et al. (2012)
Transcription factors			
Foxf1	*Foxf1*$^{+/-}$	Hypoplastic lung, fusion of right lung lobes, tracheal narrowing and fistula, ACD-like syndrome	Mahlapuu et al. (2001)
Foxm1	*Foxm1*$^{-/-}$	Late embryonic lethal, hypertrophy of αSMA^{+} cells, defective peripheral pulmonary capillary formation	Kim et al. (2005)
Foxm1	*Smmhc-Cre Foxm1*$^{-/-}$	Perinatal lethal, pulmonary hemorrhage	Ustiyan et al. (2009)
Foxc2	*Foxc2*$^{-/-}$	Abnormal lymphatic patterning, agenesis of valves	Petrova et al. (2004)
Foxo1	*Tie2-Cre Foxo1*$^{-/-}$	Endothelial cell hyperplasia, vessel thickening	Wilhelm et al. (2016)
Prox1	*Prox1*$^{-/-}$	Lethal by E15, complete lack of lymphatic capillary network	Wigle and Oliver (1999)
Hey1/2	*Hey1*$^{-/-}$; *Hey2*$^{-/-}$	Lethal after E9.5, impaired vascular remodeling and hemorrhage	Fischer et al. (2004)
Coup-tf II	*Coup-tf II*$^{-/-}$	Die circa E10, defective remodeling of primitive capillary plexus	Pereira et al. (1999)
Sox17	*Sox17*$^{-/-}$	Aberrant heart looping, enlarged cardinal vein, anterior dorsal aorta defects	Sakamoto et al. (2007)
Sox17	*Dermo1-Cre Sox17*$^{-/-}$	Die <3 weeks, decreased pulmonary microvasculature, alveolar simplification	Lange et al. (2014)

(continued)

Table 1.1 (continued)

Gene	Mouse model	Vascular phenotype	Reference
Sox18	*Sox18*$^{-/-}$	Lethal around E14.5, lack of lymphatic endothelial cell differentiation	Francois et al. (2008)
Fli1	*Fli1*$^{-/-}$	Blood vessel leak/hemorrhage	Spyropoulos et al. (2000)
Eya1	*Eya1*$^{-/-}$	Blood congestion, decreased vascular smooth muscle, blood vessel herniation	El-Hashash et al. (2011a)
Six1	*Six1*$^{-/-}$	Blood congestion, decreased vascular smooth muscle, blood vessel herniation	El-Hashash et al. (2011b)
Id1/3	*Id1*$^{-/-}$; *Id3*$^{-/-}$	Lack of branching/sprouting of blood vessels into neuroectoderm	Lyden et al. (1999)
Er71	*Er71*$^{-/-}$	Loss of Flk-1/endothelial cells	Lee et al. (2008)
Erg	*Tie2-Cre Erg*$^{-/-}$	Immature/disorganized vascular plexus	Birdsey et al. (2015)
Hoxa5	*Hoxa5*$^{-/-}$	Decreased branching, alveolar thickening, no surfactant	Aubin et al. (1997)
Nr3c1	*Col1-Cre GR*$^{-/-}$	Die at birth with respiratory distress and cyanosis, lung at pseudoglandular stage	Habermehl et al. (2011)
Nr3c1	*Dermo1-Cre GR*$^{-/-}$	Die after birth from cyanosis, lung atelectasis, developmental arrest in canalicular stage	Li et al. (2013)
Prx1	*Prx1*$^{-/-}$	Die postnatal due to cyanosis, hypoplastic lung, reduced vWF+ blood vessels	Ihida-Stansbury et al. (2004)
Runx3	*Runx3*$^{-/-}$	Die within 24 h of birth; poor alveolar expansion/vascularization	Lee et al. (2014a)

2006). RARβ signaling is essential for lung field specification while RARα and RARγ are also required for branching lung morphogenesis (McGowan et al. 2000; Desai et al. 2006). The FGF family is also critical for lung development as multiple FGFs including FGF10, FGF9, FGF18, FGF8, and receptors FGFR2B and FGFR3/4 regulate formation of the lung buds (Cardoso and Lu 2006), branching of the bronchioles (Morrisey and Hogan 2010; Herriges and Morrisey 2014), and influence development of the pulmonary vasculature (White et al. 2006). Hedgehog signaling is critical for development of the lung and other organs. SHH is derived from the endoderm and mediates expression of *Gli* genes in the mesenchyme as well as *Fgf* in the mesoderm (Warburton et al. 2000; Kumar et al. 2005). SHH is essential for lung bud formation, branching morphogenesis, and development of pulmonary vasculature (van Tuyl et al. 2007; Morrisey and Hogan 2010). *Shh*$^{-/-}$ mice have significant branching defects, and conditional deletion of *Shh* from endothelial and hematopoietic lineages (*Tie2-Cre Shh*$^{-/-}$) decreases complexity of the pulmonary vascular bed (van Tuyl et al. 2007). Conversely, TGF-β negatively regulates lung development as

a constitutively active mutant form of TGF-β_1 leads to developmental arrest in the pseudoglandular stage with poor sacculation and epithelial differentiation (Zeng et al. 2001). The PDGF family, VEGF, and retinoic acid influence alveolarization including formation of the capillary network in the alveolar region (Kumar et al. 2005). VEGF, in particular, is critical for development of lung vasculature and, indeed, for vascular formation in general. Targeted disruption of the *Vegf* gene in mice produces an embryonic lethal phenotype due to impaired blood-island formation and delayed differentiation of endothelial cells, causing abnormal blood vessel development (Carmeliet et al. 1996; Ferrara 1996). VEGF signals through tyrosine kinase receptors FLK1 and FLT1 that are expressed in endothelial cells and their progenitors. *Flk1*$^{-/-}$ mice exhibit an embryonic lethal phenotype due to disruption of vasculogenesis and loss of angioblast cells from blood islands (Shalaby et al. 1995), while *Flt1*$^{-/-}$ embryos fail to form mature blood vessels (Fong et al. 1995). Lung-specific overexpression of VEGF (*SPC-Vegf* transgenic mice) causes neonatal lethality associated with increased pulmonary vascular development and disrupted epithelial differentiation (Zeng et al. 1998). VEGF has also been shown to influence expression of *Etv2*, a transcription factor essential for development of blood vessels in the lungs and other organs (Casie Chetty et al. 2017). NR3C1, glucocorticoid receptor, signaling is critical for lung maturation as *Nr3c1*$^{-/-}$ mice die after birth due to respiratory failure and corticosteroid administration to late term or preterm babies is a long standing medical practice (Habermehl et al. 2011). Recently, microRNA (miR) have been shown to regulate lung vascular development, as silencing of either DICER or DROSHA reduces endothelial sprouting and epithelial-specific loss of *Dicer* decreases epithelial branching (Marcelo et al. 2013; Herriges and Morrisey 2014). miR-29 is the most common microRNA in adult lungs, with highest expression in the distal vasculature (Cushing et al. 2015). Disruption of miR-29 inhibits vascular smooth muscle development with increased expression of *Klf4*, *Pdgfrb*, *Fbxo32*, and *Foxo3a* (Cushing et al. 2015). The microRNA cluster miR-17-92 is highly expressed in early lung development and its overexpression driven by the *Spc* promoter results in abnormal lung development and lethality (Lu et al. 2007). Disruption of the miR-17-92 cluster causes perinatal lethality due to hypoplastic lung (Ventura et al. 2008). miR clusters miR302-367 and miR449/34 as well as miR375 have been shown to regulate cell differentiation in the later stages of lung development (Herriges and Morrisey 2014). Among microRNAs, miR-126 and miR-221 are pro-angiogenic and miR-27b promotes endothelial cell tip specification and branching of the vascular plexus (Marcelo et al. 2013). Long noncoding RNAs (lncRNAs) are another emerging player in lung development. Mice deficient in the lncRNA *Fendrr* die at birth with hypoplastic lungs and enlarged alveolar saccules (Sauvageau et al. 2013).

1.3 Transcriptional Regulation of Lung Development

1.3.1 Embryonic Stage

In order to control a process as complex as lung organogenesis, precise temporal and spatial regulation of gene expression is required. Homeobox transcription factor NKX2.1 is the earliest known marker of the primitive lung field, marking respiratory epithelial cells in the primordial lung buds. *Nkx2.1*-null mice die from respiratory failure at birth caused by severe lung hypoplasia (Kimura et al. 1996). *Nkx2.1* is regulated by FOXA2, a member of the Forkhead box (Fox) family of transcription factors, and by GATA6 (Ikeda et al. 1996; Shaw-White et al. 1999; Costa et al. 2001; Kumar et al. 2005). *Nkx2.1* expression depends on the canonical WNT/β-CATENIN pathway and BMP signaling. Disruption of either FOXA2 or GATA6 pathways blocks expression of *Nkx2.1* and inhibits lung specification (Herriges and Morrisey 2014). GATA4 also plays a role in the early specification of the lung field (Kumar et al. 2005). Mice lacking *Foxa1*/*2*, *Gata4*, or *Gata6* die at early stages of embryogenesis due to multiple developmental abnormalities, including heart defects, loss of extra-embryonic endoderm, and severely underdeveloped gut tube (Costa et al. 2001; Cardoso and Lu 2006). All these transcription factors are expressed in foregut endoderm and endoderm-derived lung epithelial cells, suggesting they control epithelial cell fate decisions during lung specification.

1.3.2 Pseudoglandular Stage

Branching morphogenesis in the pseudoglandular stage of lung development is associated with increased angiogenesis. The ETS transcription factors ER71 and FLI1 play important roles early in pulmonary vascular development (Fig. 1.1). *Fli1* is expressed in endothelial cells of the developing vasculature, and deletion of *Fli1* in mouse causes death before E12.5 related to vascular leak (Lelievre et al. 2001). In the mouse, *Er71* can be detected as early as E7, and its expression is regulated by VEGF, BMP, NOTCH, and WNT signaling pathways (Kataoka et al. 2011). *Er71*$^{-/-}$ mice die at E9.5 due to lack of endothelial cells, decreased *Fli1* and *Gata6* expression, and loss of *Flk1* (Lee et al. 2008). Both ER71 and GATA2 are critical for differentiation of hemangiogenic mesoderm into endothelial cells as opposed to hematopoetic cell lineages (Kataoka et al. 2011; Park et al. 2013). The homeobox transcription factor SIX1 and its transcriptional co-activator EYA1 are important regulators of lung vascular development. Deletion of either *Eya1* or *Six1* causes lung hypoplasia associated with decreased branching (Table 1.1), as well as disruption of FGF10 and SHH signaling (El-Hashash et al. 2011a, b). Both FGF10 and SHH are critical for epithelial–mesenchymal interactions during formation of the lung (van Tuyl et al. 2007; Morrisey and Hogan 2010). Deletion of *Eya1* or *Six1* causes vascular defects including decreased vascular smooth muscle and herniation of the blood vessels

(El-Hashash et al. 2011b). SHH plays critical roles during lung development, mediated by the GLI family of zinc finger transcription factors. Deletion or mutation of either *Gli2* or *Gli3* results in failure of normal lobulation, generating a simplified lung (Grindley et al. 1997; Motoyama et al. 1998; Costa et al. 2001). While mice with mutant *Gli1* or compound *Gli1/3* mutations have no lung phenotype, compound mutations in *Gli1/2* or *Gli2/3* cause severe lung hypoplasia (Grindley et al. 1997; Motoyama et al. 1998; Park et al. 2000; Costa et al. 2001). Although no vascular phenotype has been described in *Gli*-deficient mice, GLI2 and GLI3 inhibit FGF10, a growth factor essential for pulmonary vascular development (Warburton et al. 2000). Multiple isoforms of the IRX homeobox transcription factors, including *Irx1*, *Irx2*, *Irx3*, and *Irx5*, are expressed in the lung and play redundant roles during lung formation and branching morphogenesis. Simultaneous deletion of these *Irx*'s inhibits epithelial branching in vitro and inhibits differentiation of airway epithelial cells (van Tuyl et al. 2006). Decreased α-smooth muscle actin surrounding pulmonary vessels was observed after inhibition of the *Irx* transcription factors, supporting a role for IRX in the development of vascular smooth muscle (van Tuyl et al. 2006). Deletion of *Hoxa5* inhibited branching morphogenesis, caused alveolar thickening, and inhibited surfactant production (Aubin et al. 1997; Costa et al. 2001; Jones 2003); whereas deletion of either *Hoxa1* or *Hoxa3* caused lung hypoplasia and respiratory failure at birth (Chisaka and Capecchi 1991; Lufkin et al. 1991; Jones 2003). Consistent with a critical role of BMP signaling during pulmonary vascular development, mice lacking the BMP/SMAD targets ID (inhibitor of differentiation and DNA binding 1 and 3) died before E13.5 and exhibited vascular defects and decreased branching morphogenesis (Lyden et al. 1999; Park et al. 2013).

1.3.3 Canalicular Stage

During the canalicular stage of lung development, branching morphogenesis continues in the terminal saccules, which will later undergo sacculation to form alveoli. Vascular development is highly active during this stage, as an extensive capillary network is formed. NOTCH signaling has emerged as a major regulator of pulmonary vascular development. NOTCH signaling stabilizes angiogenic sprouts which are essential for expansion of the vascular network (Lee et al. 2014b). NOTCH signaling also influences expression of *Vegfa*, which is critical for vascular development (Crivellato 2011). Loss of VEGFA activity has been shown to result in decreased numbers of pulmonary capillaries and reduced alveolarization (Yun et al. 2016). Endothelial-specific expression of constitutively active *Notch4* caused arterial-venous shunts (Miniati et al. 2010). In support of a critical role of NOTCH in vascular development, embryos deficient in NOTCH target genes *Hey1/2* die during the early stage of lung development with impaired vascular remodeling (Fischer et al. 2004; Park et al. 2013) (Table 1.1). Furthermore, NOTCH inhibits *Sox17*, a transcription factor critical for vascular development (Lee et al. 2014b). *Sox17*-null mice die at E10.5 (Kanai-Azuma et al. 2002), while

endothelial-specific deletion of *Sox17* disrupted formation of the pulmonary microvasculature and caused alveolar simplification (Lee et al. 2014b). Conversely, endothelial overexpression of *Sox17* enhances angiogenic sprouting (Lee et al. 2014b). Mice with mesenchymal-specific *Sox17* deletion mediated by *Dermo1-Cre* die within 3 weeks of birth with a significant decrease in pulmonary microvasculature and alveolar simplification (Lange et al. 2014). Forkhead box (Fox) transcription factors are also important regulators of pulmonary vascular formation. Severe defects in pulmonary mesenchyme and undeveloped capillaries were observed in *Foxm1*$^{-/-}$ embryos causing death in utero (Wang et al. 2003; Kim et al. 2005), demonstrating FOXM1 is critical for lung development. Perturbations in *Foxm1* expression have profound effects on multiple organ systems, including the cardiovascular, gastrointestinal, and hematopoietic systems (Wang et al. 2003; Ren et al. 2010; Bolte et al. 2011, 2012; Sengupta et al. 2013). *Foxm1* deficiency inhibits development of Clara and goblet cells in airway epithelium and disrupts normal epithelial patterning in the developing lung (Wang et al. 2010, 2012; Ustiyan et al. 2012, 2016). FOXM1 stimulates proliferation of endothelial cells during formation of pulmonary vasculature and accelerates cell cycle progression in normal and neoplastic cells during tissue repair, carcinogenesis, and inflammation (Liu et al. 2011; Balli et al. 2012; Ren et al. 2013; Cheng et al. 2014; Wang et al. 2014; Gao et al. 2015; Kalinichenko and Kalin 2015; Xia et al. 2015). Conditional deletion of *Foxm1* from smooth muscle cells using *smooth muscle myosin heavy Cre* (*Smmhc-Cre Foxm1*$^{-/-}$) resulted in decreased vascular muscle formation and pulmonary hemorrhage (Ustiyan et al. 2009), supporting an important role for FOXM1 in pulmonary vascular development. FOXO1 has been shown to inhibit endothelial proliferation as mice deficient for endothelial *Foxo1* (*Tie2-Cre Foxo1*$^{-/-}$ mice) have increased rates of proliferation resulting in thicker vessels than control mice (Wilhelm et al. 2016). *Foxf1* and *Foxf2* are expressed in mesenchyme-derived cells of the lung and are regulated by SHH (Aitola et al. 2000; Bolte et al. 2015; Xu et al. 2016; Milewski et al. 2017). While *Foxf1*$^{-/-}$ mice die before E9.5 related to defects in the yolk sac and allantois (Mahlapuu et al. 2001; Kalinichenko et al. 2003b), *Foxf1*$^{+/-}$ mice frequently die at birth due to disruption of pulmonary vascular formation, a syndrome similar to alveolar capillary dysplasia (ACD) (Kalinichenko et al. 2001; Sen et al. 2014). *Foxf1*$^{+/-}$ mice are also more susceptible to lung injury indicating the importance of FOXF1 in lung injury and repair (Kalin et al. 2008; Cai et al. 2016; Pradhan et al. 2016). Mesenchymal-specific inactivation of *Pten* resulted in death at birth, decreased *Foxf1* expression, and caused pathology consistent with findings in ACD, thus implicating the PI3K/AKT pathway in development of the pulmonary vasculature (Tiozzo et al. 2012). Interestingly, overexpression of *Foxf1* in transgenic mice also causes perinatal lethality with vascular hemorrhage, immature lungs, and disrupted capillary network (Dharmadhikari et al. 2016), indicating pulmonary vascular development is dependent on dosage of the *Foxf1* gene. HIF1α and HIF2α regulate the expression of *Vegf* and thus influence normal formation of the pulmonary vasculature (Jones 2003; Galambos and deMello 2007; Park et al. 2013; Kool et al. 2014; Tibboel et al. 2015). Mice with mesenchyme-specific deletion of the *Nr3c1* nuclear receptor

failed to progress from the canalicular to the saccular stage of lung development (Li et al. 2013). These mice were cyanotic at birth and died shortly afterwards with lung morphology consistent with the canalicular stage of development (Li et al. 2013).

1.3.4 Saccular/Alveolar Stages

Considerable remodeling of lung structure occurs during the saccular and alveolar stages of lung development as the number of terminal saccules increases, complexity of the vascular network expands, and development of the pulmonary lymphatic system occurs (Costa et al. 2001; Cardoso and Lu 2006; Morrisey and Hogan 2010; Warburton et al. 2010; Herriges and Morrisey 2014; Kool et al. 2014). This period is defined by increased specialization and differentiation of respiratory epithelial cells, which differ across the proximal–peripheral gradient of the airways. PROX1 and COUP-TF II mediate formation of lymphatic vasculature and are actively expressed during the saccular stage of lung development (Pereira et al. 1999; Wigle and Oliver 1999; Galambos and deMello 2007). SOX18, known to be a critical regulator of endothelial venous cell differentiation, stimulates formation of lymphatic vessels (Francois et al. 2008; Park et al. 2013). Deletion of *Foxc2* inhibited lymphatic vessel formation (Petrova et al. 2004; Park et al. 2013), and mice lacking both *Foxc1* and *Foxc2* die during early mouse embryogenesis with defective vascular remodeling (Kume et al. 2001; Park et al. 2013). COUP-TF II blocks retinoic acid receptor signaling (Kimura et al. 2002), suggesting RAR signaling directs endothelial cells towards a vascular versus lymphatic cell fate. VEGF signaling also influences lymphatic development as deletion of *Flk1* from lymphatic vasculature (*Lyve-1-Cre Vegfr2*$^{-/-}$ mice) was sufficient to cause embryonic death at E14.5. *Lyve-1-Cre Vegfr2*$^{-/-}$ mice presented with lymphatic hypoplasia as well as decreased blood vessel formation in the yolk sac, liver, and lungs (Dellinger et al. 2013). Paired-class homeobox transcription factors PRX1 and PRX2 have been linked to pulmonary hypertension due to a role in smooth muscle proliferation (Jones 2003), and *Prx1*$^{-/-}$ mice have been shown to have decreased lung vascular smooth muscle development (Ihida-Stansbury et al. 2015). *Prx1*$^{-/-}$ mice die soon after birth from cyanosis and exhibit reduced blood vessel formation and decreased expression of *Flk-1* and *Vcam-1*, molecules essential for blood vessel formation and integrity (Jones 2003; Ihida-Stansbury et al. 2004). *Runt-related transcription factor 3* knockout mice (*Runx3*$^{-/-}$) die within the first day of life and demonstrate vascular abnormalities (Lee et al. 2014a). RUNX3 inhibits *Pecam-1*, *Vegf*, and *von Willebrand factor* expression (Lee et al. 2014a), suggesting a role in endothelial cell growth and/or maintenance. ETS1, expressed by endothelial cells during angiogenesis, regulates the expression of numerous genes involved in vascular development including *Tie1/2*, *Flt-1*, *Flk-1*, and *VE-cadherin*. Remarkably, *Ets1*$^{-/-}$ mice have no abnormalities in the vascular system, likely related to redundancy of expression of other ETS transcription factors including FLI1 and

ERG1, both known to influence expression of *VE-cadherin* and *Flt1* (Lelievre et al. 2001; Grzenda et al. 2013). ERG in particular has been shown to be critical for vascular development as endothelial deletion of *Erg* (*Tie2-Cre* $Erg^{-/-}$) causes death before E9.5, prior to lung vascular development, and mice heterozygous for endothelial *Erg* have decreased levels of *VE-cadherin* in lung endothelial cells (Birdsey et al. 2015).

1.4 Foxf1 Transcription Factor Is a Critical Regulator of Pulmonary Vascular Development

Alveolar Capillary Dysplasia with Misalignment of Pulmonary Veins (ACD/MPV) is one of the most severe human congenital disorders. This syndrome, which occurs in neonates and in early infancy, is associated with impaired lobulation, loss and displacement of pulmonary capillaries, abnormalities in formation of pulmonary arteries and malposition of veins, which leads to pulmonary hypertension, lung edema, cyanosis, and respiratory failure (Bishop et al. 2011). ACD/MPV patients frequently present with other congenital malformations of the genitourinary, gastrointestinal, and cardiovascular systems. The severity of congenital abnormalities and progressive respiratory insufficiency in ACD/MPV infants usually results in death shortly after birth (Bishop et al. 2011). Recently, heterozygous deletions and point mutations in the *FOXF1* gene locus were identified in up to 50% of ACD/MPV patients (Stankiewicz et al. 2009; Dharmadhikari et al. 2015). These clinical studies demonstrate a critical role for FOXF1 in development of the capillary network of the human lung.

FOXF1 (HFH-8 or FREAC-1) is a member of the Forkhead Box (Fox) family of transcription factors which share an evolutionary conserved Winged helix/Forkhead DNA binding domain. *Foxf1* is expressed in endothelial and mesenchymal cells of yolk sac, placenta, and allantois, and $Foxf1^{-/-}$ mice die in the early embryonic period at approximately E8.5 with vascular abnormalities in the extra-embryonic mesoderm (Peterson et al. 1997; Mahlapuu et al. 2001; Kalinichenko et al. 2002). *Foxf1* haploinsufficiency in mice causes an alveolar capillary dysplasia-like syndrome, associated with lung hypoplasia, fusion of lung lobes, loss of pulmonary capillaries, and various developmental defects in the mesenchyme of gallbladder, esophagus, and trachea (Kalinichenko et al. 2001, 2002; Mahlapuu et al. 2001). A subset of $Foxf1^{+/-}$ mice die at birth, in association with ACD and progressive respiratory insufficiency (Kalinichenko et al. 2001). The remarkable similarities between mouse and human phenotypes associated with mutations in *Foxf1* indicate these mice provide a useful model to study the pathogenesis of ACD and to develop therapeutics to intervene with this congenital pediatric disorder. In fact, *Foxf1* promoter and coding regions have been found to be highly conserved from mouse to humans (Kim et al. 2005); however, *Foxf1* haploinsufficiency in mice is less severe than the syndromes seen in humans, since 50–70% of $Foxf1^{+/-}$ mice survive

after birth (Kalinichenko et al. 2001). Although these mice usually reach adulthood, they are susceptible to lung injury, pulmonary hemorrhage, and exhibit abnormal lung and liver repair (Kalinichenko et al. 2002, 2003a).

Foxf1 is a downstream target of SHH signaling and *Shh*$^{-/-}$ mice lack *Foxf1* expression (Mahlapuu et al. 2001). SHH-mediated activation of *Foxf1* occurs through direct binding of GLI transcription factors to the *Foxf1* gene promoter region (Madison et al. 2009; Hoffmann et al. 2014). Loss of GLI binding sites in the *FOXF1* gene locus were found in ACD/MPV patients (Szafranski et al. 2013), emphasizing the importance of the SHH/GLI/FOXF1 signaling pathway in the regulation of pulmonary vascular development. FOXF1 plays a critical role in migration of endothelial and mesenchymal cells through activation of *Integrin β3* and *Notch-2* (Kalinichenko et al. 2004; Malin et al. 2007). Mice with *Foxf1*-floxed alleles were recently generated (Hoggatt et al. 2013; Ren et al. 2014). Inactivation of *Foxf1* in endothelial cells using either *Tie2-Cre* or *Pdgfb-CreER* transgenes disrupted the development of the pulmonary vasculature (Ren et al. 2014; Cai et al. 2016), indicating FOXF1 functions in an endothelial cell-autonomous manner during pulmonary angiogenesis. FOXF1 promotes VEGF, PDGF, and ANGPT/TIE2 signaling in the developing vasculature through direct transcriptional activation of *Flk1, Pdgfb*, and *Tek* genes.

1.5 Summary

Pulmonary vascular morphogenesis is mostly mediated by angiogenesis, instructed by paracrine signaling between endodermally derived epithelial progenitor cells and mesenchymal cells via processes mediated by VEGFs, ANGIOPOIETINs, PDGFs, FGFs, RETINOIC ACID, TGF-β, SHH, WNT, and NOTCH, which in turn control the activities of multiple transcription factors. Recent evidence supports important roles for the FOX, SOX, GATA, ETS, PRX, HIF, and PROX families of transcription factors in pulmonary vascular development. From a clinical perspective, FOXF1 transcription factor serves as a critical regulator of the pulmonary capillary network as haploinsufficiency of the *Foxf1* gene in mice and humans causes Alveolar Capillary Dysplasia. Mutations in the *FOXF1* locus lead to respiratory insufficiency in preterm infants associated with severe abnormalities in formation of the pulmonary vascular network. Better understanding of the signaling and transcriptional regulatory networks controlling development of the pulmonary vasculature will identify new therapeutic targets needed to treat congenital and acquired pulmonary disorders.

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Chapter 2
Comparative View of Lung Vascular Endothelium of Cattle, Horses, and Water Buffalo

David Schneberger, Ram S. Sethi, and Baljit Singh

Abstract Endothelium plays an important role in maintaining the vascular barrier and physiological homeostasis. Endothelium also is fundamental to the initiation and regulation of inflammation. Endothelium demonstrates phenotypic and functional heterogeneity not only among various organs but also within an organ. One of the striking examples would be the pulmonary endothelium that participates in creating blood–air barrier. Endothelium in large pulmonary blood vessels is distinct in structure and function from that lining of the pulmonary capillaries. This chapter focuses on the comparative aspects of pulmonary endothelium and highlight unique differences such as the presence of pulmonary intravascular macrophages among select species.

2.1 Introduction

The pulmonary endothelium is a dynamic and metabolically active layer of squamous endothelial cells that is ideally placed to mediate lung homoeostasis (Millar et al. 2016). The pulmonary capillary is made up of a few (usually 2–3) thin, squamous endothelial cells (Dornan and Meban 1985; King et al. 2004), and capillary endothelial cells constitute 30–50% and 46–50% of the total cell population in the alveolar septal wall

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in human (Crapo et al. 1982) and nonhuman primate (Crapo et al. 1983), respectively. Horse lung showed an average of 252.4 capillaries per centimeter of lung alveolar wall with endothelial cells constituting 49.5% of total tissue cells in the alveolar septal wall (Gillespie and Tyler 1967). Endothelium is the gatekeeper of the tissues and regulates traffic of circulating cells and molecules into the tissues (Cotran 1989).

Endothelial cells of the lung can be easily split into two subgroups, one comprising the lining of larger blood vessels such as arteries and veins and another population that constitutes capillary endothelial cells of the microvasculature, each with distinct functions. Both subsets of endothelial cells contain rough endoplasmic reticulum, numerous vesicles such as caveolae, a low number of mitochondria, and filaments around the basal cell surface amongst other common cellular features (King et al. 2004). Filaments are more common in arterial cells which also exhibit projections from the apical surface, particularly near cellular junctions (King et al. 2004). As there is transition from arterial to microvascular endothelial cells, there is a flattening and elongation of the cells, with a greater restriction of cytoplasm near the flattened periphery. Many of the organelles are excluded from the flattened parts and concentrated around the nucleus. While apical projections were present, they were not as pronounced in this population (King et al. 2004; Ochoa et al. 2010).

A key intracellular feature of endothelial cells are dark bodies in the cytoplasm referred to as Weibel–Palade bodies (WPb) which contain a variety of secretable products such as von Willebrand Factor (vWF), interleukin (IL)-8, factor XIIIa, and P-selectin. While these bodies are present in arterial endothelial cells they are not present in the capillary endothelial cells, though these factors are still secreted by these cells as well (Fuchs and Weibel 1966; Lowenstein et al. 2005). Upon injury or another activating stimulus, these bodies fuse with the cellular membrane and degranulate, releasing their contents into circulation and on the surface of the endothelial cells. vWF, a substrate of XIIIa, plays a role in adhesion of platelets to the endothelium and also serves as a carrier for coagulation factor VIII (functions reviewed in Wang and Eikenboom 2010). IL-8 on the other hand is a strong neutrophil chemoattractant and activator, which is likely to cause damage to the endothelium (discussed later; Fig. 2.1). vWF can bind to platelets and initiate clot formation (Ochoa et al. 2010). While this is one of the most obvious and recognizable difference between both endothelial cell populations, a host of other differences exist.

Another function of the endothelium is the active regulation of vascular permeability and relaxation of arterial vessels. Relaxation is controlled by nitric oxide (NO) release from arterial endothelial cells (Furchgott and Vanhoutte 1989; Furchgott and Zawadzki 1980) which in turn prompts smooth muscle to release guanylate cyclase, increasing cGMP and vasodilation (Furchgott and Vanhoutte 1989). This mechanism is observed during exercise (Pelletier and Leith 1993), even at rest (Rees et al. 1989), and can be prompted by shear force of circulating blood (Kaiser and Sparks 1986; Koller et al. 1994). In hypoxia, removal of endothelial cells from vessels significantly reduces arterial NO production and the resulting vasoconstriction (around 65–70%), indicating a role for these cells in this phenomenon as well (MacEachern et al. 2004). However, such removal in human and sheep completely abrogated this response (Demiryurek et al. 1991, 1993), suggesting that this function is somewhat less

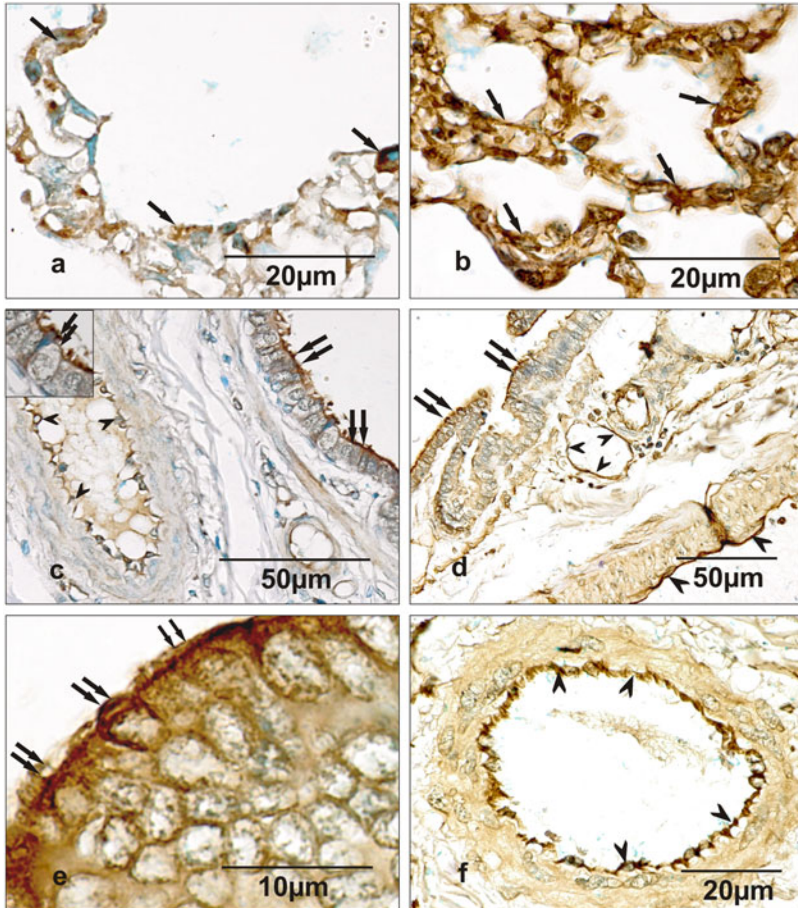

Fig. 2.1 (a) IL-8 expression (arrows) is seen in the alveolar septa of normal and (b) inflamed lungs. (c and e) The airway epithelium (double arrows) and vascular endothelium (arrowheads) of the normal lungs reacts weakly for IL-8 compared with (d) the intense expression in the airway epithelium (double arrows) and (f) the vascular endothelium (arrowheads) of the inflamed lungs. IHC. Reprinted with permission from J Comp Pathol. 2011 Feb–Apr;144(2–3):135–44. doi: https://doi.org/10.1016/j.jcpa.2010.08.003

important in horses and cattle than in other species (MacEachern et al. 2004). The role of NO in this phenomenon may be more complex though as in exercise models of lung injury, inhibition of NO production in horses increased damage seen in the lungs, suggesting mechanisms other than just vessel relaxation (pressure) likely play a role in endothelial damage (Kindig et al. 2000).

2.2 Circulation Constraints in the Lung

Maintenance of vascular integrity is an important function of endothelial cells in the lung. They can however often fail during exertion (Pascoe et al. 1981). Several studies have focused on exercise induced pulmonary hemorrhage in the lungs of race horses, where blood pressure can become very high (Pascoe et al. 1981; West et al. 1993). Arterial pressures can reach up to 120 mmHg in exercising horses (West et al. 1993), and capillary threshold pressure can reach between 75 and 100 mmHg in thoroughbreds (Birks et al. 1997). By comparison, the capillary threshold in rabbits is below 40 mmHg, and 70 mmHg in dogs (Fu et al. 1992; Tsukimoto et al. 1991). While we do not know of any study that examines molecular structural differences between rabbit and horse lung that can account for these differences, it seems likely that such differences must exist to allow for such increased pressures in the capillaries. A study estimating canine and equine capillary strength based on morphology, while accurately predicting increased strength over rabbits, also underestimated actual strength in equines (Birks et al. 1994), suggesting such molecular differences should be present.

Response to pressure in endothelial cells is carried out by the calcium channel TRPV4 (Jian et al. 2008; Yin et al. 2008). Increased pressure elevates cellular calcium, but blockage of TRPV can abrogate most of this change (Jian et al. 2008; Yin et al. 2008). Activation of the channel can also increase lung endothelial permeability (Alvarez et al. 2006). Increase in pressure is also shown to induce exocytosis of endothelial vesicles such as WPb (Kuebler et al. 1999). As these bodies include proteins such as vWF, P-selectin, and IL-8, pressure and calcium control could also be considered mechanisms of inflammation in these cells. Even eNOS production in these cells is controlled partially by calcium and can act as a negative feedback on TRPV4 induced calcium influx (Yin et al. 2008).

Finally, endothelial cells are constrained by the competing requirements of gas diffusion and blood pressure. Thus, it is no surprise that their capillary cell morphology takes on a more flattened structure compared to arterial endothelial cells to improve gas exchange. Increased ability to handle pressure will come at a cost of increased thickness, which will also trade off gas exchange efficiency. Thoroughbred horses in particular fall into this conundrum. While bred for work and speed, cardiac output has been increased, but the lungs often cannot handle this output pressure, such that alveolar bleeding is a common occurrence (West 2000). In the end, selection pressure may remain on improving endothelial cell thinness to maintain gas diffusion efficiency, and increase of extracelluar matrix toughness to improve strength of the alveoli (West 2000). While improvement of endothelial cell junctions would be expected, disruptions when they happen are observed frequently in the cells and not at junctions (Costello et al. 1992), and often seal quickly upon relief of pressure (Elliott et al. 1992).

Capillary endothelium is surrounded by mainly alveolar type I epithelial cells and occasional type II cell (Townsley 2012). Further, capillary endothelium is connected to other cellular constituents of the alveolar septal wall via fibroblasts

by intercellular junctions (Sirianni et al. 2003) and pericytes by myoendothelial junctions (Michel et al. 1995). Most of these cell junctions are not random but strategically placed at the interface between the thick and the thin sides of the septal wall to provide structural stability and minimize the distensibility, especially during the increase in the mass of surrounding tissue (Walker et al. 1995).

Capillary endothelium lacks large gap junctions unlike endothelial junctions in extra-alveolar arteries and veins, but contains occasional small gaps in capillary inter-endothelial cell junctional complexes (Schneeberger 1982). Further, a third adjoining endothelial cell forms a flap overlaying the borders of these cells and this flap has specific organization of junctional strands (Walker et al. 1994). These strands run parallel instead of perpendicular to the plane of the overlying cell to provide some protection against transendothelial fluid movement during increased intravascular pressure as the increased hydrostatic force results in “sealing” of this junctional complex (Walker et al. 1994).

Endothelial cells are capable of allowing diffusion of a number of molecules across the cellular barrier, but studies in several species have shown a general resistance to diffusion, particularly in lung capillary endothelial cells. Capillary endothelial cells of the lung show lower permeability to water and solutes compared to vascular endothelial cells (Kelly et al. 1998; Parker and Yoshikawa 2002; Parker et al. 2006). Studies of hydraulic conductance also show values much lower for microvascular endothelial cells suggesting tighter junctions and lower diffusion (Kelly et al. 1998; Parker et al. 2006; Ofori-Acquah et al. 2008). This makes sense when considering the need to maximize diffusion of oxygen across the cell membrane and minimize solute and fluid transport, the last of which could cause tissue or alveolar edema problems if not closely controlled. Both arterial and microvascular endothelial cells have about the same amount of intracellular actin. However, the chemical disruption of actin wtih cytochalasin D is much harder in microvascular endothelial cells (Ofori-Acquah et al. 2008).

A reason for this more restrictive permeability is alterations in localized cAMP expression in endothelial cells. High levels of cAMP are generated at the membrane of microvascular endothelial cells by adenylate cyclase 6 which enhances barrier function (Ludwig and Seuwen 2002). This adenylate cyclase is calcium regulated and thus permeability can be increased by increased intracellular (cytosolic) calcium (Cioffi et al. 2002; Stevens et al. 1995; Sayner and Stevens 2006; Sayner et al. 2006), though a specific cAMP effector of barrier strength is not fully resolved (Prasain and Stevens 2009). One such example is endothelial response to thrombin. Thrombin is a pro-inflammatory molecule that binds to PAR receptors (PAR-1 and PAR-3) and activates endothelial cells (reviewed in Minami et al. 2004). Amongst these cellular changes is Gq activation, which leads to calcium release from the endoplasmic reticulum resulting in calcium depletion which is replenished by opening calcium channels (TRPC1 and TRPC4 subunits). This increase in free cytosolic calcium thus disrupts the endothelial cell barrier (Cioffi et al. 2009). Another ion channel, TVRP4, is selectively expressed in lung microvasculature but not the arterial endothelial cells (Alvarez et al. 2006). Activation of this channel

leads to leakage into the alveoli, but in this case leakage is due to sloughing of cells and/or loss of attachment to cell matrix (Alvarez et al. 2006).

Neuropeptides play important roles in the regulation of respiratory function and topographical inflammation and are localized in both motor and sensory neurons in the mammalian respiratory tract (Lundberg et al. 1988). Substance P (SP) and calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibers are present around pulmonary blood vessels throughout the respiratory tract of calves and cows (Nishi et al. 2000). These fibers are few in number in the lung compared to nasal and laryngeal mucosae and tracheal bronchus, and are more numerous in calves than in cows (Nishi et al. 2000). Co-localization of SP and CGRP in most of the nerve fibers suggests that these nerve fibers are involved in the regulation of the bovine respiratory tract.

2.3 Inflammation and Immunity

Endothelial cells are located at the interface between blood and tissues not only to gate the traffic of molecules and cells across the vessel wall but also contribute to hemostasis, inflammatory reactions, and immunity (Mantovani et al. 1992). When not activated, endothelial cells can secrete a variety of products that can aid in inhibiting clotting and inappropriate activation of innate immune functions (reviewed in Pober and Sessa 2007). Amongst these is NO, which can inhibit platelet aggregation and adhesion in addition to being a vasorelaxant as mentioned earlier (Sessa 2004). In this state, P-selectin is sequestered within the cell (Bonfanti et al. 1989), and other leukocytes adherence molecules such as E-selectin, VCAM, and ICAM-1 are suppressed to a greater degree (Pober and Sessa 2007).

During inflammation, there is binding to cell-surface receptors by one of a number of potential molecules (e.g., histamine, thrombin, LPS, etc.). For those that bind to G-protein receptors, there is a release of cytosolic Ca^{2+} that will result in increased endothelial permeability as discussed earlier (Pober and Cotran 1990; Pober and Sessa 2007) causing release of WPb (Birch et al. 1992, 1994). Calcium also activates phospholipase A2 which catalyzes arachidonic acid formation and subsequently its conversion to COX1 resulting in increased blood flow (Egan and FitzGerald 2006). A by-product of arachidonic acid formation, lysophosphatidylcholine, at the same time acts as a platelet activating factor. When this is combined with the released P-selectin it leads to neutrophil attachment, integrin activation, and the extravasation of said neutrophils across the endothelial barrier (Prescott et al. 1984; Lorant et al. 1991; Pober and Sessa 2007).

Another activated protein, RHO, also aids in contraction of cellular actin filaments, resulting in creation of gaps between endothelial cells, especially post-capillary venules (Heltianu et al. 1982). Passage of most cells through the endothelial barrier appears to be between such endothelial cells and supported by increased expression in these intercellular gaps of PECAM1 and CD99 (Marchesi 1961; Schenkel et al. 2002). Cytokines such as TNF-α and IL-1 can also induce a similar response

(reviewed in Pober and Sessa 2007), but leading to transcriptional activation of NF-kB and AP-1, which activates a number of the same effector pathways already mentioned (Pober and Sessa 2007). Secretion of IL-8 will lead to activation, firm attachment, and diapedesis of neutrophils to the endothelial cells, but still requires E-selectin to tether such cells (Pan et al. 1998).

Specific to pulmonary microvascular capillary endothelial cells is a 1G T-type calcium channel. Activated by thrombin, the channel causes membrane depolarization, but in this case does not increase permeability but instead promotes P-selectin expression for neutrophil recruitment (Wu et al. 2003, 2009). Thus, calcium again appears to play a very important role in induction of inflammatory responses in endothelial cells, particularly with regard to P-selectin expression (Kuebler et al. 1999; Parthasarathi et al. 2006).

2.4 Toll-Like Receptors

Toll-like receptors (TLRs), the mammalian homologues of the Drosophila Toll family, are critical for recognition of conserved pathogen-associated molecular patterns in bacteria and viruses by immune cells (Aderem 2001). TLRs are expressed in the endothelium to activate the immune system when encountering specific antigen (Xu et al. 2011). TLR4 recognizes lipopolysaccharides (LPS) and plays important roles in host defense against bacterial infections (Lymboussaki et al. 1998; Takeda et al. 2003). We reported the presence of weak TLR4 immunoreactivity in the vascular endothelium in normal lung of cattle (Wassef et al. 2004) and buffalo (Sethi et al. 2011), which is consistent to what is observed in humans (Faure et al. 2000). Some TLR4 staining was reported in peribronchiolar blood vessels and alveolar septal endothelial cells in horse lungs (Singh Suri et al. 2006). TLR4 activates endothelium and induces recruitment of leukocytes in lung along with expression of adhesion molecules such as P-selectin and vascular cell adhesion molecule-1 (Andonegui et al. 2002, 2003). Interestingly, TLR4 appeared unchanged or reduced in the vascular endothelium of inflamed lungs from calves and Water buffalo infected with *Mannheimia haemolytica* and *Pasteurella multocida*, respectively (Wassef et al. 2004; Sethi et al. 2011). Lack of TLR4 in vascular endothelium during *Mannheimia haemolytica* and *Pasteurella multocida* infection may be a protective mechanism against unwanted inflammation.

Similarly, early studies with LPS treatment of horse lungs showed endothelial cells to be fairly unaffected by the exposure. This was, however, in the absence of immune cells such as neutrophils. While this study primarily looked at electron microscopic examination of endothelial cells, it does suggest that endothelial cells maintain morphology through exposure. By contrast, cell death was common in the presence of neutrophils, suggesting that when other immune cells are present endothelial cells are sensitive to products of these cells in the horse (Turek et al. 1987). Later experiments have also suggested TNF-α and IL-1b secretion by these cells to LPS in a whole animal exposure system (Parbhakar et al. 2005).

TLR9 identifies bacterial DNA and is a key player in bacterial DNA induced cell signaling (Bauer and Wagner 2002). TLR9 is not only activated by unmethylated CpG motifs within ssDNA, but also by CpG motifs in nucleic acids released during vascular apoptosis and necrosis (Krogmann et al. 2016). We observed that pulmonary vascular endothelium of water buffalo did not show any TLR9 immunopositive reactivity which is similar to expression of TLR4 (Sethi et al. 2011) (Fig. 2.2). However, unlike TLR4, TLR9 expression was increased in the vascular endothelium during *P. multocida* induced lung inflammation in water buffalo (Sethi et al. 2011). The data suggest that increased TLR9 expression may handle the DNA released either from the phagocytosed bacteria or from the dying extracellular bacteria at the later stages of infection. However, the contradictory finding of TLR9 agonism in vascular biology remains the potential area of research. In horse and cattle, expression of TLR9 was clearly shown in vascular endothelial cells (Fig. 2.3), with some possible capillary staining, though if present it was much lighter in comparison (Schneberger et al. 2009, 2011).

Recently, TLR10 mRNA upregulation in THP-1 cell line has been linked with reactive oxygen species induced during hypoxia (Kim et al. 2010). TLR10 is expressed in lungs of human although at very low levels and immune cells viz neutrophils, macrophages, and dendritic cells (Chuang and Ulevitch 2001). We recently reported immuno-histochemical and immuno-electron microscopic data indicating expression of TLR10 in the pulmonary vascular endothelium of cattle along with other veterinary species (Balachandran et al. 2015). However, TLR10 immunohistological expression was much reduced in the *M. haemolytica* infected animals compared to the controls (Balachandran et al. 2015). The data suggest implications of TLR10 during *M. haemolytica* infection in terms of entry of bacteria to establish infection in cattle lung.

2.5 Pulmonary Intravascular Macrophages

One of the most striking differences between species in the lung capillaries is the presence of pulmonary intravascular macrophages (PIMs) attached to the microvascular endothelial cells of the lung. This population of macrophages colonizes the lung shortly after birth and are maintained through life in animals such as in horses, Artiodactyla, odontoceti, and cats, while in other species they may be induced under certain circumstances (reviewed in Schneberger et al. 2012). They are attached strongly to the endothelial cell layer through a number of darkly staining structures, which may complicate creating primary cell cultures. These attachments to the endothelial cells are unknown but are thought to be a glycosyl-phosphatidyl inositol anchor (Atwal et al. 1992; Singh et al. 1995; Singh and Atwal 1997). Attachment is seen on the thicker side of the endothelial cell, possibly to reduce interference with gas exchange, (Winkler 1988) and the cells range from 20 to 80 μM in size, with horse PIMs being the largest. While similar to other macrophages, PIMs possess a unique decoration of lipid/lipoprotein vesicles at or near the

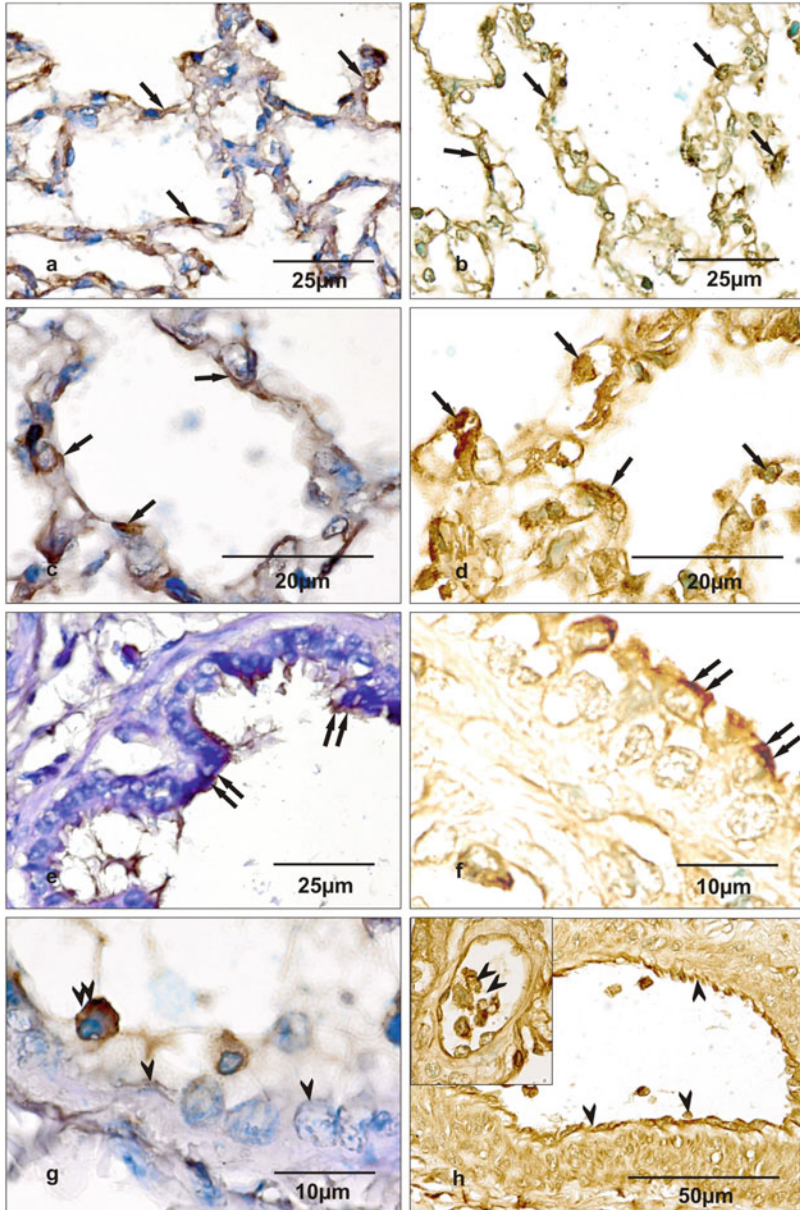

Fig. 2.2 (a) Normal lung section shows weak expression of TLR9 in alveolar septa (arrows) and (b) airway epithelium (double arrows), (c) but not in the endothelium of large blood vessels (arrowheads). (d) TLR9 expression was increased in the alveolar septa (arrows), (e) airway epithelium (double arrows) and (f) the endothelium of large blood vessels (arrowheads) in inflamed lungs. The blood cells (double arrowheads) in the control (c) and the infected lung (f) were also positive for TLR9 expression. IHC. Reprinted with permission from J Comp Pathol. 2011 Feb–Apr;144(2–3):135-44. doi: https://doi.org/10.1016/j.jcpa.2010.08.003

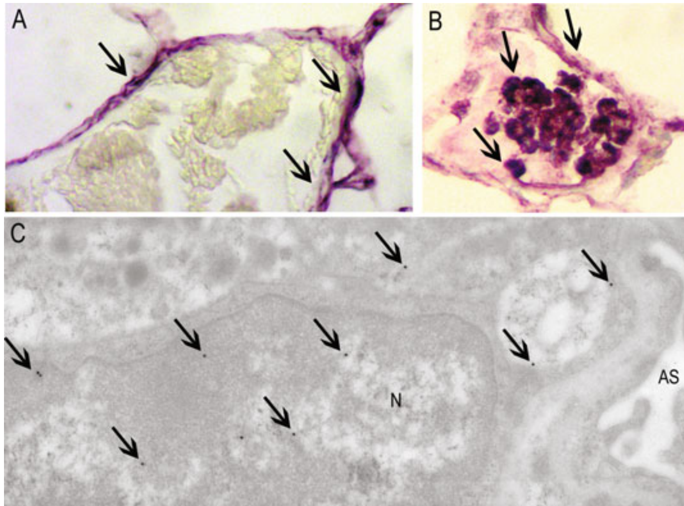

Fig. 2.3 TLR9 immunostaining of vascular endothelium in lungs of control (A) and LPS-treated horses (B) (noted by arrows). Immuno-electron microscopy (C) showed TLR9 staining in nucleus (N) of capillary endothelial cells in the lung (arrows). Original magnification A-B: ×400, C: ×10,000. Reprinted with permission from Anat Rec (Hoboken). 2009 Jul;292(7):1068–77. doi: https://doi.org/10.1002/ar.20927

cell surface membrane which are believed to help in phagocytosis (Atwal et al. 1992; Singh and Atwal 1997). Recently, PIMs were also identified in Water buffalo (Sethi et al. 2011; Fig. 2.4). Like other macrophage populations, PIMs are decorated with a number of TLR receptors (Wassef et al. 2004; Singh Suri et al. 2006; Schneberger et al. 2009, 2011; Sethi et al. 2011) (Fig. 2.5) and play a role in lung inflammation (Singh and de la Concha-Bermejillo 1998; Singh et al. 2004; Parbhakar et al. 2005), and phagocytosis (Atwal and Saldanha 1985). In disease, abrogation of PIMs can greatly reduce symptoms in recurrent airway obstruction in horses (Aharonson-Raz et al. 2012). Recently, we have reported the recruitment of PIMs in dogs that died due to acute necrotizing pancreatitis (Vrolyk et al. 2017). These data raise the intriguing possibility of PIM recruitment in domestic animal species that may not have constitutive PIMs and that the recruited PIMs may predispose these to higher susceptibility for lung inflammation and disease.

2.6 Culture of Endothelial Cells

Isolation and culture of equine endothelial cells has been described in detail (Lamar et al. 1986; MacEachern et al. 1997). Arterial endothelial cells are obtained by surgical removal of vessels and flushing them with PBS supplemented with antibiotics to remove any residual blood and potential bacterial contamination. Warmed DMEM with collagenase added is instilled in vessels which are clamped shut and

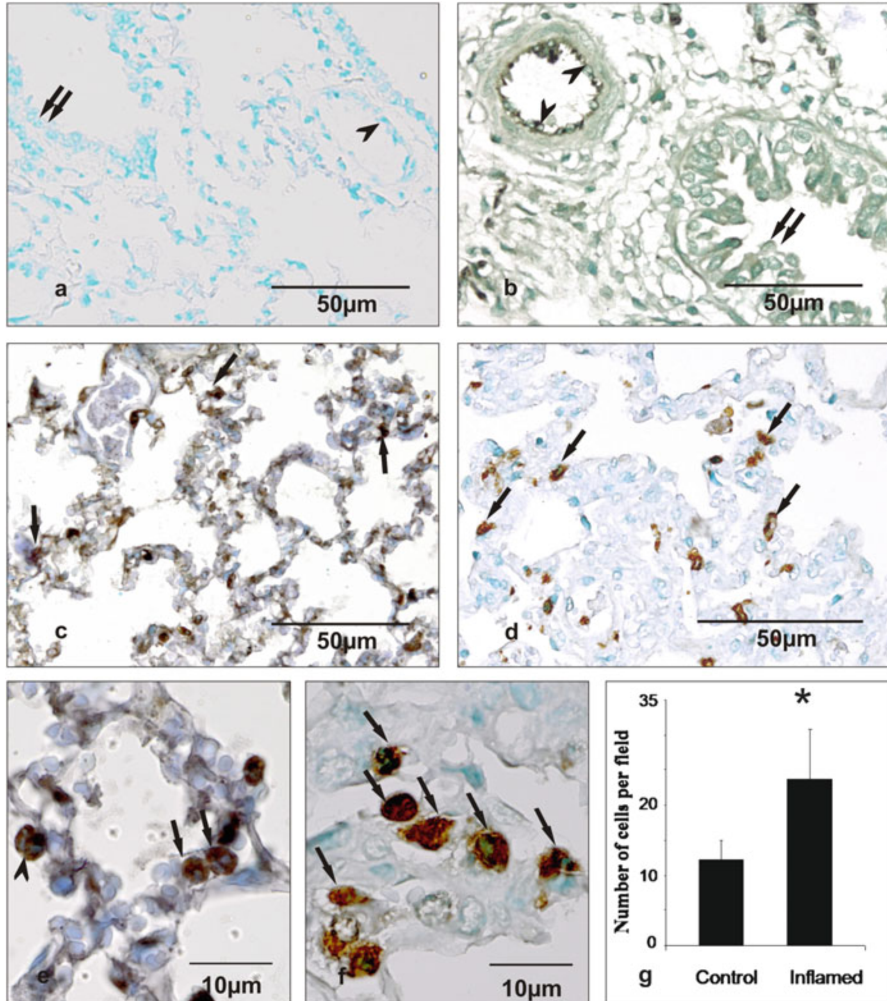

Fig. 2.4 (a) Lung section stained without primary antibody does not show any colour development in airways (double arrows) or bloodvessels (arrowheads). (b) Antibody specific for Factor VIII-related antigen labels vascular endothelium (arrowheads), but not the airways (double arrows). Macrophage antibody reacts with septal cells (arrows) in the normal lungs (c) and the infected lungs (d). (e) High magnification shows intravascular location of labelled macrophages (arrows) in the normal and (f) the infected lungs. An alveolar macrophage (arrowhead) is seen in (e). (g) The number of septal macrophages was significantly increased (P ¼ 0.001) in the inflamed lungs compared with the normal lungs. Reprinted with permission from J Comp Pathol. 2011 Feb–Apr;144(2–3):135–44. doi: https://doi.org/10.1016/j.jcpa.2010.08.003

incubated for 30–40 min at 37 °C. After incubation, vessels are rinsed emptied, media saved, and further washed with PBS and antibiotics. This wash is added to growth media and centrifuged to remove fluid, replacing it with final culture media of DMEM plus fetal calf serum and antibiotics. This is then cultured on 1% gelatin

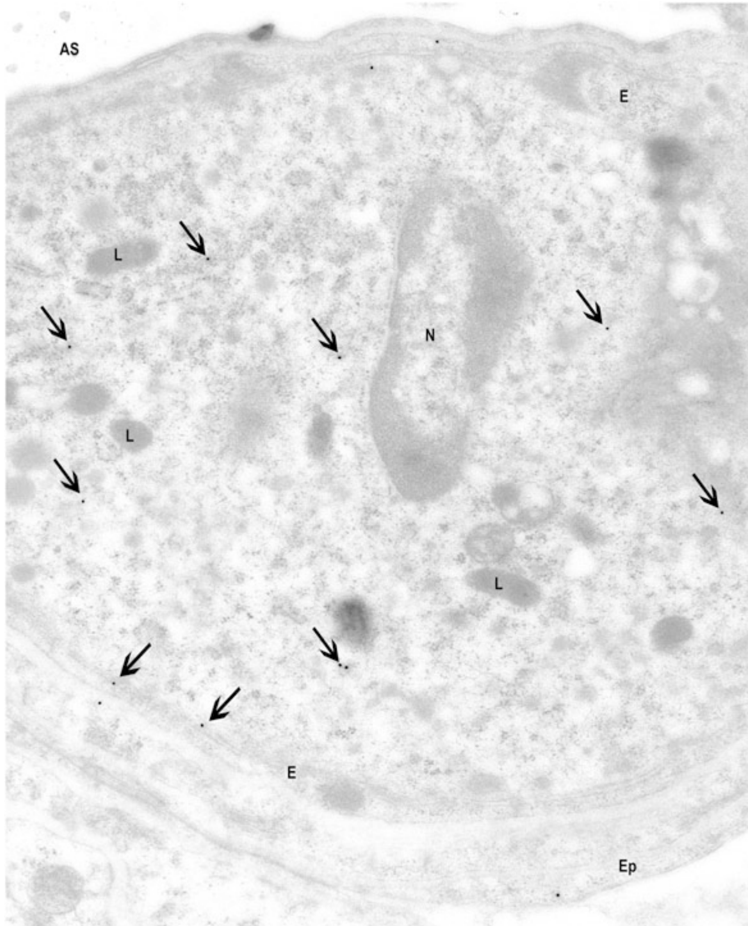

Fig. 2.5 TLR9 staining observed in a PIM and endothelium (E: arrows). L: Lysosomes; Ep: Epithelium; AS: Alveolar Space. Original magnification ×10,000. Anat Rec (Hoboken). 2009 Jul;292(7):1068–77. doi: https://doi.org/10.1002/ar.20927

coated plates, washing cells with PBS and replacing with fresh media until cells reach required density (MacEachern et al. 1997 for more details; Lamar et al. 1986).

This method yields primarily epithelial cells, but some smooth muscle cells will also be harvested in the process. Use of plasma-derived fetal bovine serum in media (10–20%) was able to inhibit growth of these smooth muscle cells, however, as opposed to equine-derived serum which stimulated this population as well as endothelial epithelium. It is believed that a lack of platelet-derived growth factors caused this inhibition of smooth muscle cells (Lamar et al. 1986). Cell type is verified by staining for vWF (Lamar et al. 1986).

Isolation procedures for lung microvascular endothelial cells will follow harvest and isolation techniques seen with capillaries in other tissues. Tissue is aseptically

obtained and minced or otherwise mechanically disrupted in Hank's balanced salt solution, often with addition of antibiotics similar to arterial methods to ensure sterility. This suspension is then treated with collagenase to digest connective tissue and heated for 30 min at 37 °C before passing through a nylon mesh. Filtrate is combined with equal an equal volume of DMEM with 10% fetal bovine serum. After 10 min, cells are centrifuged at low speed, resuspended in DMEM+FBS, and passed through a 30 μM filter. The filter is washed for clumps of endothelial cells which are saved, while flow through is discarded (containing erythrocytes and stromal cells). Harvested cells are washed, re-centrifuged, re-suspended in HBSS and 10 ml gently layered onto 35 ml HBSS + 5% BSA in centrifuged and left stand at room temperature for 15 min. The bottom 20–25 ml containing enriched endothelial cells is saved while the upper layer containing stromal cells is discarded. Cells are centrifuged, resuspended in DMEM + 10% FBS plus antibiotics and endothelial cell growth supplement and plated into dishes coated with fibronectin (Bochsler et al. 1989).

While some microvascular cultures methods will suggest vWF as a method of detection of these cells, expression in microvascular endothelial cells is less robust, and there are no signs of WPb in these cells. Some other suggested markers include uptake of low density lipopeptide and angiotensin converting enzyme in bovine models (Chung-Welch et al. 1988). While not specifically tested in equine, CD34 has also been proven effective at labeling endothelial cells (Muller et al. 2002), and unlike vWF, it appears to be more strongly expressed in microvascular endothelial cells. Similarly, discrimination of different lung endothelial cells has been done using lectins that selectively bind to each endothelial population in several species including cattle (Schnitzer et al. 1994; Magee et al. 1994; Abdi et al. 1995), though these can vary between species, and to our knowledge no screening study of these has yet been done for the horse.

2.7 Conclusions

There are good amounts of data on the morphology and function of lung vascular endothelium in domestic animals. However, there are significant gaps in the molecular phenotyping of lung vascular endothelium in domestic animals. This will always remain the case because of the challenge of creating knock-outs or abilities to functionally block a molecule for precise functional phenotyping. The strength of the data obtained from domestic animal species is their closer physiological relevance to human's pulmonary physiology. There are, however, instances where the data on cells such as PIMs is opening new areas to investigate mechanisms of lung inflammation in rodent models and humans. Therefore, an increased focus on the comparative physiology of domestic animal species and less reliance on rodent models may provide more translational data to understand human lung vascular physiology. The increased use of methods such as electron microscopy especially the correlative light and electron microscopy will yield more precise localization of molecules of functional interest in lung capillary endothelium.

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Chapter 3
The Pulmonary Vascular Barrier: Insights into Structure, Function, and Regulatory Mechanisms

Kaushik Parthasarathi

Abstract Pulmonary blood vessels act as a well-regulated barrier to the flux of fluid and solutes between the lumen and the air space. Perturbation of the barrier function results in excessive fluid leak into the interstitium and alveoli, and impairs gas exchange. Recent studies provide deeper insight into the precise control mechanisms involved in the regulation of the barrier. This chapter will highlight these mechanisms and discuss the current understanding on the fluid and solute transport pathways across the vascular endothelial layer. In addition, the chapter summarizes the contributions of extra-endothelial structures such as pericytes and glycocalyx in regulating fluid flux across pulmonary vessels. The chapter concludes with an analysis on the impact of pulmonary endothelial heterogeneity and experimental models on current interpretations of barrier function and regulatory mechanisms.

3.1 Introduction

The pulmonary vasculature forms a semi-permeable barrier and acts as the first line of defense that limits the movement of fluid and macromolecules from blood into the interstitium. When this protective barrier is impaired, fluid leaks across the vascular endothelium, resulting in accumulation of the fluid in the interstitial space and subsequently entering the pulmonary air spaces. Excessive leak that overwhelms the fluid clearance mechanisms of the pulmonary epithelial barrier (Vadasz and Sznajder 2017) leads to pulmonary edema.

Fluid flux across the pulmonary vascular barrier and development of pulmonary edema could be of either cardiogenic or non-cardiogenic origin. Cardiogenic pulmonary edema results from impairment of cardiac function, including myocardial

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infarction and congestive heart failure, which lead to elevation of hydrostatic pressure gradients in pulmonary microvessels (Cosentini et al. 2009). The increased pressure differences result in the leakage of fluid with low protein content, typically less than 65% compared to that in plasma into the interstitial space (Ware et al. 2010). In contrast, non-cardiogenic edema results from alterations in the permeability properties of the pulmonary vascular barrier. In response, fluid with protein content similar to that in plasma leaks into the interstitium (Ware et al. 2010; Weidenfeld and Kuebler 2017). Excessive accumulation of proteinaceous fluid in air spaces of the lung is a characteristic feature of acute lung injury and its more severe form, acute respiratory distress syndrome (Gonzales et al. 2015; Matthay et al. 2017; Wang et al. 2017). The accumulating fluid impairs gas exchange, resulting in arterial hypoxemia and hypercapnia (Matthay et al. 2012). The ensuing tissue hypoxia can in itself induce inflammation (Eltzschig and Carmeliet 2011; Eltzschig et al. 2014) and result in organ dysfunction. Given the important role of the pulmonary vascular barrier in limiting fluid flux, maintenance and tight regulation of the barrier is critical to maintaining proper lung function and homeostasis.

This chapter provides a broad outline of the current understanding of the features of the pulmonary vascular barrier, mechanisms that result in failure of the interendothelial junctions, and the role of the actin cytoskeleton. Mechanisms leading to the lung vascular barrier breakdown and emerging therapeutic mechanisms aimed at reversing excessive fluid flux are briefly discussed. Several recent reviews provide additional in-depth information on specific topics and are referred to throughout the article.

3.2 Transcellular Fluid–Flux Pathway

3.2.1 Aquaporins

Fluid and solute flux across the pulmonary vasculature could utilize either the paracellular or transcellular pathway. The transcellular fluid flux is considered to contribute minimally to the transport of water across the endothelial barrier. Transport via this pathway is mediated either via aquaporins or caveolae. Lung endothelial plasma membranes express aquaporin-1 (Schnitzer and Oh 1996; Bai et al. 1999; King et al. 2002). However, the role of aquaporin-1 as a mediator of osmotically driven water transport across the pulmonary vascular endothelium has remained controversial, due to the limited loss of fluid transport functions in aquaporin null mice (Verkman 2007). However, newer evidence point to a modification in the expression of aquaporin-1 in human lungs with diffuse alveolar damage (Pires-Neto et al. 2016). Whether the newer studies support or discount the role of aquaporin-1 in lung fluid balance remain to be seen.

3.2.2 Caveolae

In contrast to aquaporins, there is a growing body of evidence that suggest a role for caveolae in lung fluid balance. Caveolae are plasma membrane-associated flask-shaped pits, averaging 55 nm in diameter (Richter et al. 2008). Caveolae are abundant in multiple cell types including endothelial cells and are involved in diverse functions including mechanosensing, lipid regulation, and endocytosis (Heimerl et al. 2008; Hansen and Nichols 2010; Sinha et al. 2011; Parton and del Pozo 2013). Loss of caveolae results in cardiovascular dysfunction due to impaired nitric oxide and calcium signaling (Drab et al. 2001). In lungs, absence of proteins critical to the formation of caveolae causes dysregulation of endothelial proliferation and fibrosis (Drab et al. 2001; Hansen et al. 2013).

Endothelial caveolae play an important role in albumin transcytosis via endocytosis, trafficking across the cell, and exocytosis at the basolateral plasma membrane. Signaling associated with caveolae-mediated albumin transcytosis is increasingly better understood since the seminal descriptions of the process by Palade (Palade 1953, 1961; Milici et al. 1987). Caveolae coat proteins, caveolin-1, caveolin-2, and caveolin-3, mediate caveolae-dependent signaling (Le Lay and Kurzchalia 2005). Recent reviews provide extensive details on the structure and function of caveolins (Maniatis et al. 2012; Sowa 2012; Hansen et al. 2013; Parton and del Pozo 2013). Lack of caveolin-1 results in loss of caveolae in endothelial cells, which leads to reduced transcytosis of albumin (Li et al. 2013). Interestingly, loss of caveolin-1 results in elevated permeability via the paracellular route (Schubert et al. 2002), indicating that the two pathways regulating endothelial permeability are likely intertwined.

Caveolae-mediated albumin transcytosis is mediated by interaction of caveolin-1 with GTP-ases, rac, and dynamin (Armstrong et al. 2012) and requires integrated actions by actin cytoskeleton-associated proteins, including filamin A (Sverdlov et al. 2009). The transcytosis rate in pulmonary microvascular endothelial cells can be modulated by inflammatory agents such as lipopolysaccharide, thrombin, high-mobility group box protein 1, and paraquat, which act through intermediates such as nuclear factor-κB, acid spingomyelinase, advanced glycation end products, and C-src, respectively (Tiruppathi et al. 2008; Wang et al. 2015; Kuebler et al. 2016; Shang et al. 2016; Huang and He 2017). The findings from these reports suggest that albumin transcytosis plays a key role in augmenting transvascular fluid flux under inflammatory conditions. As discussed in other sections of this chapter, paracellular pathways for fluid flux are also enhanced by proinflammatory mediators. While arguments of the dominance of one mechanism over another have been posited, it is very likely that both mechanisms may act in tandem toward regulating fluid flux across the endothelial barrier. Thus, resolution of inflammation-induced increase in fluid leak may require a holistic approach targeting the elevated fluid flux via both para- and trans-cellular routes.

3.3 Paracellular Fluid–Flux Pathway

3.3.1 Adherens Junctions

Fluid flux via the paracellular route across the pulmonary vascular barrier is regulated by a complex set of interacting proteins. The interendothelial junction is populated by junctional complexes that include adherens junctions, tight junctions, and gap junctions. Adherens junctions of the vascular endothelium are primarily composed of vascular endothelial (VE)-cadherin, the extracellular domain of which binds homotypically at the interendothelial junctions (Taveau et al. 2008). Disruption of this VE-cadherin-mediated cell–cell interaction results in augmented permeability across the microvessel barrier (Corada et al. 2002), suggesting the critical role of VE-cadherin in the maintenance of this barrier. On the cytoplasmic side, VE-cadherin forms a complex with several catenin subtypes including α, β, γ, and p120 catenin. The catenins are targets for cell signaling during inflammation and thus serve as regulatory switches in the modulation of microvessel permeability. Recent reviews provide extensive details on the structure of VE-cadherin and its interaction with catenins (Quadri 2012; Vogel and Malik 2012; Dejana and Orsenigo 2013; Gavard 2014). Coupling between the VE-cadherin–catenin complex and actin cytoskeletal network lends stability to the adherens junction complex (Dorland and Huveneers 2017). Dynamic interaction between the actin cytoskeleton and the VE-cadherin–catenin complex (Oldenburg and de Rooij 2014; Schnittler et al. 2014) facilitates both the physiological and pathological modulation of the interendothelial junction strength.

Given the complex interplay among various components of the adherens junctions, it is no surprise that perturbations of any one component elicited via changes in protein expression can lead to disassembly of the junctions, and, thus, disruption of the pulmonary vascular barrier (Gao et al. 2000; Dejana and Orsenigo 2013). In addition to expression changes, redistribution of junctional components from the plasma membrane sites could also induce similar disruptions (Sawant et al. 2011). In this regard, redistribution of VE-cadherin from the membrane and, thus, loss of VE-cadherin homophilic interactions could lead to loss of barrier integrity. Relevant mechanisms that initiate the redistribution have been studied extensively (Iyer et al. 2004; Fainaru et al. 2008; Herwig et al. 2008; Ieguchi et al. 2013). Modulations in cytosolic calcium levels play an important role in inducing changes in VE-cadherin homophilic interaction and its redistribution. Calcium is essential for the maintenance of VE-cadherin homophilic interactions, with low cytosolic calcium levels leading to disassembly of VE-cadherins (Gao et al. 2000). However, calcium plays the role of a double-edged sword as an increase in endothelial cytosolic Ca^{2+} also leads to destabilization of VE-cadherin interactions.

In this context, mechanisms for cytosolic calcium increase include store-operated calcium entry, which is activated in response to depletion of endoplasmic calcium stores. Blocking store-operated calcium entry by inhibiting the proteins that mediate this process, including STIM1, the endoplasmic store calcium sensor, and Orai1, the

plasma membrane pore-forming component, (Ambudkar et al. 2017), both attenuate fluid leak across the pulmonary microvascular endothelium (Wang et al. 2016; Yazbeck et al. 2017). Interestingly, modulating events both upstream and downstream of STIM1-Orai1 also bears upon endothelial barrier strength. Inositol 1,4,5-trisphosphate, the messenger involved in triggering calcium release from endoplasmic reticulum stores, which is an early event leading to store-operated Ca^{2+} entry (Wu et al. 2014), causes clustering of its receptors on the endoplasmic reticulum. Inhibiting the receptor clustering blocks endoplasmic reticulum calcium release and blunts endothelial permeability (Geyer et al. 2015). Transient receptor potential channels facilitate calcium entry and a subset of these are triggered by store-operated calcium entry. One such channel that is thought to be downstream of STIM1 and Orai1 is transient receptor potential canonical 1 (Ambudkar et al. 2017), whose function is relevant in the context of pulmonary microvessels (Sundivakkam et al. 2012). Activation of this channel and the resulting increase in cytosolic calcium leads to perturbation of the microvascular barrier (Ahmmed and Malik 2005). However, the exact contributions of transient receptor potential channels to microvascular permeability and the specific mechanisms remain to be teased out further (Villalta and Townsley 2013; Malczyk et al. 2017). The presence of multiple transient receptor potential channels in the pulmonary vasculature, the heterogeneity in channel expression among the different regions of the pulmonary vasculature, and the overlap of triggering events complicate the delineation of channel contribution to pulmonary microvessel barrier disruption.

Increase in endothelial cytosolic calcium activate different signaling pathways that impact on the integrity of the adherens junctions. For an extensive delineation and discussion of the pathways, the reader is referred to comprehensive reviews on the topic (Tiruppathi et al. 2006; Ambudkar et al. 2017). Recent studies show that the integrity of adherens junctions is modulated precisely via tyrosine phosphorylation of the various components of these junctions, through the action of kinases, which in themselves are activated by elevated levels of endothelial cytosolic calcium (Adam 2015). For example, increases in cytosolic calcium by thrombin activate protein kinase C subtypes, which phosphorylate p120 catenin (Lucas et al. 2012; Vandenbroucke St Amant et al. 2012; Bijli et al. 2016). In addition to p120 catenin, phosphorylation of β-catenin and indeed VE-cadherin itself has also been shown to result in disassembly of adherens junctions (Cai et al. 2017; Muramatsu et al. 2017; Soni et al. 2017). Interestingly, tyrosine phosphorylation of VE-cadherin and catenins is only one of the multiple mechanisms associated with adherens junction disruption. Internalization of VE-cadherin and enzymatic cleavage of VE-cadherin also contribute to disassembly of adherens junctions and are reviewed elsewhere (Dejana et al. 2008). Intriguingly, it has recently been shown that phosphorylation of VE-cadherin in itself is only a necessary, but not a sufficient condition to induce disruption of adherens junctions (Orsenigo et al. 2012). In addition to phosphorylation, it has been posited that the presence of inflammatory agents is also required in order for an increase in microvessel permeability to occur. These reports suggest that our understanding of the barrier regulation via modulating adherens junction proteins is still incomplete and requires further elucidation.

3.3.2 Tight Junctions

Intertwined among the VE-cadherin interendothelial bridges are tight junction proteins, including claudins, occludins, and junction adhesion molecules (Tsukita et al. 2001; Dejana 2004). The cytoplasmic domain of tight junctions is linked to adaptor proteins including zona-occludins and cingulin (Bazzoni et al. 2000). Similar to adherens junctions, interaction among the transmembrane and cytoplasmic components of the tight junctions and the actin cytoskeleton regulates the strength of the microvessel barrier (Dejana 2004).

3.3.3 Actin Cytoskeleton

As can be evinced from the above discussion, the integrity and stability of both adherens and tight junctions is modulated by the actin cytoskeleton. In endothelial monolayers, stability of the junctional protein complexes is concomitant with the presence of cortical actin (Gulino-Debrac 2013). Disruption of the cortical actin layer in tandem with an increase in stress fiber formation increases stresses on the junctional complexes, resulting in their disassembly (Schnoor et al. 2017). The link between the junctional complexes and actin cytoskeleton is mediated mainly by actin-binding proteins zona occludin-1 and α-catenin, for tight and adherens junctions, respectively (Garcia-Ponce et al. 2015). The specific mechanisms via with these binding proteins function is still under much debate. The debate is much more rigorous since a classic study by Yamada et al. dented a long standing paradigm that α-catenin interacted simultaneously with cadherin and actin (Yamada et al. 2005). It is now emerging that the interaction may depend on the specific form of α-catenin involved (Desai et al. 2013). The complexity of making these determinations exemplifies the dynamic nature of the interactions between junctions and the endothelial cytoskeletal framework. In addition to α-catenin and zona occludin-1, a number of other actin-binding proteins, including vinculin and epithelial protein lost in neoplasm, modulate the actin-junctional complex interactions. For a detailed analysis of the mechanisms associated with these proteins, the reader is referred to other comprehensive reviews on this subject (Oldenburg and de Rooij 2014; Garcia-Ponce et al. 2015).

3.3.4 Small GTPases

Contractility of the actin cytoskeleton in itself is modulated by the Rho family of small GTPases (Duluc and Wojciak-Stothard 2014). In addition to the Rho family, the superfamily of small GTPases includes Rab and Ras families (Takai et al. 2001),

which too play a role in mediating endothelial permeability. Overexpression of Rab4 and Rab9, members of the Rab family involved in the regulation of intracellular vesicle trafficking, blunt endocytic uptake of VE-cadherin and thus disruption of the pulmonary endothelial barrier (Chichger et al. 2016). In contrast to Rab-4 and -9, knockdown of Rab11a protects against VE-cadherin recycling, and thus, limits fluid leak across pulmonary microvessels (Yan et al. 2016). Thus, the functional contribution of Rab GTPases to barrier enhancement or disruption appears to be heterogeneous among members of the family. Knockdown of R-Ras, a member of RasGTPases, also increases endothelial barrier permeability (Ichimiya et al. 2015; Vahatupa et al. 2016). In contrast to Rab- and Ras-GTPases, more detailed information is available on the role of RhoGTPases, likely due to their direct impact on the actin cytoskeletal dynamics (Wojciak-Stothard and Leiper 2008; Duluc and Wojciak-Stothard 2014). The Rho/Rac/CDC42 subfamily of the RhoGTPases act as intermediaries for a number of proinflammatory stimuli that disrupt the pulmonary vascular barrier. RhoA and its effector protein Rho kinase are activated by proinflammatory agents, such as histamine and lysophosphatidic acid (Mikelis et al. 2015; Cai et al. 2017). In contrast to Rho, activation of Rac and CDC42 blunt increases in pulmonary endothelial permeability. Rac1 mediates the endothelial barrier enhancing effects of sphingosine-1-phosphate (Singleton et al. 2005; Schlegel and Waschke 2014), and a reduction in Rac1 activity is associated with asymmetric methylarginine-induced pulmonary endothelial barrier breakdown (Wojciak-Stothard et al. 2009). Thus, a complex interplay among the small GTPases in lung endothelial cells allows for the dynamic regulation of the actin cytoskeleton and, thus, plays a role in both pulmonary endothelial barrier disruption and post-injury repair of the barrier.

In addition to RhoGTPases, cytosolic calcium increases via endoplasmic store calcium release could also modulate actin cytoskeleton dynamics. Induction of actin stress fibers in endothelial monolayers in response to thrombin and other proinflammatory agents that increase cytosolic calcium levels is well described (Park et al. 1999; Chiang et al. 2009; Shinde et al. 2013; Absi et al. 2014; Parker et al. 2015). Recent findings suggest that the responses to thrombin treatment are similar in in situ lung microvessels (Escue et al. 2017). Herein, the data revealed that thrombin infusions into microvessels increased endothelial F-actin levels, which in turn was dependent on the release of calcium from endoplasmic stores. However, direct evidence of the mechanisms that tie increased F-actin levels with calcium increase induced by store calcium release is lacking and needs to be elucidated. One possibility is to explore the role of heat shock protein 27, which regulates the actin cytoskeleton by stabilizing the cortical actin and limiting stress fiber formation (Piotrowicz and Levin 1997; Mounier and Arrigo 2002; Sawada et al. 2015). Inactivation of heat shock protein 27 by p38 mitogen-activated protein kinase in a calcium-dependent manner (Huot et al. 1997; An et al. 2005; Evans and Zhao 2017) could be a likely mechanism for actin stress fiber formation in response to proinflammatory mediators.

3.3.5 Gap Junctions

Gap junctions, the third interendothelial junctional complex, act as channels between adjacent cells (Kumar and Gilula 1996; Saez et al. 2003). These channels facilitate the movement of small molecules (<1 kDa) between the connected cells. Connexins, the primary components of gap junctions, assemble into hemichannels or connexins on the plasma membrane (Parthasarathi and Quadri 2009). Connexons, which are hexamers of connexins, diffuse along the plasma membrane toward the intercellular junction. Therein, the hemichannels from apposing plasma membranes then dock together to form a functioning channel. Connexin expression is ubiquitous, and accordingly, connexins are involved in wide-ranging roles from development to apoptosis (Cea et al. 2016; Buo et al. 2017; Uzu et al. 2017). In lung microvessels, gap junctions act as conduits for interendothelial transfer of calcium (Parthasarathi et al. 2006). Thus, gap junction-mediated transfer of calcium increases from one vessel to another could induce proinflammatory responses at the destination vessel. While three connexin subtypes, connexin37, connexin40, and connexin43, predominate the pulmonary vasculature, inhibition of connexin43-containing gap junctions completely abrogates the inter-vascular calcium transfer (Parthasarathi et al. 2006). These data suggest the importance of connexin43 in proinflammatory responses in lung microvessels. In addition, endothelial connexin40 has also been shown to facilitate intercellular propagation of hypoxia-induced membrane depolarization, resulting in vasoconstriction of upstream vessels (Wang et al. 2012).

However, emerging evidence suggest that in addition to their conduit function, gap junctions could act as regulators of permeability in microvessels (Parthasarathi 2012; Soon et al. 2016). Increases in microvascular permeability initiated by instillation of hydrochloric acid in adjoining alveoli are reversibly blocked by inhibiting connexin43-dependent intercellular communication using gap peptides specific to connexin43 (Parthasarathi 2012). This blunting of permeability increases is evident not only at the single microvessel level but also in larger regions of a lung subjected to injury via intra-tracheal acid instillation (Parthasarathi and Bhattacharya 2011). An intriguing nature of these findings is that the increased strength of the barrier is synchronous with the loss of gap junction communication. This raises the question on how loss of the conduit function of gap junctions impacts on the barrier protective function of adherens and tight junctions. It is possible that the physical interaction of gap junctions with tight and adherens junction proteins (Nagasawa et al. 2006; Chang et al. 2014; Ambrosi et al. 2016; Radeva and Waschke 2017) could underlie the functional interaction among these junctions. In brain and retinal endothelial cells, it has been posited that physical interaction between zona occludin-1 and connexin43 underlies the reduction in barrier strength when the interendothelial gap junction communication is blocked (Nagasawa et al. 2006; Tien et al. 2013). Thus, studies using endothelial cells of the systemic vasculature support the possibility of physical and functional interaction between gap junctions and other junctional proteins, albeit in a diametrically inverse direction. Loss of gap junction communication weakens the endothelial

barrier in the systemic circulation, while it strengthens the barrier in the pulmonary circulation, suggesting that the barrier regulatory mechanisms in the two circulations may be dissimilar. Support for functional interaction between gap and adherens junction in pulmonary microvessels comes from studies to determine the long-term effects of endotoxin. Airway instillation of endotoxin in rats caused an acute increase in microvessel permeability, which declined to baseline levels in 5 days (Kandasamy et al. 2015). In situ immunofluorescence of VE-cadherin and connexin43 expression in lung microvessels following endotoxin treatment revealed that VE-cadherin levels declined in the acute phase and returned to baseline levels, while connexin43 levels increased in the acute phase, but declined to near zero in 5 days (Kandasamy et al. 2015). The interaction between the two proteins is further supported by findings that show an increase in VE-cadherin levels in lung microvessels in response to in vivo knockdown of connexin43, elicited by tail vein injection of connexin43 shRNA. However, the intermediary between the loss of gap junction communication and increase in VE-cadherin expression remains to be delineated.

3.4 Glycocalyx

The luminal side of the vascular endothelium is layered with a variety of macromolecules, together termed the glycocalyx (Collins et al. 2013). The major constituent molecules of the glycocalyx are proteoglycans and glycosaminoglycan, whose composition is continually modified via self-assembly and shedding (Reitsma et al. 2007; Woodcock and Woodcock 2012). The glycocalyx extends into the interendothelial cleft and forms a physical and functional barrier against the movement of proteins across the vasculature (Reitsma et al. 2007; Dull et al. 2012). In systemic vessels, damage to the glycocalyx, as assessed by penetration of dextran molecules into the glycocalyx layer or presence of glycocalyx components in plasma, increases under inflammatory conditions such as sepsis and due to the presence of inflammatory mediators such as TNF-α (Henry and Duling 2000; Steppan et al. 2011), leading to increased microvascular permeability (Salmon and Satchell 2012). However, in the pulmonary microcirculation, evidence of a role for glycocalyx in permeability regulation has been at best, indirect. In human lung endothelial cell monolayers, knockdown of glycocalyx components increases fluid flux across the monolayer (Wu et al. 2017). Enzymatic degradation of sialic acid, a glycocalyx component that confers a protective negative charge, increases endothelial monolayer permeability and alveolar fluid filling in isolated lungs (Cioffi et al. 2012). Inhibiting enzymatic degradation of glycocalyx components by lipopolysaccharide limits the development of pulmonary edema (Wang et al. 2017). Thus, while these studies suggest the possibility that the glycocalyx may be involved in regulating lung microvessel permeability, additional studies are needed to strengthen this hypothesis and shed more light on the specific mechanisms and pathways involved.

3.5 Pericytes

Pericytes are located on the abluminal side of pulmonary microvessels and maintain close contact with the endothelium (Schallek et al. 2013). Their location and morphology pose difficulties in identifying and studying them in situ. However, a recent consensus has emerged that cells in close proximity to the endothelium and those that show reactivity to two markers, platelet-dependent growth factor receptor-β and nerve-glial antigen 2, could be classified as pericytes (Navarro et al. 2016). This newer definition has facilitated pericyte identification and characterization. The proposal that pericytes play a role in regulating fluid and solute flux across lung microvessels was presented more than 20 years ago (Lonigro et al. 1996). The ability of lung pericytes in culture to contract in the presence of certain inflammatory agents (Speyer et al. 1999; Donoghue et al. 2006; Kerkar et al. 2006), suggest that they may play an active, rather than passive role in permeability regulation in the lung. However, in vivo evidence that directly support a role for pericytes in pulmonary microvessel permeability remains elusive. Indirect evidence in this regard include recent data showing that lipopolysaccharide treatment results in loss of pericytes, microvessel dysfunction, and attendant increase in fluid leak in mice (Zeng et al. 2016). Further, experiments using endothelial-pericyte coculture in a three-dimensional culture model reveal that the presence of pericytes enhances endothelial junctional protein coupling and reduction in permeability (Bichsel et al. 2015). In contrast to these studies, recent findings that ablation of pericyte-like cells in mouse lungs using diphtheria toxin does not modify the protein content in bronchoalveolar lavage (Hung et al. 2017a), oppose the notion that pericytes are involved in permeability regulation in lungs. These divergent findings could be due to the emerging role of pericytes as active mediators of inflammatory responses. Accordingly, in response to lipopolysaccharide treatment, pericytes in murine lungs have been shown to secrete chemokines (Hung et al. 2017b). Hence, it is possible that pericytes play a more active role in an inflammatory milieu. Additional novel approaches are thus needed to specifically address the role pericytes in lung microvessel permeability.

3.6 Microvessel Heterogeneity

To fully appreciate the signaling mechanisms relevant to the pulmonary endothelial barrier that have been elucidated thus far, two caveats need to be borne in mind. One is the heterogeneous nature of the vascular endothelium, both in terms of the molecular properties and function. In a broad sense, it is well established that significant differences exist between the pulmonary, systemic, and lymphatic vasculature (Potente and Makinen 2017). Juxtaposing characteristics of the vasculatures include hypoxia-induced changes in vascular dimensions, sites of leukocyte retention and migration, and proteoglycan expression (McMahon et al. 2002;

Paffett and Walker 2007; Calabrese et al. 2011; Gane and Stockley 2012). It has been shown recently that inflammatory stimuli elicit heterogeneous responses in different vascular beds (Scott et al. 2013). Even within the pulmonary vasculature, a great degree of phenotypic heterogeneity is evident (Stevens 2011). Expression of proteins including, endothelial nitric oxide synthase, nucleosome assembly protein-1, transient receptor potential channels, and Weibel–Palade bodies differ between pulmonary arteries and microvessels (Stevens 2005; Clark et al. 2008; Cioffi et al. 2009; Ochoa et al. 2010). Further, vascular barrier strength differs between extra-alveolar vessels and septal capillaries (Lowe et al. 2007). Interestingly, endothelial heterogeneity extends even to single pulmonary microvessels. Within single post-capillary venules, some endothelial cells may act as pacemaker cells (Ying et al. 1996). The pacemakers may have a greater number of organelles and thus, exhibit exaggerated responses to inflammatory stimuli in comparison to their neighboring endothelial cells (Parthasarathi et al. 2002; Parthasarathi 2012). In addition to pulmonary microvessels, pulmonary arteries too exhibit regional differences in reactivity (Stack et al. 2016). The extensive inter- and intra-vascular heterogeneity behooves us to limit broad generalizations of data on signaling and mechanisms captured using vessels or cells from a single pulmonary bed. By the same token, the heterogeneous nature of the pulmonary vasculature needs to be implied when extending molecular and functional properties specific to a subsection of the pulmonary vasculature to other subsections.

3.7 Endothelial Monolayers Versus Intact Lung Models

A second caveat involves the differences between data derived using pulmonary endothelial cells and vessels in situ. Due to methodological difficulties associated with characterizing signaling mechanisms in lung vasculature in situ, the bulk of the reported studies are based on monolayers of endothelial cells isolated from pulmonary arteries or microvessels. Alternatives to monolayer-based elucidations include the isolated blood-perfused lung preparation and surgically implanted thoracic window preparation, wherein the endothelial cell mechanisms can be observed in situ (Tabuchi and Kuebler 2008; Parthasarathi 2012; Kandasamy and Parthasarathi 2014). Though these alternatives do have their own limitations, they offer the added advantage of being able to specify the specific subsection of the pulmonary vasculature from which the data is obtained and thus limit errors due to endothelial heterogeneity (Kandasamy and Parthasarathi 2014). A second advantage of the alternative strategies is that the endothelial cells remain in their native environment. The disadvantages of the intact lung preparations are that they are complex and expensive, which likely limits their use. However, though widely utilized, growing evidence indicate that signaling mechanisms delineated using endothelial cells grown in monolayers differ significantly from those defined using in situ endothelial cells (Kandasamy et al. 2013; Uhlig et al. 2014; Aman et al. 2016). In addition to endothelial heterogeneity, a possible reason for the differences in signaling could

be that endothelial cells in situ interact with other cells and the matrix within their microenvironment (Aman et al. 2016), a feature that is not available for cells in culture. These differences raise the question on how representative are the data derived using endothelial monolayers. Our studies using isolated lung preparations show that the overall signaling pathways defined in single microvessels concur with that from endothelial monolayers. However, the functional, temporal, and spatial aspects of the signaling are different between the two systems (Kandasamy et al. 2013; Escue et al. 2017). To mitigate these issues, alternate experimental approaches are needed for studies on pulmonary endothelial signaling. Recent advances, such as endothelial–epithelial cocultures, transwell culture methods, and microfluidic platforms (Cao et al. 2016; Chonan et al. 2017; Kim et al. 2017), may well provide a middle path that facilitates generation of more physiologically accurate data.

3.8 Conclusion

Dysregulated increases in pulmonary microvascular permeability contribute to reduced quality of life and increased mortality in affected patients. In addition to microvessels, pathological increases in permeability also occur in pulmonary arteries, which could play a role in pulmonary artery hypertension (Zhou et al. 2016; Satoh et al. 2017). Thus, blunting and eventually reversing pathophysiological disruptions of the pulmonary vascular barrier is key step to restoring normal lung function. Toward this end, several potential targets have been proposed, though their success in mitigating lung injury in clinical practice remains elusive (Bosma et al. 2010; Confalonieri et al. 2017). Pharmacological therapies remain unsuccessful (Matthay et al. 2017) and could be due to the possibility that the underlying causes of endothelial barrier dysfunction are heterogeneous. Intriguingly, several of the proposed therapeutic possibilities have been proven to successfully mitigate vascular dysfunction in animal and cell-based models and yet have failed to yield similar success in clinical trials (Network 2000; Jerng et al. 2006; Rice et al. 2011; Schwartz et al. 2015; Evans and Zhao 2017; Nagendran et al. 2017). Thus, newer therapies based on novel approaches, which take into account pulmonary vascular heterogeneity discussed above, are critically needed. In this regard, use of mesenchymal stem cells toward repairing the endothelial barrier shows promise with success in preclinical trials (Huppert and Matthay 2017). Similar novel and well-designed approaches could well serve to improve quality of life and increase survival in patients afflicted by pulmonary microvascular dysfunction.

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Chapter 4
Pulmonary Endothelial Cell Apoptosis in Emphysema and Acute Lung Injury

Eboni Chambers, Sharon Rounds, and Qing Lu

Abstract Apoptosis plays an essential role in homeostasis and pathogenesis of a variety of human diseases. Endothelial cells are exposed to various environmental and internal stress and endothelial apoptosis is a pathophysiological consequence of these stimuli. Pulmonary endothelial cell apoptosis initiates or contributes to progression of a number of lung diseases. This chapter will focus on the current understanding of the role of pulmonary endothelial cell apoptosis in the development of emphysema and acute lung injury (ALI) and the factors controlling pulmonary endothelial life and death.

List of Abbreviations

AAT	Alpha1-anti-trypsin
ADA	Adenosine deaminase
ADP	Adenosine-5′-diphosphate
APC	Activated protein C
ARDS	Acute respiratory distress syndrome
ARs	Adenosine receptors
ATF6	Transcription factor 6
ATGs	Autophagy-related genes
ATP	Adenosine-5′-triphosphate
BALF	Broncheoalveolar lavage fluid
CD39	ecto-5′-nucleotidase
CD73	ecto-5′-nucleotidase
CHOP	C/EBP homologous protein
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoke
CSE	Cigarette smoke extract

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DAMPs	Damage associated molecular patterns
EC	Endothelial cells
ECM	Extracellular matrix
eIF2α	Eukaryotic initiation factor 2α
ENT1/2	Equilibrative nucleoside transporter 1/2
ER	Endoplasmic reticulum
FAC	Focal adhesion complexes
FAK	Focal adhesion kinase
GSH	Glutathione
ICMT	Isoprenylcysteine-O-carboxyl methyltransferase
IRAK-1	Interleukin (IL)-1 receptor associated kinase
IRE1	Inositol-requiring enzyme 1
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MLKL	Mixed lineage kinase domain-like protein
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation factor 88
PERK	Pancreatic ER kinase like ER kinase
RBC	Red blood cells
RIPK1/3	Receptor-interacting protein kinase 1 and 3
ROS	Reactive oxygen species
S1P	Sphingosine 1-phosphate
SAH	S-adenosyl-L-homocysteine
SAHH	S-adenosyl-L-homocysteine hydrolase
SAM	S-Adenosyl-L-Methionine
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
TRAF-6	TNF receptor associated factor-6
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VEGFR2	VEGF receptor type 2

4.1 Overview of Cell Death

4.1.1 Apoptosis

Apoptosis is a term first used by Kerr et al. in 1972 to describe a genetically determined energy-dependent active form of programmed cellular suicide. Apoptosis is characterized by well-ordered morphologic and molecular features including: cell surface exposure of phosphatidylserine, plasma membrane blebbing, cell shrinkage, cytoskeletal rearrangement, collapse of nuclear membrane, chromatin condensation, DNA fragmentation, and formation of membrane bound fragments known as "apoptotic bodies" (Kerr et al. 1972). Cell surface-exposed phosphatidylserine acts as a chemoattractant for phagocytes to engulf and clear apoptotic bodies (Henson and Tuder 2008). Apoptosis serves to eliminate unwanted, aged, harmful, injured, or infected cells. Due to limited release of intracellular contents, minimal inflammation occurs (Savill et al. 2002). However, if ingestion of apoptotic bodies by monocytes, macrophages, and dendritic cells (efferocytosis) is impaired, inflammation and

autoimmunity may be enhanced (Gaipl et al. 2006). Apoptosis plays an essential role in the maintenance of tissue homeostasis and embryonic development. Further, during embryonic development, the timing of apoptosis is genetically determined. Excessive or inadequate apoptosis can, however, contribute to the pathogenesis of a variety of human diseases. Apoptosis is triggered by external stressors (e.g., death ligands, ultraviolet, and γ radiation) and/or internal stimuli (e.g., oxidants, DNA damage, increased Ca^{2+}). Apoptosis is processed by two fundamental signaling pathways: the death receptor-mediated extrinsic pathway and the mitochondria-dependent intrinsic pathway (Olson and Kornbluth 2001; Thorburn 2004). Extrinsic pathway-activated caspase-8 can truncate and activate BID, thus activating the intrinsic pathway (Li et al. 1998). The details on regulation of apoptosis have been reviewed (Harrington et al. 2007; Subramanian and Steer 2010; Ola et al. 2011). Therapies targeting regulators of apoptosis have been used in preclinical and clinical trials for a variety of diseases including the treatment of cancers (Goldar et al. 2015).

4.1.2 Necrosis

Necrosis is a passive and caspase-independent cell death, characterized by cell swelling, mitochondrial degeneration, impaired ATP generation, lysosomal leakage, early rupture of plasma membranes, random fragmentation/degradation of DNA, and leakage of cellular contents into the surrounding environment (Henriquez et al. 2008). Necrosis is usually induced by nonspecific and non-physiological stress. Further, inhibition of caspases leads to necrosis (Henriquez et al. 2008). Due to release of potentially pro-inflammatory and pro-immunogenic cellular contents into surrounding tissues, necrosis often induces inflammation, autoimmune responses, and is often seen concomitant with apoptosis.

4.1.3 Necroptosis

Necroptosis describes a type of active, regulated, and programmed necrosis dependent upon the serine/threonine kinase activity of receptor-interacting protein kinase 1 and 3 (RIPK1/3) (Linkermann and Green 2014). Necroptosis and apoptosis share several upstream signaling elements including death receptors caspase 8 and FLIP. When caspase-8 is inhibited, RIPK1 is activated and forms an intracellular complex with RIPK3 to assemble the necrosome, leading to phosphorylation of mixed lineage kinase domain-like protein (MLKL) and ultimately cell death. Unlike apoptosis, necroptosis promotes harmful innate and adaptive immunologic responses by releasing damage associated molecular patterns (DAMPs). Thus, the reduction of necroptosis might be beneficial by minimizing the release of DAMPs

and proinflammatory responses. Necroptosis is, however, a defense mechanism against invading microbes, including viral infections, and promotes the death and removal of virally infected cells. Therefore, blockade of necroptosis may increase susceptibility to viral infections particularly in patients with suppressed immunity. A number of inhibitors of necroptosis, such as necrostatin (specific inhibitor for RIPK1) and necrosulfonamide (specific inhibitor for human MLKL), have been described, providing potential therapeutic tools for treatment. Given the complex role of necroptosis, tissue and cell-specific targeting therapy is needed.

4.1.4 Endoplasmic Reticulum Stress-Induced Apoptosis

The endoplasmic reticulum (ER) is the site of posttranslational modifications and folding of secreted and membrane proteins. A variety of insults, such as ER Ca^{2+} chelators, reducing agents, glucose starvation, glycosylation antagonists, and protein mutations, can disrupt ER protein folding and lead to an accumulation of unfolded or misfolded proteins in the ER, thus initiating ER stress (Schroder and Kaufman 2005). Cells respond to ER stress by the unfolded protein response (UPR). The UPR includes three arms: pancreatic ER kinase (PKR)-like ER kinase (PERK)/ eukaryotic initiation factor 2α (eIF2α), transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (Schroder and Kaufman 2005). Through the UPR, cells attempt to restore ER homeostasis in order to maintain cell survival by inhibiting global protein synthesis (to reduce the loading of client protein to the ER for folding), enhancing ER protein folding capacity, and promoting ER-associated degradation of misfolded or unfolded proteins (Schroder and Kaufman 2005).

Prolonged ER stress causes cell death due to simultaneous activation of multiple apoptotic pathways by the UPR (Szegezdi et al. 2006). PERK-induced phosphorylation of eIF2α can lead to apoptosis by induction of pro-apoptotic transcription factor, C/EBP homologous protein (CHOP), which suppresses expression of anti-apoptotic protein, Bcl-2. Activated IRE1 activates c-Jun N-terminal kinase (JNK), which causes apoptosis by phosphorylation and thus inactivation of Bcl-2 and by phosphorylation and thus activation of pro-apoptotic protein, Bim. In addition, increased Ca^{2+} in the ER activates the death effector, Bax/Bak in the ER membrane, causing movement of Ca^{2+} from the ER to the mitochondria leading to mitochondrial-dependent apoptosis. ER membrane-localized caspase-12 (rodent) and caspase-4 (human) have also been implicated in ER-stress-induced apoptosis (Szegezdi et al. 2003; Kim et al. 2006). Caspase-12/-4 are cleaved and thus activated by the Ca^{2+}-dependent protease, m-calpain, by ER stress (Groenendyk and Michalak 2005). However, other studies have suggested that ER stress-induced apoptosis depends upon the apoptosome and not caspase-12/-4 (Obeng and Boise 2005; Di Sano et al. 2006).

Cell fate determination is not well understood when both survival (adaptive) and apoptotic pathways are simultaneously activated. It has been proposed that persistent ER stress causes apoptosis due to sustained induction of CHOP and instability of the adaptive pathway (Lin et al. 2007). It has also been suggested that cells

survive mild ER stress because of the short half-life of pro-apoptotic proteins, compared to pro-survival proteins (Rutkowski et al. 2006). Robust prolonged ER stress causes apoptosis due to the induction of CHOP excessive to its degradation (Rutkowski et al. 2006).

4.1.5 Autophagy-Associated Cell Death

Autophagy is a dynamic and continuous process by which cells dispose of damaged or unneeded cellular proteins or organelles (mitochondria) by self-digestion to generate intracellular nutrients. During physiological conditions, autophagy is suppressed by mammalian target of rapamycin (mTOR), thus inhibiting the expression of autophagy-related genes (ATGs). Upon external or internal stress: including nutrient starvation, growth factor deprivation, hypoxia, ischemia, or mitochondrial aging, mTOR is inhibited thus initiating autophagy. Autophagy is a multistep sequential process, consisting of the formation of double-membrane vesicles that sequester unwanted cargo (proteins or mitochondria) in autophagosomes, fusion of autophagosomes with endosomes or lysosomes to form amphisomes or autolysosomes, and digestion of cargo by proteases (Hotchkiss et al. 2009; Choi et al. 2013). Autophagy is an evolutionarily conserved housekeeping process that allows recycling of damaged proteins and organelles in order to maintain homeostasis. Impairment in any step of autophagy causes cellular nutrient deficiency and/or accumulation of damaged proteins and organelles leading to cell death (Hotchkiss et al. 2009). Whether autophagy promotes cell survival or death may depend on cell type and setting (Gustafsson and Gottlieb 2008).

4.1.6 Assessments of Cell Death

Based on the unique characteristics of different types of cell death, a variety of assays have been developed to assess the specific types of cell death in vivo and in vitro. Different types of cell death may share common characteristics at different stages of cell death; therefore, it is often necessary to use multiple assays to confirm cell death. The details on the assessments of cell death have been extensively reviewed (Harrington et al. 2007; Henson and Tuder 2008; Lu and Rounds 2009; Klionsky et al. 2016) and will not be discussed in this review.

4.2 Pulmonary Endothelial Cell Apoptosis

Balance of endothelial cell survival and death is crucial for angiogenesis, vessel regression, and barrier function. Due to the unique position of endothelial cells (EC) at the interface of circulating blood and surrounding tissues, EC may be exposed to various environmental stress including: hypoxia, hyperoxia, oxidants, lipopolysaccharide (LPS), and cigarette smoke (CS), or internal stress including: adenosine, ceramide, tumor necrosis factor (TNF)-α, and angiotensin II. Apoptosis is a pathophysiological consequence of these stimuli. However, a variety of biomechanical and biochemical factors are involved in the anti-apoptotic processes. For example, physiological levels of shear stress and cyclic strain, vascular endothelial growth factor (VEGF), focal adhesion kinase (FAK), activated protein C (APC), and sphingosine 1-phosphate (S1P) protect EC against apoptosis. The pro- and anti-apoptotic effects of these mediators have been reviewed (Harrington et al. 2007; Lu and Rounds 2009); therefore, this review will focus on the current understanding of endothelial pro-survival factors (VEGF and FAK) and apoptosis-inducing stress (adenosine, cigarette smoke, and LPS) in the lungs.

4.2.1 Vascular Endothelial Growth Factor

EC express abundant VEGF, which promotes EC survival and maintains normal alveolar structure (Voelkel et al. 2006). Expression of both VEGF and VEGF receptor type 2 (VEGFR2) are decreased in lung tissue of patients with chronic obstructive pulmonary disease (COPD) (Kasahara et al. 2001). This diminished VEGF/VEGFR2 signaling is inversely associated with increased lung EC apoptosis (Kasahara et al. 2001). Lung-targeted inhibition of VEGF or VEGFR2 causes alveolar septal cell apoptosis in mice (Kasahara et al. 2000; Tang et al. 2004). Our group has also shown that blockade of VEGFR2 causes cultured pulmonary artery EC apoptosis in vitro (Lu 2008). These results indicate that VEGF signaling is essential for lung EC survival.

4.2.2 Focal Adhesion Kinase

EC are linked to the basement membrane through binding of cell surface expressed integrins to extracellular matrix (ECM) proteins at focal adhesion complexes (FAC) (Hynes 1992). As anchorage-dependent cells, EC undergo detachment-initiated apoptosis, referred to as anoikis, upon loss of adhesion to underlying basement membrane. FAK, a non-receptor tyrosine kinase and an essential component of FAC, is activated upon integrin engagement of ECM (Guan et al. 1991; Guan and Shalloway 1992; Parsons 2003). FAK provides survival signaling for anchorage-dependent cells such as cultured fibroblasts (Hungerford et al. 1996). Similarly, EC

isolated from FAK-null embryos undergo apoptosis (Ilic et al. 1995, 2003). Endothelium-specific deletion of FAK (Cre/FAK^{flox}) is embryonic lethal and causes EC apoptosis (Shen et al. 2005; Braren et al. 2006). Guan and colleagues (Guan et al. 1991; Guan and Shalloway 1992) have demonstrated that FAK tyrosine kinase activity is essential for FAK activity. FAK promotes cell survival by recruiting proteins containing SH2 domain including Src and phosphatidylinositol-3-kinase (PI3K) (Schaller et al. 1994). The activated PI3K recruits and activates Akt (Khwaja et al. 1997), which promotes cell survival via phosphorylation and thus inhibition of pro-apoptotic protein, Bad (Kennedy et al. 1997). FAK also promotes survival by activation of NF-κB and ERK signaling pathways (Huang et al. 2007). Additionally, FAK can translocate to the nucleus and inhibit p53 transcriptional activation and enhance p53 degradation, leading to protection against apoptosis (Ilic et al. 1998).

4.2.3 Adenosine

Adenosine is generated from adenosine-5′-triphosphate (ATP) and adenosine-5′-diphosphate (ADP) by extracellular ecto-5′-nucleotidases, CD39 and CD73, and is metabolized by adenosine deaminase (ADA). Extracellular adenosine exists in low concentrations (40–600 nM) under physiological conditions and is increased due to platelet degranulation, cell necrosis, activation of CD39 and/or CD73, or inhibition of ADA (Thompson et al. 2004; Eltzschig et al. 2006; Volmer et al. 2006; Eckle et al. 2007). Increased extracellular adenosine can interact with cell surface G-protein coupled adenosine receptors (ARs) (Feoktistov et al. 2002; Wyatt et al. 2002; Umapathy et al. 2010). Activation of adenosine receptors, specifically A_3-mediated signaling, has been shown to protect against apoptosis and tissue injury (Rivo et al. 2004; Chen et al. 2006; Matot et al. 2006).

However, sustained increased adenosine in ADA-deficient mice enhances alveolar cell apoptosis (Zhou et al. 2009). We have also shown that prolonged exposure to adenosine causes apoptosis of cultured lung EC (Lu et al. 2013). The injurious effect of adenosine is mediated by equilibriative nucleoside transporters. EC predominantly express equilibriative nucleoside transporter 1 (ENT_1) and ENT_2 (Archer et al. 2004). Upon sustained exposure, adenosine may be taken up into cells by ENTs. Further, similar to other G-protein coupled receptors, prolonged engagement of ARs causes receptor desensitization and internalization (Fredholm et al. 2001). This concept is supported by findings that sustained increased adenosine in ADA-deficient mice enhances alveolar cell apoptosis via a mechanism independent of adenosine receptor, $A_{2B}R$ (Zhou et al. 2009). In addition, sustained exposure to adenosine causes endothelial cell apoptosis; this effect is prevented by inhibition of $ENT_{1/2}$ however exacerbated by inhibition of either $A_{2A}R$ or $A_{2B}R$ (Lu et al. 2013). These results are consistent with the concept that $ENT_{1/2}$-facilitated intracellular adenosine uptake and subsequent metabolism mediates adenosine-induced EC apoptosis, whereas AR-mediated signaling limits apoptosis (Simonis et al. 2009).

Once intracellular, adenosine reacts with homocysteine and generates S-adenosyl-L-homocysteine (SAH) by inhibition of SAH hydrolase (SAHH). SAH

induces endothelial cell apoptosis independent of homocysteine (Sipkens et al. 2012). SAH is also a product of carboxyl methylation with S-adenosyl-L-methionine (SAM) as a methyl donor. We have demonstrated that exogenous adenosine causes lung EC apoptosis via increased ratio of intracellular SAH to SAM (Rounds et al. 1998). The increased ratio of SAH to SAM suppresses carboxyl methyltransferase activity. Isoprenylcysteine-O-carboxyl methyltransferase (ICMT) is a major methyltransferase for carboxyl methylation of small GTPase, Ras (Clarke 1992), which is a posttranslational modification essential for membrane localization and activation of Ras (Boivin and Beliveau 1995; Fleming et al. 1996; Kranenburg et al. 1997; Michaelson et al. 2001). We have shown that exogenous adenosine causes lung EC apoptosis in part by ICMT inhibition-mediated inhibition of Ras carboxyl methylation and activation (Kramer et al. 2003).

SAM is a precursor to glutathione (GSH) and is synthesized exclusively in the cytosol (Reytor et al. 2009) and also transported into mitochondria (Agrimi et al. 2004). Exogenous SAM has been shown to elevate GSH levels in vivo and prevent alcohol-induced mitochondrial oxidative stress and dysfunction as well as liver and lung injury in animal models (Holguin et al. 1998; Bailey et al. 2006; Cederbaum 2011). p38 is a redox-sensitive protein (Matsuzawa and Ichijo 2008). Reactive oxygen species (ROS)-mediated p38 activation has been implicated in extracellular ATP-induced macrophage apoptosis (Noguchi et al. 2008) and H_2O_2-induced EC apoptosis (Machino et al. 2003). Activation of p38 has also been implicated in homocysteine-induced apoptosis of endothelial progenitor cells (Bao et al. 2010) and cardiomyocytes (Wang et al. 2011). We have shown that sustained exposure to exogenous adenosine causes mitochondrial defects and endothelial apoptosis via mitochondrial oxidative stress-induced activation of p38 (Lu et al. 2012, 2013). Active p38 causes apoptosis by direct phosphorylation, and thus inhibition of Bcl-2 (De Chiara et al. 2006; Farley et al. 2006) and by increasing mitochondrial translocation of Bax (Capano and Crompton 2006). Future studies are needed to address whether sustained adenosine exposure reduces mitochondrial SAM, thus leading to mitochondrial oxidative stress via increased ratio of SAH to SAM in the cytosol.

In summary, adenosine displays seemingly paradoxical effects on lung EC life and death. Acute exposure protects EC against apoptosis via AR-mediated signaling, whereas prolonged exposure causes EC apoptosis via $ENT_{1/2}$-mediated intracellular adenosine uptake and subsequent metabolism and mitochondrial oxidative stress.

4.2.4 Cigarette Smoke

Lung EC apoptosis is significantly elevated in human smokers with emphysema (Kasahara et al. 2001) and mice with mild emphysema caused by CS exposure (Sakhatskyy et al. 2014). We (Sakhatskyy et al. 2014) and others (Tuder et al. 2000; Damico et al. 2011) have shown that CS extract (CSE) causes cultured lung macro- and microvascular EC apoptosis in vitro. The mechanisms underlying CS-induced lung EC apoptosis are rather complicated and involve FAK, p53, UPR, and autophagy.

FAK is a survival signal for anchorage-dependent cells (Hungerford et al. 1996). Tyrosine 397 phosphorylation of FAK is essential for its activation (Schaller et al. 1994). CSE decreases FAK phosphorylation at tyrosine-397 in an oxidative stress-dependent manner (Lu et al. 2011)—essential in CSE-induced EC apoptosis (Sakhatskyy et al. 2014). FAK also promotes cell survival via suppression of p53 (Ilic et al. 1998). Further, activation of p53 has contributed to CSE-induced pulmonary EC apoptosis (Damico et al. 2011). Thus, we speculate that CSE causes lung EC apoptosis via oxidative stress-mediated inhibition of FAK and subsequent activation of p53.

The UPR is an important mechanism of the elimination of ER stress and enhanced cell survival (Schroder and Kaufman 2005). The UPR is activated in lung tissue of smokers who do not have emphysema (Kelsen et al. 2008). The UPR is also activated by CSE in cultured human bronchial epithelial cells and 3T3 fibroblasts (Hengstermann and Müller 2008; Jorgensen et al. 2008) and cultured pulmonary EC (Sakhatskyy et al. 2014). Using mouse models of CS exposure, we have demonstrated a strong link between impairment of eIF2α signaling with lung EC apoptosis (Sakhatskyy et al. 2014). Future studies are necessary to determine if impaired eIF2α signaling contributes to lung EC apoptosis.

Autophagy is increased in response to deficiencies in extracellular and intracellular nutrients. Enhanced autophagy is observed in the lung tissue of smokers with emphysema (Chen et al. 2008). Autophagy is also activated by CSE exposure in lung epithelial cells and fibroblasts (Kim et al. 2008) as well as lung EC (Sakhatskyy et al. 2014). Increased autophagy has contributed to CS-induced alveolar epithelial cell apoptosis in mice (Chen et al. 2010). In contrast, increased autophagy has also been shown to protect against pulmonary endothelial cell apoptosis induced by cadmium, a component of cigarette smoke (Surolia et al. 2015). We have reported that autophagy was not altered in the lung tissue of a mouse strain susceptible to CS-induced lung EC apoptosis and emphysema (Sakhatskyy et al. 2014). The role of autophagy in CS-induced apoptosis may be dependent on cell types and stimuli.

Due to open structure and limited repair capacity, mitochondrial DNA is 50 times more sensitive to oxidative damage than nuclear DNA (Yakes and Van Houten 1997). Oxidative stress-induced mitochondrial DNA damage triggers mitochondrial dysfunction and apoptosis of lung EC (Ruchko et al. 2005). The role of mitochondrial DNA damage in CS-induced lung EC apoptosis remains to be studied.

4.2.5 Lipopolysaccharide

LPS, also known as lipoglycans or endotoxin, is a component of the outer envelope of gram-negative bacteria and elicits pro-inflammatory responses. It is well established that LPS-induced EC activation, dysfunction, and apoptosis play an important role in bacterial sepsis and endotoxemia. In the blood circulation, LPS binds to soluble CD14 via LPS-binding protein (LBP), followed by engagement of toll-like receptor

(TLR)-4. This engagement results in the recruitment of adaptor, myeloid differentiation factor 88 (MyD88), and subsequent activation of interleukin (IL)-1 receptor associated kinase (IRAK)-1, TNF receptor associated (TRAF)-6, NF-kB, and MAPK pathways (Desch et al. 1989; Wang et al. 2001; Bannerman and Goldblum 2003).

NF-kB has been shown to transcriptionally upregulate anti-apoptotic genes such as IAP-1, IAP-2, and FLIP (LaCasse et al. 1998; Bannerman et al. 2004). However, suppression of NF-kB has minimal effect on LPS-induced EC apoptosis (Zen et al. 1999). This is due to FADD/MyD88-dependent negative regulation of LPS-induced NF-kB activation (Martin et al. 2005; Zhande et al. 2007); Fas is no longer able to activate MyD88, thus stimulating LPS/TLR4/NF-kB signaling (Martin et al. 2005). LPS also stimulates MyD88-independent signaling of endothelial apoptosis (Dauphinee and Karsan 2006). Heterotrimeric Gi/Go proteins play a role in LPS-induced TLR signaling independent of the MyD88-dependent pathway, leading to MAPK, Akt, and IFN activation of endothelial cells (Dauphinee et al. 2011). Whether LPS-induced stimulation of heterotrimeric G coupled proteins plays a role in EC apoptosis is unknown. LPS can activate the BID-dependent intrinsic pathway of apoptosis in lung EC (Wang et al. 2007). Conversely, LPS has been shown to upregulate mRNA of anti-apoptotic molecules, thus preventing EC apoptosis (Hu et al. 1998). LPS-induced intrinsic apoptosis and cytoprotection in disease states are not well understood and require further study.

4.3 Pulmonary EC Apoptosis in Lung Diseases

Apoptosis has been shown to ameliorate or exacerbate lung injury. Pulmonary EC apoptosis plays an important role in physiological processes including vasculogenesis and angiogenesis during lung development. Pulmonary EC apoptosis may also initiate or contribute to the progression of a number of lung diseases, as reviewed elsewhere (Harrington et al. 2007; Lu and Rounds 2009). In this review, we will focus on the role of pulmonary EC apoptosis in development of emphysema and Acute Lung Injury (ALI).

4.3.1 Emphysema

Chronic obstructive pulmonary disease (COPD), a progressive respiratory condition consisting of emphysema and chronic bronchitis, is the fourth leading cause of death worldwide and may become the third leading cause of death by 2030 based on prediction by the World Health Organization (Khaltaev 2005). The prevalence of COPD in the United States in 2013 was estimated to be 6.4% (15.7 million adults) (Wheaton et al. 2015). COPD is also an important contributor of mortality and disability in the United States (Murray et al. 2013). Further, COPD-related medical costs were estimated at $32 billion in the USA in 2010 with an additional $4 billion

in costs due to absence from work (Ford et al. 2015). α1-antitrypsin (AAT) deficiency and other genetic predispositions contribute to the development of COPD (Sandford et al. 1997). However, tobacco smoke remains the leading cause of this devastating disease. Indoor air pollution (such as biomass fuel used for cooking and heating), outdoor air pollution, and occupational dusts and chemicals also increase the risk of COPD (Diette et al. 2012). Although the pathology of COPD has been well defined, the pathogenesis of the disease initiation and progression is not understood. Currently, there is no specific treatment available to reverse COPD.

Emphysema, a common and debilitating manifestation of COPD, is characterized by alveolar airspace enlargement, loss of alveolar capillary septa, and resultant impaired gas exchange. Several hypotheses have been proposed to explain alveolar wall damage in emphysema. Protease/anti-protease imbalance has been accepted as a major mechanism for emphysematous lung destruction (Shapiro 1995, 1999; Shapiro et al. 2003; Taraseviciene-Stewart and Voelkel 2008). It is believed that neutrophil elastase and macrophage matrix metalloproteinases enzymatically degrade elastin in alveolar septa, leading to emphysema (Taraseviciene-Stewart and Voelkel 2008). This notion is supported by findings that patients with genetic deficiency of the anti-protease, AAT, develop emphysema (No Authors 1997). Additionally, intra-tracheal instillation of proteases causes an emphysema phenotype in rats (Pastor et al. 2006). However, less than 5% of emphysema patients have AAT deficiency. Inflammatory cell infiltration is also seen in human emphysema. However, lung inflammation in pneumonia or acute lung injury does not usually result in emphysema. This suggests that inflammation may not be sufficient by itself for the development of emphysema. Oxidant stress and immunological injury also play a role in the pathogenesis of emphysema (Taraseviciene-Stewart and Voelkel 2008). Emerging evidence has highlighted a role of apoptosis, particularly EC apoptosis, in the initiation and progression of emphysema (Kasahara et al. 2000, 2001; Giordano et al. 2008).

Lung tissue from patients with emphysema displays increased apoptosis of both epithelial and endothelial cells in the alveolar septa (Kasahara et al. 2001; Imai et al. 2005). Bcl-2 single-nucleotide polymorphisms have been associated with severity of human emphysema (Sata et al. 2007). We have shown that lung EC apoptosis is elevated in a mouse model of emphysema induced by CS exposure (Sakhatskyy et al. 2014). Interestingly, induction of alveolar cell apoptosis by intratracheal instillation of the active caspase-3 causes emphysema in rats (Aoshiba et al. 2003). Additionally, inhibition of VEGF signaling causes alveolar septal cell apoptosis and emphysema in mice (Kasahara et al. 2000; Tang et al. 2004). Similarly, intra-tracheal instillation of C_{12} ceramide triggers alveolar endothelial and epithelial cell apoptosis and emphysema-like changes in mice (Petrache et al. 2005). Further, lung EC-targeted induction of apoptosis led to emphysema and enhanced oxidative stress and lung inflammation (Giordano et al. 2008). More importantly, inhibition of apoptosis using pan-caspase inhibitors prevented the emphysematous changes induced by either ceramide (Petrache et al. 2005) or blockage of VEGF signaling (Kasahara et al. 2000; Tang et al. 2004). These results

support a central role of lung EC apoptosis in the development of emphysema. Anti-protease, AAT, inhibits CSE-induced pulmonary EC apoptosis in vitro by direct interaction with caspase-3 (Aldonyte et al. 2008). Overexpression of AAT also inhibits lung endothelial apoptosis and attenuates emphysema caused by either active caspase-3 or blockade of VEGF signaling (Petrache et al. 2006). These studies suggest that lung EC apoptosis is a critical step in the pathogenesis of emphysema.

Inhibition of FAK causes emphysema-like change in rat lungs (Mizuno et al. 2012). We have shown that CS exposure for 3 weeks enhanced pulmonary EC apoptosis and decreased FAK activity in mice susceptible to CS-induced emphysema (Sakhatskyy et al. 2014). Further studies are necessary to address whether reduced FAK activity contributes to CS-induced lung EC apoptosis and emphysema in humans in vivo. We have shown that CS exposure increases lung tissue adenosine levels in mice, an effect associated with lung EC apoptosis and early emphysema (Lu et al. 2013). Sustained increased adenosine in ADA-deficient mice also enhances alveolar cell apoptosis and causes emphysema in mice (Zhou et al. 2009). ADA expression and activity are reduced in the lung of smokers with COPD (Zhou et al. 2010). Whether chronically elevated adenosine contributes to CS-induced lung endothelial cell apoptosis and development of emphysema remains to be investigated.

Ceramide is upregulated in emphysematous lungs of patients and animal models, as well as in cultured pulmonary EC exposed to CSE (Petrache et al. 2005). This increase in ceramide is associated with enhanced alveolar cell apoptosis (Petrache et al. 2005). Interestingly, intratracheal instillation of C_{12} ceramide triggers air-space enlargement and apoptosis of alveolar EC and type II epithelial cells (Petrache et al. 2005). Further, inhibition of de novo ceramide synthesis significantly attenuated lung cell apoptosis and emphysema induced by VEGFR2 blockade (Petrache et al. 2005). These results suggest that ceramide is also an important mediator of alveolar cell apoptosis and emphysema (Petrache et al. 2005).

Only 10–15% of smokers develop emphysema. The mechanism underlying increased susceptibility to emphysema remains unclear. The UPR is elevated in the lungs of smokers without evidence of emphysema (Kelsen et al. 2008). Nrf2, a redox-sensitive, antioxidant transcription factor, is activated by eIF2α, a branch of UPR (Digaleh et al. 2013). Nrf2 knockout mice demonstrate enhanced susceptibility to cigarette smoke-induced emphysema in comparison to wild-type mice (Iizuka et al. 2005). We have shown that active eIF2α was significantly reduced in the lungs of AKR mice with mild emphysema induced by CS (Sakhatskyy et al. 2014). Future studies are needed to address whether Nrf2 is reduced in the lungs and whether inadequate induction of Nrf2 contributes to development of emphysema.

Autophagy is significantly increased in lung tissue of patients with COPD; the degree of autophagy positively correlates with the clinical severity of disease (Chen et al. 2008). Increased autophagy has contributed to CS-induced alveolar epithelial cell apoptosis and emphysema in mice (Chen et al. 2010; Mizumura et al. 2014). In contrast, increased autophagy protects against pulmonary endothelial cell apoptosis and emphysema induced by cadmium, a component of cigarette smoke

(Surolia et al. 2015). We have reported that autophagy was not altered in lung tissue of a mouse strain with increased lung EC apoptosis and mild emphysema induced by CS (Sakhatskyy et al. 2014). Thus, the role of autophagy in regulating lung EC apoptosis and early onset of CS-induced emphysema needs further study.

4.3.2 Acute Lung Injury

ALI and its more severe form, acute respiratory distress syndrome (ARDS), are life-threatening disorders clinically characterized by severe hypoxemia and pulmonary bilateral infiltrates. In the United States, ARDS affects approximately 190,000 patients annually (Rubenfeld et al. 2005). ARDS accounts for 3.6 million associated hospital days (Rubenfeld et al. 2005; Adhikari et al. 2010). The global impact of ARDS has been difficult to assess due to varying definitions of the broad clinical phenotypes and limited data. Thus, ARDS remains an underreported disease of treated incidence, as opposed to actual incidence, in the undeveloped world (Buregeya et al. 2014). Although the mortality rate of ARDS has decreased to around 30–40% due to lung protective ventilation strategies (Amato et al. 1998; Villar et al. 2006), ARDS remains a deadly syndrome without a specific cure. Currently, there are no pharmacological interventions available to reduce the mortality of ARDS.

Sepsis, bacterial and viral pneumonia, and trauma remain the leading risk factors for the development of ARDS. Emerging evidence from epidemiologic studies, animal models, and cultured cell models have suggested that both active and passive cigarette smoke exposure modifies the susceptibility for development of ALI and ARDS (Iribarren et al. 2000; Calfee et al. 2011; Lu et al. 2011, 2013; Hsieh et al. 2014; Borgas et al. 2016).

The pathophysiology of ARDS is characterized by increased permeability of the alveolar-capillary barrier, influx of protein and inflammatory cell-rich fluid into the alveolar space, attenuated gas exchange between alveolar-capillary barrier, and dysregulated inflammation. Increased permeability of the microvascular endothelium and alveolar epithelium promotes edema formation, and this concept has been accepted as an important mechanism for the initiation of ARDS (Matthay et al. 2012). It is well established that polymorphonuclear cells (PMN) and immunological injury also play a significant role in the pathogenesis of ARDS (Perl et al. 2011). PMN accumulation is observed in the broncheoalveolar lavage fluid (BALF) (Pittet et al. 1997) and lung biopsies of early ARDS patients (Bachofen and Weibel 1977, 1982). Further, neutrophilia has been correlated with exacerbation of sepsis-induced ALI (Steinberg et al. 1994). However, ARDS may also develop in neutropenic patients, and neutrophil activation and migration may be observed in human lungs without injury (Martin et al. 1989; Downey et al. 1999). This suggests that inflammation may not be sufficient by itself for the development of ARDS.

Emerging evidence has suggested a role of pulmonary cell apoptosis in the initiation and progression of ARDS. The death receptor, Fas, and its ligand, FasL

system, is an important death receptor-mediated extrinsic pathway of apoptosis. FasL is expressed and released by inflammatory cells, including neutrophils and lymphocytes, whereas Fas is expressed on the surface of lung EC, alveolar and bronchial epithelial cells, Clara cells, and alveolar macrophages. Fas and FasL are increased in pulmonary edema fluid and in lung tissue of patients with ARDS (Albertine et al. 2002). Silencing of Fas/FasL reduces lung cell apoptosis and ALI in a mouse model of sepsis (Perl et al. 2005, 2007). Soluble FasL (sFasL) is a cleaved form of FasL by metalloproteinases and is increased in BAL fluid of patients with ARDS (Matute-Bello et al. 1999). sFasL released from inflammatory cells is capable of inducing lung epithelial cell apoptosis (Matute-Bello et al. 1999). The role of Fas/FasL in lung EC apoptosis is not yet clear. Robust pulmonary endothelial cell apoptosis has been observed in patients with severe ARDS (Abadie et al. 2005) and in mice with ALI induced by LPS (Fujita et al. 1998). Sepsis-induced ARDS in mice indicates evidence for pulmonary microvascular endothelial cell death as a cause of barrier dysfunction and edema (Gill et al. 2014, 2015). Inhibition of apoptosis using a broad-spectrum caspase inhibitor prolonged survival of mice exposed to LPS (Kawasaki et al. 2000). Since apoptosis of alveolar endothelial, epithelial, and interstitial inflammatory cells occurs during ALI, future studies are needed to address the role of apoptosis of specific cells in initiation of ALI/ARDS.

Apoptosis has been thought of to be a non-inflammatory means of removing injurious cells, thus facilitating lung repair. However, there is increasing evidence indicating that Fas/FasL-mediated lung epithelial apoptosis results in release of pro-inflammatory cytokines (such as TNF-α and TGF-β1), leading to inflammation and progression from ARDS to fibrosis (Chapman 1999). Whether pulmonary endothelial cell apoptosis occurs during initiation or progression of pulmonary fibrosis is unknown.

The role of necroptosis in development of ARDS is yet to be determined. Of interest, a recent study of blood transfusion-related acute lung injury indicates that banked red blood cell (RBC) transfusion enhances susceptibility to lung inflammation and ARDS in critically ill transfused patients and mice through necroptosis of lung EC and subsequent release of DAMPs (Qing et al. 2014).

4.4 Conclusions and Perspectives

Cell life and death are tightly regulated by survival signaling and death inducing programs. Pulmonary EC apoptosis significantly contributes to the development of emphysema and ALI/ARDS, as depicted in Fig. 4.1. Pan-caspase inhibitors have been used to inhibit lung cell apoptosis and prevent emphysema and ALI in animal models. However, use of such drugs to treat apoptosis-associated lung diseases may be problematic due to breakdown of tissue homeostasis and activation of necroptosis (Linkermann and Green 2014). The therapeutic potential of drugs that modulate cell death is dependent upon cell type-specific, tissue-specific, and

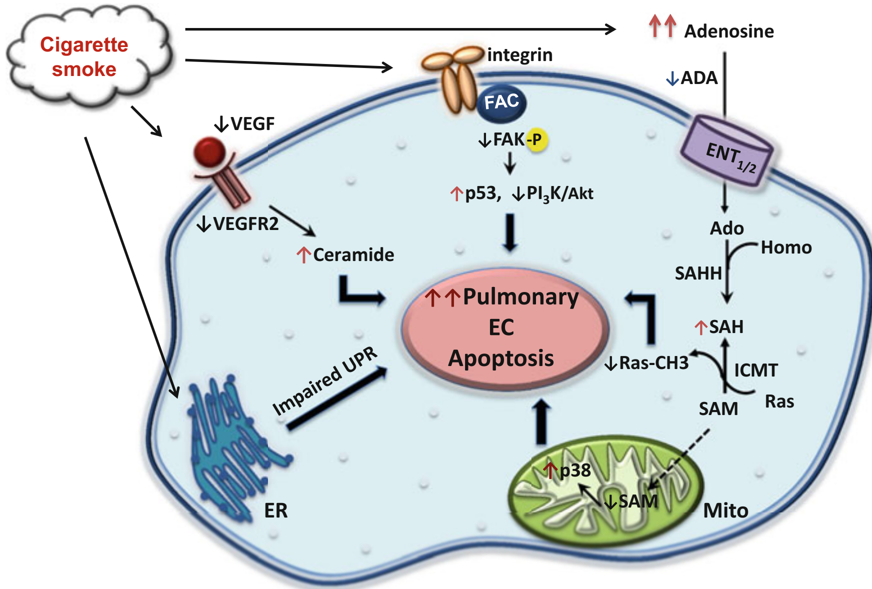

Fig. 4.1 Signaling pathways to CS-induced pulmonary endothelial cell apoptosis. Multiple signaling pathways are involved in CS-induced pulmonary endothelial cell apoptosis. (1) CS reduces VEGF/VEGFR2 signaling, leading to induction of ceramide and consequent apoptosis; (2) CS reduces FAK activation, leading to activation of p53 and inhibition of PI3K/Akt signaling, which results in apoptosis; (3) CS causes mitochondrial oxidative stress and mitochondrial dysfunction, leading to apoptosis; (4) CS elevates adenosine levels, leading to inactivation of Ras and mitochondrial oxidative stress, resulting in apoptosis; (5) CS impairs unfolded protein response, leading to apoptosis

vascular bed-specific actions. Thus, drugs acting locally and with cell type specificity are needed. Areas where research is needed include: (1) apoptosis susceptibility of different EC (conduit artery versus microvascular versus progenitor); (2) role of apoptosis of specific lung cells in initiation and/or progression of lung diseases; (3) role of necrosis and necroptosis in development of lung diseases, such as emphysema and ALI.

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