HYPOXIA INDUCIBLE FACTOR
A BREATH OF FRESH AIR IN LUNG DEVELOPMENT

Hypoxie Induceerbare Factor
Frisse lucht in longontwikkeling

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CONTENTS

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Chapter 5
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Groenman FA, van Heijst A, de Rooij JD, Hulsbergen-van de Kaa CA, Rottier R, de Krijger RR, Tibboel D. Pulmonary hypertension of the newborn: Different causes of onset, same final common pathway? Submitted.

Chapter 7
Groenman FA, de Krijger RR, Hammond S, Michalsky MP, van Heijst A, Hulsbergen-van de Kaa CA, Rottier R, Tibboel D. Alveolar Capillary Dysplasia; angiogenesis gone awry. Submitted.

chapter 1

General Introduction

The Molecular Basis for Normal and Abnormal Lung Development

ABSTRACT

Our understanding of lung development in the past two decades has moved from an anatomical to a histological basis and, most recently, to a molecular basis. Tissue interactions specify tracheal and lung primordia formation, program branching morphogenesis of the airway epithelium and regulate epithelial differentiation. In addition, lung development is influenced by mechanical and humoral factors. The regulatory molecules involved in morphogenetic signaling include growth and transcription factors and extracellular matrix molecules. These morphogenetic signals are responsible for lung patterning and differentiation. We will provide a brief overview of molecular signaling during early respiratory formation, airway branching, pulmonary vascularization and epithelial differentiation. We will then review aberrant morphogenetic signaling in human lung abnormalities, such as tracheoesophageal fistula, congenital diaphragmatic hernia, pulmonary hyperplasia, alveolar capillary dysplasia, congenital cystic adenomatoid malformation and bronchopulmonary dysplasia.

INTRODUCTION

Our understanding of the molecular basis of lung development has been growing rapidly in the past decade. Now, the challenge is to integrate the identified signaling pathways into a morphogenetic map, which then can be used to model aberrant lung development and its consequences to the neonatal lung. This review will briefly outline the current state of knowledge of the molecular basis of normal fetal lung development and then discuss the importance of these molecules in aberrant human lung development. For simplicity, we focus our review mainly on growth and transcription factors.

NORMAL LUNG DEVELOPMENT

Lung development can be divided into six stages:[1]

1. *embryonic period*, a ventral diverticulum from the primordial foregut elongates caudally to form the prospective trachea, which branches and gives rise to the main bronchi of the two lungs;
2. *pseudoglandular period,* branching morphogenesis of the airways down to the terminal bronchioli;
3. *canalicular period,* respiratory bronchioli appear, and vascularization of peripheral mesenchyme increases;
4. *saccular period,* the peripheral air spaces expand in length and become sacculi, and at the end of this period, the saccular walls contain a double capillary network;
5. *alveolar period,* secondary septa appear on the existing primary septa in the saccular lung to form alveoli, and
6. *microvascular maturation,* the double capillary layer of the immature alveolar septa is reduced to a single capillary layer.

The first four stages of lung development take place in utero, while the last two stages primarily occur postnatally. Each of the stages is governed by a variety of regulatory signals, including epithelial-mesenchymal interactions, pO_2 tensions, physiological mechanical forces as well as humoral factors.

Embryonic Period

In human, early lung formation lasts from day 26 to 7 weeks after conception. During the fourth week of gestation, a midventral groove appears in the single foregut tube. This deepens and then constricts, thereby forming a separate ventral trachea and a dorsal esophagus. The ventral trachea elongates and, subsequently, bifurcates to form two separate bronchial lung buds.[2] These primary bronchi continue to grow into the splanchnic mesenchyme. Interactions between the mesenchyme and invading epithelium[3,4] fine tune the temporal-spatial expression pattern of signaling and transcriptional molecules [for a review, see 5]. Important growth factors are sonic hedgehog (Shh) and fibroblast growth factors (FGFs),

specifically FGF10.[6-8] Important transcription factors are hepatocyte nuclear factor-3β (HNF-3β or Foxa2),[9,10] Hox genes,[11,12] Gli genes[13–15] and thyroid transcription factor-1 (TTF-1 or Nkx2.1).[16,17] Several of these regulatory factors are also implicated in esophageal-tracheal separation as well as left-right asymmetry development, including Shh,[18] Gli,[13] Foxj1a[19] and Pitx-2.[20,21]

Early pulmonary organogenesis

FGF10 is a crucial factor in the initiation of lung development. Mice deficient in this factor show no lung development below the trachea.[7] FGFs are generally produced by the pulmonary mesenchyme and have their receptors in the lung epithelium. Deletion of the FGFr2-IIIb receptor, which binds FGF10, also resulted in an arrest of lung development after tracheal formation.[22] A similar phenotype was noted in mice overexpressing a dominant-negative FGFr2 in lung epithelial cells using the surfactant protein (SP)-C promoter.[23] Although a deletion of FGF7, which also binds to FGFr2-IIIb, has no significant effect on lung development,[24] targeted overexpression of FGF7 in developing mouse lung epithelium resulted in abnormal lung morphogenesis that resembles cystic adenomatoid malformation.[25] Shh is a widely studied morphogen with major impact on early lung development and is expressed in the pulmonary epithelial cells.[13,26,27] Shh binds to a mesenchymal multiple-pass membrane-spanning receptor, Patched1 (Ptch1).[13,26-28] Shh can also bind to another surface membrane protein, namely hedgehog-interacting protein 1 (Hip1).[26] Activation of the Ptch receptor releases inhibition of a membrane-bound receptor known as Smoothened (Smo).[26] Activation of Smo results in an upregulation of the downstream pathway. Activation of Hip1, on the other hand, results in attenuation of the Shh pathway.[26] Activation of the Shh pathway modifies the activity of the Gli family of transcriptional regulators.[13] Three Gli genes have been described in mice: Gli1, Gli2 and Gli3. Knockout studies in mice underline the importance of Gli2 and Gli3 in lung bud formation. Mice deficient in Gli2 exhibit foregut defects such as tracheoesophageal fistula (TEF) with esophageal atresia (EA).[29] Gli2–/–;Gli3–/– mice have no lung, trachea or esophagus at all,[14] which is a more severe phenotype than a knockout of Shh alone. Lung buds do develop in these Shh null mutants, but there is an almost complete failure of branching morphogenesis.[6,30] Furthermore, the Shh–/– mice demonstrate EA/stenosis, TEF and decreased mesenchymal proliferation.[31] Overexpression studies of Shh show an increased mesenchymal and epithelial proliferation.[15] Hip–/– mice show an increase in hedgehog signaling and a decrease in branching morphogenesis with a relative increase of mesenchymal cells,[26] which correlates with the overexpression studies of Shh.[14] A recent study suggests that besides regulating branching morphogenesis, Shh also affects the differentiation of the peripheral lung mesenchyme towards the smooth muscle cell lineage.[8]

Another crucial player in early lung development is TTF-1. Disruption of this gene in mice results in severe hypoplasia of the thyroid and lung, with a developmental arrest at the pseudoglandular stage of lung development[17] and a complete

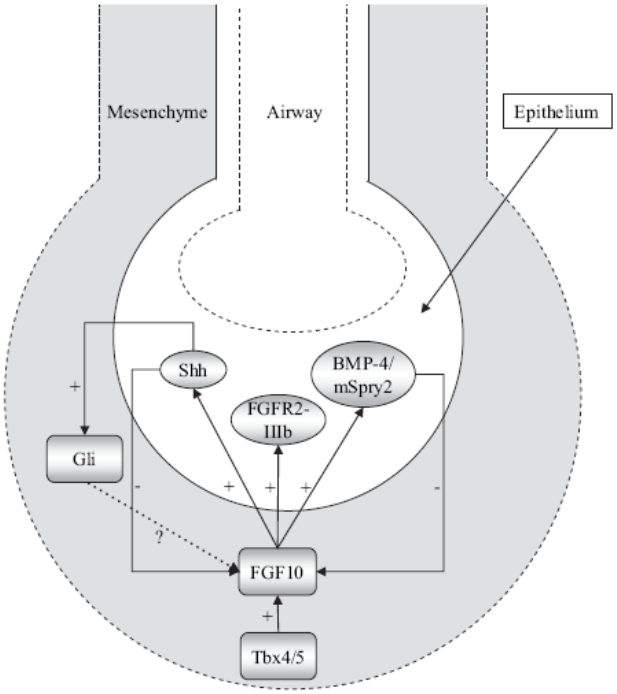
tracheoesophageal defect.[32] A deletion of the TTF gene has also been described in humans. These infants suffered from thyroid dysfunction and respiratory failure.[33,34]

The aforementioned factors all seem to influence each other. It has been proposed that Shh is a target of FGF10/FGFr2b signaling.[35] The mesenchymal FGF10 upregulates the epithelial expression of Shh, which in turn signals back to the mesenchyme via the Gli pathway and by downregulation of FGF10 expression.[35] A similar negative feedback loop is found for bone morphogenetic protein 4 (BMP-4) and sprouty 2 (mSpry2).[36,37] These factors are expressed at the tips together with Shh. Both these factors inhibit branching and proliferation and are stimulated by FGF10. Once stimulated, they signal back negatively to FGF10.[36] FGF10 seems to set up a negative feedback loop so that the epithelium can control the extent of its outgrowth. This feedback loop may be controlled by T-box genes (Tbx) 4 and 5.[36] If both Tbx genes are knocked down by antisense oligonucleotides in lung explant culture, FGF10 expression is suppressed and branching comes to a halt.[36] This suggests that Tbx4/5 stimulate FGF10 expression (see *figure 1.1* for an overview of epithelial-mesenchymal interactions). Recent reports suggest that Nkx molecules are involved in the activation of Tbx proteins.[38,39] Retinoic acid (RA) plays a crucial role in the developmental processes of almost every organ.[40] Mice deficient in the RA receptor (RAR)-α and RAR-β show a variety of lung development defects such as agenesis of the left lung and lung hypoplasia.[41] Furthermore, RA may regulate Hox genes.[42] Clusters a and b of this family of homeobox-containing transcription factors are expressed in the developing lung.[43] Mouse studies for Hox show that Hoxa5–/– mice have laryngotracheal malformations and lung hypoplasia;[44] Hoxa1–/–; Hoxb1–/– mutants suffer from severe lung hypoplasia.[45]

Development of left-right asymmetry

At around 5 weeks of gestation, five lobes can be seen in the human lung, namely two on the left and three on the right side. The development of this left-right asymmetry is vital for correct development and positioning of intrathoracic and intraabdominal organs. A number of factors influencing the development of this left-right asymmetry have been identified, including Shh,[18] FGF8,[46] N-cadherin,[47] activin-β, activin receptor IIB[48,49] and Foxj1.[19] Interestingly, all these factors influence the expression patterns of nodal and lefty-1,2[50] which belong to the transforming growth factor-β (TGF-β) family. Lefty-1 knockout mice have left pulmonary isomerism with bilateral expression of nodal and lefty-2.[51] In addition, these mice misexpress the downstream homeobox transcription regulator Pitx-2. Pitx-2 normally promotes leftness in this organ. Mice deficient in Pitx-2 show right pulmonary isomerism and altered cardiac positioning.[21]

Figure 1.1 Epithelial-mesenchymal interactions



Tbx4/5 interacts with mesenchymal FGF10, which then stimulates its receptor FGFr2-IIIb in the epithelium. FGF10 also upregulates epithelial Shh, BMP-4 and mSpry2. These factors then signal back to FGF10 in a negative manner. Shh also upregulates Gli genes in the mesenchyme. Interaction between Gli genes and FGF10 has not been demonstrated.

Pseudoglandular Period

The pseudoglandular period takes place from week 5 to week 17 of gestation in the human. Branching of the epithelial buds is a hallmark in this period as the conductive airway tree down to the terminal bronchioles is formed. Arterial branches accompany the newly formed airway branches (conventional arteries), and smaller supernumerary arteries appear. Veins run in the mesenchymal septa and extend in a plane between each generation of dichotomous airway branching. Epithelial cells start to differentiate with the appearance of ciliated cells, goblet cells and basal cells. Mesenchymal cells begin to form cartilage and smooth muscle cells.[1]

Induction of terminal airway branching

The TGF-β superfamily of proteins appears to be responsible for a large part of the counterregulatory balance in pulmonary development. TGF-β1, 2, and 3 have been shown to be expressed early in lung development, along with their TGF receptor and their putative downstream signaling molecules, the Smad family.[52] Downregulation of TGFr2 and Smad2,3,4 in mouse lung explants has been associated with increased branching morphogenesis.[53,54] Overexpression of TGF-β1 in distal pulmonary mouse epithelium resulted in a pseudoglandular arrest in development. These findings underline the negative influence which TGF-β1 has on branching.[55] FGFs also play an important role in the branching morphogenesis. In

distal lung epithelium, FGF7 and FGF10 are positive regulators of the branching morphogenesis.[37] Other transcription factors involved in the branching morphogenesis are GATA-6 and N-myc. Decreased branching morphogenesis is seen when GATA-6 is inhibited.[56] Surprisingly, overexpression of GATA-6 in distal epithelium also results in a disrupted branching morphogenesis.[57] This suggests that a balanced GATA-6 in this phase of lung development is required for normal branching. N-myc is expressed in pulmonary epithelium, and mice with a targeted disruption of this gene demonstrate lung hypoplasia and decreased branching.[58]

Physical factors

In situ, several physical forces are exerted on the developing lung, including (a) fetal breathing movements (FBM), (b) peristaltic airway contractions and (c) lung fluid circulation. FBM can be observed as early as 10 weeks of gestation in the human[59] and are an important factor for lung growth [for a review, see 60]. This is supported by the observation that myogenin-deficient mice which cannot produce normal FBM in utero have lungs that show a significant decrease in lung:body weight ratio compared with controls. These mice not only display a decrease in lung proliferation, but also an increase in apoptosis.[61] This observation holds true in humans, as children with severe congenital hypotonia and insufficient respiratory effort frequently have concomitant pulmonary hypoplasia.[62] FBM may also be inhibited by a lack of amniotic fluid. Children born after a loss of amniotic fluid prior to 24 weeks gestational age frequently suffer from pulmonary hypoplasia as well.[63] Prenatal tracheal ligation results in a hyperplasia of the lung,[64] presumably by increasing the volume of lung liquid by preventing its efflux from the lungs. This increases the internal stretch on the pulmonary epithelium. Although the lungs are hyperplastic, this process has the detrimental effect of disrupting the differentiation of epithelial cells.[65]

Canalicular Period

In the period from 16 to 26 weeks of gestation, the airway branching pattern is completed, and the prospective gas exchange region starts to develop. Respiratory bronchioli appear, interstitial tissue decreases, vascularization of the mesenchyme increases and distal cuboidal epithelium differentiates into type I and type II cells.

Pulmonary vascularization

Vascularization of the lung increases dramatically due to the formation of capillaries in the primitive interstitium. Interstitial endothelial cells form an extensive vascular network that follows the airspaces in close physical approximation. The newly formed capillaries lean against the epithelium, and the prospective air-blood barrier develops.[1] Genetic analyses have demonstrated that cell-extracellular matrix and cell-cell interactions as well as growth and transcription factors are involved in vascular development. Especially the vascular endothelial growth factor (VEGF), a potent mitogen for endothelial cells, appears to be a key player in pulmonary vascular development.[66,67] VEGF is known to upregulate endothelial nitric oxide

synthase (eNOS). Mice with an eNOS deficiency fail to increase lung weight and volume;[68] furthermore, the lungs of these mice have capillary hypoperfusion and a decrease in number of arteriolar branches. They also display misalignment of pulmonary veins, a feature seen in alveolar capillary dysplasia as well.[69] Upstream of VEGF are hypoxia-inducible factors (HIF), a family of transcriptional regulators upregulated under hypoxic conditions.[70-72] The HIF transcriptional complex is a heterodimer composed by one of the three alpha subunits (HIF-1α , HIF-2α or HIF-3α) and a beta subunit (HIF-1β) also known as the aryl hydrocarbon nuclear translocator. The regulation of HIF by oxygen occurs through modifications of the alpha subunit, whereas the beta subunit is a constitutive nuclear protein, and its activity is not affected by hypoxia. Under hypoxic conditions, the alpha subunit is stable, allowing it to accumulate in the nucleus, where, upon binding to HIF-1β, it recognizes HIF-response elements within the promoter regions of hypoxiaresponsive target genes, including VEGF.[73] HIF-1α–/– mice have severe cardiovascular defects and die in utero,[74] underscoring the influence of HIF on vascularization. Recent studies have shown that a low oxygen environment enhances pulmonary vascularization.[75] Antisense knockdown of HIF-1α and VEGF revealed that pulmonary vascular development may be rate limiting for epithelial branching morphogenesis in vitro,[75] Inhibition of neovascularization with endothelial monocyte-activating polypeptide (EMAPII) resulted in a canalicular arrest in pulmonary development,[76] also suggesting that branching of the lung and vascular development go hand in hand.

Pulmonary epithelial differentiation

The epithelial cells lining the airways differentiate into more than 20 different cell types with highly specific functions. Pulmonary neuroendocrine cells (PNECs) are the first cells to differentiate in humans. PNEC development is controlled by basic helix-loop-helix transcriptional factors. Mice deficient in Mash1 do not develop PNECs,[77] whereas Hes1-deficient mice have increased numbers of PNEC.[78] Both Hes1 and Mash1 are downstream components of the Notch-signaling pathway.[79] Recent antisense knockdown experiments have implicated Notch1 in regulating pulmonary neuroendocrine differentiation.[80] Pod-1 is another transcriptional basic helix-loop-helix protein of importance to cell fate and differentiation. Mice deficient in this factor die at birth with severely hypoplastic lungs, a lack of acinar tubules, increased Clara cell secretory protein (CCSP) and decreased SP-C expression.[81] Other transcription factors implicated in the process of epithelial cell differentiation are TTF-1, Foxa2 and GATA-6. TTF-1 has been shown to regulate the transcription of SP-A, B, C and CCSP,[82-85] whereas Foxa2 modulates the expression of SP-B and CCSP.[86,87] GATA-6 acts synergistically with TTF-1 to influence the activity of the SP-C promoter.[88]

Saccular Period

The most important steps in this period towards ex utero life are the growth of the pulmonary parenchyma, the thinning of the connective tissue between the airspaces

and the maturation of the surfactant system. Apoptosis plays an important role in the condensing of the connective tissue between the airspaces and is mediated by proapoptotic factors such as TGF-β and antiapoptotic factors such as insulin-like growth factor (IGF)-1, nitric oxide and secreted apoptosis-related proteins; these are all found in the alveolar environment [for a review, see 89]. Glucocorticoid receptors (GRs) are present in the mesenchyme of the developing fetal lung.[90] Their abundance increases with advancing gestation and peaks just prior to cellular differentiation, thus further supporting the hypothesis of glucocorticoid importance in late gestation.[91] GR knockout is associated with fatal neonatal respiratory insufficiency.[92] The requirement for glucocorticoid-GR stimulation in lung development is confirmed by the observation of a similarly severe lung phenotype in mice with a targeted mutation in the corticotrophin-releasing hormone gene.[93] In contrast to previous reports showing glucocorticoids influencing lung branching in vitro,[94] the loss of GRs in mice has no influence in early lung branching (*figure 1.2*). Genes regulated by glucocorticoids in the lung include surfactant proteins, TGF-β3 and the retinoid responsive gene midkine (MK).[90,93,95,96] Indeed, TGF-β3 null mice exhibit a similar delayed phenotype as observed in GR-negative mice.[92,97] It has been shown that prenatal administration of glucocorticoids is beneficial for the treatment of lung hypoplasia in sheep,[98] and administration of glucocorticoids to pregnant women at risk of preterm birth has been used as a therapy for the improvement of newborn lung function.[99]

Alveolar Period and Microvascular Maturation

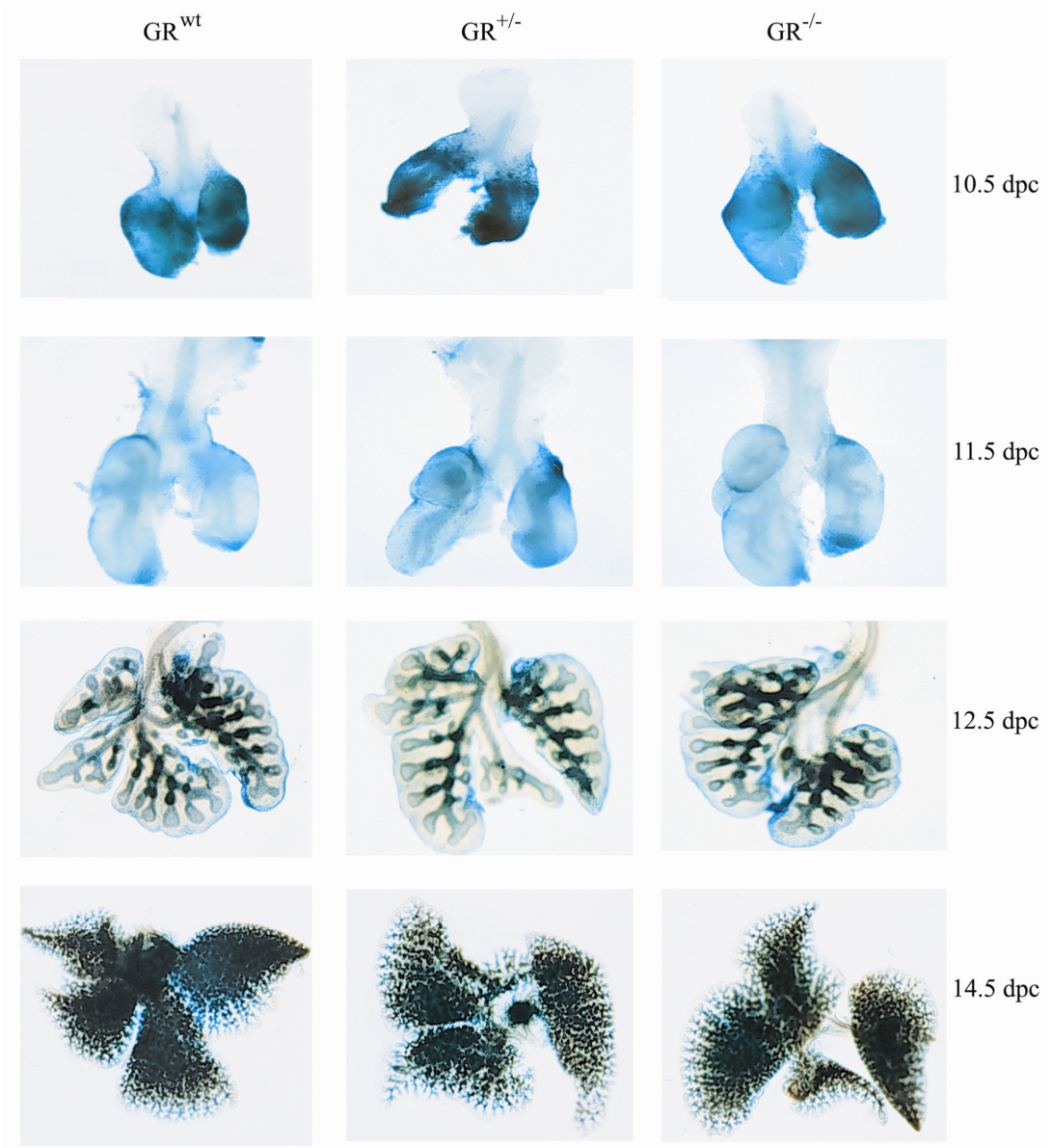
This period predominantly takes place after birth. Alveoli are formed by septation of the pulmonary saccules that form the immature lung. The septation finds its origin in low ridges arising in the saccular wall. These ridges form the interalveolar walls or secondary septa. The secondary septa contain a double capillary layer separated by a sheet of connective tissue. During microvascular maturation, the lung matures in a lung with a single-layer capillary bed.[1] Factors that play a role in the alveolarization process are Foxa2,[100] GATA-6,[101] platelet-derived growth factor A (PDGF-A),[102] TTF-1[103] and RAR.[104] Factors important for the vascular maturation are VEGF,[105,106] MK[107] and Foxf1.[108]

Alveolar period

The number of secondary septa is decreased in mice with a conditional lung-specific knockout of Foxa2, pointing to a crucial role of Foxa2 in alveolarization.[100] Overexpression of GATA-6 using the SP-C promotor resulted in normal lung morphology at E18.5; however, during the neonatal period, defects in alveolar septation occurred.[101] Mice lacking PDGF-A die either in early gestation or within the first weeks of life.[102] Animals dying postnatally have an emphysematous appearance and an absence of septa and alveoli. Normal lungs have smooth muscle cells (positive reactivity for smooth muscle α-actin staining) in their alveolar septa, while PDGF-A null mutants display a deficiency in alveolar staining for smooth muscle α-actin.[102] Overexpression of TTF-1 results in a normal prenatal lung formation;

however, the postnatal lung shows emphysema, severe inflammation and fibrosis.[103] RARs also play a critical role in alveolarization, as overexpression of a dominant negative RAR-α during neonatal development results in a decrease of alveolar epithelial cells and an inhibition of SP-B synthesis.[104]

Figure 1.2 (*for color figures see page 153)* Normal early lung branching in GR knockout mice



GR mice were crossed with cordon blue(C101)-LacZ transgenic mice, which displays LacZ gene expression specifically in epithelial cells. X-gal staining of lungs of GR/C101-LacZ mice at E10.5–14.5 visualized the complex branching pattern which was not affected by loss of GR signaling.
GR^{wt} *Wildtype mice*
GR+/– *heterozygous mice*
GR–/– *GR null mice*

Microvascular maturation

Some genes known to play a role in vascular development, such as VEGF and MK, contain HIF-response elements, suggesting that oxygen homeostasis in the postnatal lung may play a role in the microvascular maturation. VEGF expression is upregulated in lung cells by stretch,[106] supporting a role for vascularization in stretch-induced lung growth. Therefore, VEGF seems a promising candidate for therapeutic use in patients with pulmonary hypoplasia. However, caution should be taken, since a conditional postnatal overexpression of VEGF leads to increased neonatal mortality in mice, due to vascular leak and pulmonary hemorrhage.[104] Mice which survive into adulthood display pulmonary hemosiderosis and emphysema.[105] MK is a RA-responsive growth factor that is shown to induce angiogenesis, cell growth and cell migration. High MK levels are seen during alveolarization and vascular maturation, suggesting a role for MK in these processes.[107] Mice exposed to hypoxia during postnatal lung development display elevated MK levels with remodeling of peripheral vessels. MK production in the epithelium is regulated by hypoxia in a HIF-1α-dependant manner.[107] In mice with decreased Foxf1 expression there is a disruption in the epithelial-mesenchymal interface with increased apoptosis and decreased SP-B expression. This results in alveolarization and vasculogenesis defects associated with pulmonary hemorrhage.[108]

DEVELOPMENTAL DISORDERS OF THE RESPIRATORY SYSTEM

Tracheoesophageal Fistula

Relatively common malformations of the foregut are TEF, esophageal and tracheal atresia. These occur in isolation and as part of more complex syndromes such as Pallister-Hall, VACTERL and Smith-Lemli-Opitz. The inheritance pattern is thought to be multifactorial although there are reported cases of familial TEF.[109,110] A failure of the dorsal esophagus to divide from the ventral trachea will result in TEF. Most commonly, the result is a blind-ending esophageal pouch with a fistula joining the distal esophagus to the trachea. The aberrant signaling mechanisms involved in the production of TEF have been much speculated about, as there appears to be a large concert of signals involved in the partition of the trachea from the early foregut. These signals include FGF10, Shh, Foxf1, Gli2, Gli3, RARs and TTF-1. Mice deficient in these factors show a variety of tracheoesophageal defects: FGF10–/– mice show defects in tracheoesophageal separation and a blind-ending trachea; Shh–/– mice show a tracheoesophageal septum defect; Foxf1+/– mice show narrowing of the trachea and esophagus with EA and a fistula-like fusion between esophagus and trachea; Gli2–/– mice show tracheoesophageal separation defects with esophageal and tracheal stenosis; Gli2–/–;Gli3–/– mice show no esophagus, trachea or lung; Gli2–/–;Gli3+/– mice show a single tracheal tube connecting to the stomach and esophageal atresia; RAR-α–/–;RAR-β2–/– and RAR-α1–/–;RAR-β–/– mice show tracheoesophageal septum defects and tracheal cartilage malformations, and TTF-1–/– mice show a tracheoesophageal septum defect.[6,7,14,30,32,111,112] The

observations on the Shh and Gli mutant mice suggest that the Shh pathway plays a major role in the development of TEF. Indeed, disrupted Shh signaling has been implicated in congenital syndromes exhibiting TEF, esophageal and tracheal atresias.[113,114] Others have also attributed human TEF to defective Shh signaling.[115,116] Studies using the adriamycin-induced rat model of EA and TEF have shown defective FGF signaling. There is a marked absence of FGF1,[117,118] FGF7 and its receptor FGFr2-IIIb.[119] Upon adding FGF1 to lung bud cultures of fetuses of adriamycin-treated rats, branching occurred, but these lung buds did not respond to FGF7 and FGF10.[117] The latter is most likely due to the absence of their common receptor FGFr2-IIIb. In human TEF samples there is an absence of Shh signaling in the developing fistula tract;[116] however, the fistula does express TTF-1 and Foxa2, suggesting that the fistula derives from respiratory precursors.[120]

Congenital Diaphragmatic Hernia

Congenital diaphragmatic hernia (CDH) is a relatively common disorder of fetal chest development occurring in 1 in 2,000 - 5,000 live births. This defect may also be an isolated anomaly or occur as part of a larger syndrome. The communication between the diaphragm and the pleural and peritoneal cavities is intact until the 8th week of gestation, and it is generally believed that the etiology of CDH lies in a defect in closure of this pleuroperitoneal canal.[121] From the nitrofen-induced model of CDH in rodents comes an alternative hypothesis suggesting the primary problem to be pulmonary hypoplasia and that the defect in the diaphragm is secondary.[122] Difficulties with ventilation due to pulmonary hypoplasia are the leading causes of morbidity and mortality in these children. CDH may also cause the vascular bed to develop poorly,[123] with the net result being pulmonary hypertension. Chang et al[124] recently showed a downregulation of VEGF expression in the nitrofen-induced rat model of CDH. FGF7 and FGF10 have also been shown to be downregulated in the same model.[125] Furthermore, pulmonary Shh expression in human CDH is delayed, peaking in the canalicular/saccular period as opposed to its peak in the pseudoglandular phase in normal development.[126] TGF-β does not seem to play a role in the murine nitrofen-induced model of CDH.[127] Other growth factors, including IGF,[128] epidermal growth factor and TGF-α,[129] have been shown to be misexpressed in human CDH hypoplastic lung. The alterations in expression of regulatory signaling molecules suggest a persistence of the fetal stage of pulmonary airway development in CDH.[128,129] Another interesting player is the RA pathway. RA reduces the percentage of CDH after nitrofen ingestion in rats,[130] and plasma levels of retinol and retinol-binding protein have been shown to be decreased in a small number of infants with CDH.[131] In the nitrofen-induced CDH model, Hoxb5 is shown to persist in later stages of development, again suggesting a delayed development and maturation compared with normal lungs.[132] In human CDH, an increased GR expression has been observed implicating a role for glucocorticoids in the hypoplastic lung.[133] TTF-1 has been shown to be decreased in the nitrofen rat model of CDH;[134] however, in human CDH samples, no differences in TTF-1 expression

were seen.[135] Although the exact pathogenesis of CDH is unknown, findings so far point to aberrant signaling during pulmonary morphogenesis.

Pulmonary Hyperplasia

Pulmonary hyperplasia is a much less common phenomenon than pulmonary hypoplasia, but carries significant morbidity and mortality for affected babies. It can also provide important insights into developmental patterns of the lung. The most common initiator of pulmonary hyperplasia is tracheal obstruction, which leads to a marked overgrowth of the lungs. The pathogenesis of this overgrowth is not well understood; however, it seems to be more complicated than simply a mechanical phenomenon related to the inability for lung liquid to escape the lungs. In an elegant study using fetal sheep, Moessinger et al[136] decreased the right fetal lung volume by drainage of lung fluid while the volume of the left lung was expanded by mainstem bronchus ligation leading to lung fluid retention. This procedure created an unilateral pulmonary hyperplasia and contralateral pulmonary hypoplasia. Interestingly, biochemical indices of lung maturation, including total phospholipids, phosphatidylcholine and disaturated phosphatidylcholine content expressed per unit of tissue DNA, were no different when comparing the hypoplastic, hyperplastic and control lungs. These data suggest that fetal lung cell proliferation is influenced by local distension with lung fluid, but that maturation of the alveolar epithelium is under systemic control. Tracheal obstruction has been studied in both humans[137,138] and animals[139-143] as a possible therapy for antenatal management of congenital diaphragmatic hernias. Lungs of CDH patients who received this fetal treatment showed an enlarged lung sonographically, but histologically, the lung still showed low radial alveolar counts and increased alveolar size. The increase in lung size may be attributed to emphysema and mucous fluid pooling.[144] Prolonged tracheal occlusion leads to an almost complete loss in type II epithelial cells and a subsequent loss of surfactant production.[65] In an animal model, removal of the tracheal clip 1 week prior to delivery reversed the negative effects tracheal occlusion has had on type II epithelial cells.[145] However, with the removal of the clip, premature delivery and infection are always a great risk. Corticosteroids were employed to succumb these risks, and a maternal injection with steroids together with the removal of the clip 2 days before birth resulted in an almost complete recovery of type II cells.[146] In an animal model of tracheal occlusion, an upregulation of FGF7 was seen after ligation of the trachea.[142] Lambs with a surgically created CDH had lower IGF-1 levels than control lambs. However, tracheal occlusion of lambs with CDH restored IGF-1 levels to control levels.[141] Fetal tracheal occlusion in the lamb surgical model of CDH also resulted in an upregulation of TGF-β2.[143]

Alveolar Capillary Dysplasia

Alveolar capillary dysplasia (ACD), with or without misalignment of the pulmonary veins, is a fatal lung disease characterized by persistent pulmonary hypertension of the neonate. These infants die despite intensive medical support such as inhaled

nitric oxide and extracorporeal membrane oxygenation. The initial response to inhaled nitric oxide is usually good, but often persistent pulmonary hypertension of the neonate reoccurs.[147] The number of capillaries is reduced greatly, causing a decrease in blood-air interface. Furthermore, the veins are often misaligned, sharing an adventitial sheath with the bronchoarterial bundle.[148] The alveolar epithelium is surrounded with mesenchyme containing small blood vessels but no capillaries.[149] It seems that the vessels fail to grow towards the type I cell and fuse to form a thin blood-air interface. Recently, it has been reported that a majority of eNOS null mice exhibit an ACD phenotype.[69] Nitric oxide has been reported to induce angiogenesis via induction of VEGF.[150] A deficiency of eNOS in infants with ACD might prevent the capillaries to grow towards the alveoli. There are many other factors governing the development of the pulmonary vascular tree, and their interplay, both spatial and temporal, needs to be further elucidated. More than 50% of patients with ACD have associated congenital anomalies, not all associated with vascular malformations, suggesting there are other factors involved in the development of ACD.

Congenital Cystic Adenomatoid Malformation of the Lung

Congenital cystic adenomatoid malformation (CCAM) and bronchopulmonary sequestration (BPS) are congenital lung malformations characterized by abnormal airway patterning during lung branching morphogenesis.[151] BPS is caused by the branching of an accessory diverticulum of the foregut, caudal from the branching of the normal lung bud, while CCAM is formed by abnormal branching of immature bronchioles.[151] The histologic similarities between CCAM and BPS suggest a similar defect in early lung development. One of the potential genes of influence for these developmental disorders is Hoxb5. Throughout gestation, its expression in CCAM and BPS maintains a level typical for early lung development.[151] Four different types of CCAM have been identified, the first three showing a bronchial type of epithelium and type 4 an alveolar-acinar type of epithelium.[152] This suggests that the arrest in development between the different types took place in different stages in lung development. The arrest of the first three types happened in the pseudoglandular phase of lung development and the arrest of type four in the saccular phase of development.[152] Another explanation might be that there is no arrest per se, but more of a distal development and maturation disorder. Overexpression of FGF7 using the SP-C promotor in mice results in a lung with CCAM-like lesions.[25] This observation raises the possibility that disruption of FGF7-dependent interactions between epithelium and mesenchyme may be involved in the pathogenesis of CCAM in humans.

Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD) is the chronic lung disease of infancy which likely is a consequence of ventilator and oxygen therapy for acute respiratory failure after premature birth.[153] During long-term follow-up, children with BPD have varying degrees of persistent respiratory problems, including airflow limitation, gas

trapping, exercise intolerance, pulmonary hypertension and others. Histologically, the lungs display a decreased number of distal respiratory units, abnormal microvessels, saccular emphysema and atelectasis.[154] Thus, important features of BPD are decreased alveolarization and abnormal vessel growth.[155] This suggests that a disruption in vascular growth goes together with a decrease in distal lung branching. Indeed, blockage of VEGFR signaling has been shown to result in reduced pulmonary vascularization and alveolarization of the neonatal rat and mouse lung.[156-158] Infants dying of BPD have decreased levels of pulmonary VEGF,[159] and infants developing BPD have lower levels of VEGF in their tracheal aspirate than controls.[160] VEGF is one of many downstream targets of the hypoxia-inducible regulator HIF. Whether HIFs are expressed in the BPD lung remains to be investigated. HIF-1α null mutants die early in gestation,[161] whereas mice deficient in HIF-2α die from respiratory problems after birth.[162] Compared with control mice, lungs of HIF-2α-deficient mice have decreased VEGF protein levels. Their lungs display a normal number of alveoli, but there are abnormally thick alveoli with abnormal capillaries.[162] When exogenous VEGF was injected either intraamniotic or intratracheally 1 day before preterm delivery, it significantly improved lung maturation and decreased mortality due to RDS in the HIF-2α-deficient mice. In addition, VEGF has been shown to stimulate epithelial cell proliferation in human fetal lung in vitro.[163] Thus, VEGF administration may be a potential treatment for the regeneration of alveoli. Steroids are often used for maturation of the lung, but there have been reports of associations of steroids and adverse neuro-developmental outcomes.[164] Low doses of dexamethasone upregulated VEGF expression; however, high doses of dexamethasone seemed to suppress VEGF expression,[162] indicating that excessive amounts of dexamethasone counteracts the beneficial effects of VEGF.

A vast array of other growth factors are suggested to play a role in BPD, including IGFs, epidermal growth factors, TGF-α, PDGFs, FGFs and TGF-β [for a review, see 165]. The complex interactions of all these growth factors needs to be understood in order to appreciate the impact they have on BPD. It is plausible that transcription factors, such as Foxa2, GATA-6 and TTF-1, implicated in alveolarization and microvascular maturation, also play a role in the development of BPD.

CONCLUSION

Molecular signaling during fetal lung development is an intricate and complex concert of various growth factors, transcription factors and morphogens. Lung disease is one of the leading causes of mortality and major morbidity in the newborn period. An understanding of the basis of lung development is vital in order to understand the aberrant development (*table 1.1*) and, subsequently, to contemplate new and innovative therapies for the treatment of newborn lung disease.

Table 1.1 Stages of lung development when aberrations occur

Stage	Developmental disorder	Important factors
Embryonic	CCAM, BPS, TEF, CDH	Shh, Ptch, Smo, Hip, FGFs, Foxa2, Foxj1a, Foxf1, Hoxa/b, Gli2/3, TTF-1, Pitx-2, BMP-4, mSpry2, Tbx4/5, RA, RAR, Nodal, Lefty1-2
Pseudoglandular	ACD, CDH	TGF-β, Smad, FGFs, GATA-6, N-myc, Shh, BMP-4, mSpry2, VEGF, HIFs
Canalicular	ACD	VEGF, eNOS, HIFs, Mash1, Hes1, Pod-1, TTF-1, Foxa2, GATA-6
Saccular		TGF-β, IGF-1,GC,GR, MK, HIFs, VEGF
Alveolar/micro-vascular maturation	BPD	Foxa2, GATA-6, PDGFs, IGFs, EGFs, TGFs, FGFs TGFβ, TTF-1, RAR, VEGF, MK, Foxf1, HIFs

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chapter 2

OUTLINE OF THE THESIS

THE INFLUENCE OF HYPOXIA INDUCIBLE FACTOR ON LUNG DEVELOPMENT

HYPOXIA INDUCIBLE FACTOR AND LUNG DEVELOPMENT

Lung disease is a major cause of mortality and severe morbidity in the newborn period and remains a significant challenge facing the neonatologist, pediatric surgeon and pediatric intensivist despite the availability of various treatment modalities.

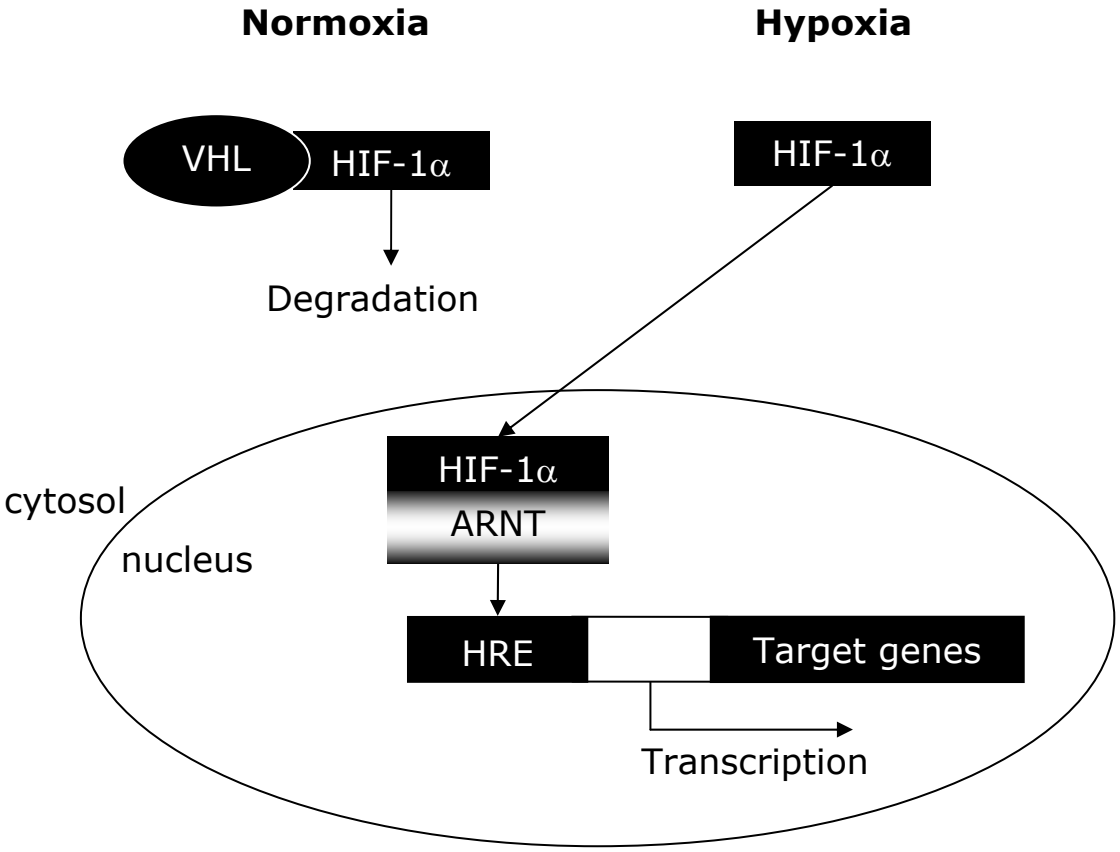
Present therapeutic modalities are administration of oxygen through artificial ventilation (either conventional, high frequency oscillation ventilation [HFO] or experimental liquid ventilation), inhaled nitric oxide (iNO), and intra-tracheally or intravenously administered prostacyclin (PGI_2) is available as a last resort. When these options fail extracorporeal membrane oxygenation (ECMO), and most recently phosphodiesterase inhibitors are available (for review see (1)).

Developmental aberrations *in utero* lie at the origin of some of these disorders, including idiopathic persistent pulmonary hypertension of the neonate (PPHN), congenital diaphragmatic hernia (CDH) and other causes of pulmonary hypoplasia, alveolar capillary dysplasia (ACD), or congenital cystic adenomatoid malformation of the lung (CCAML). Also abnormal adaptation of the fetus to *ex utero* life may lead to pulmonary hypertension (PH) or occasionally bronchopulmonary dysplasia (BPD).

Prenatal lung development

Molecular signalling during fetal lung development is an intricate and complex concert of various growth factors, transcription factors and morphogens. Normal pulmonary development takes place in a relatively hypoxic environment of the uterus[2] that is beneficial for lung organogenesis, including vascular development.[3,4] In most mammalian systems the cellular responses to oxygen alterations are mediated through a highly conserved hypoxia-inducible factor (HIF) family of transcriptional regulators. In relative hypoxia, the hypoxia-sensitive HIF-α accumulates and dimerizes in the nucleus with its constitutive counterpart ARNT (Aryl Hydrocarbon Nuclear Translocator, HIF-1β). Subsequently HIF recognizes HIF-response elements (HRE) within the promoter regions of hypoxia-responsive target genes (*figure 2.1*). HIFs are known to control over 500 genes, many of which are involved in endothelial cell proliferation and survival, such as VEGFR2, VEGF, eNOS, and iNOS.[5-9]

Figure 2.1 HIF accumulates in hypoxic conditions and activates its target genes

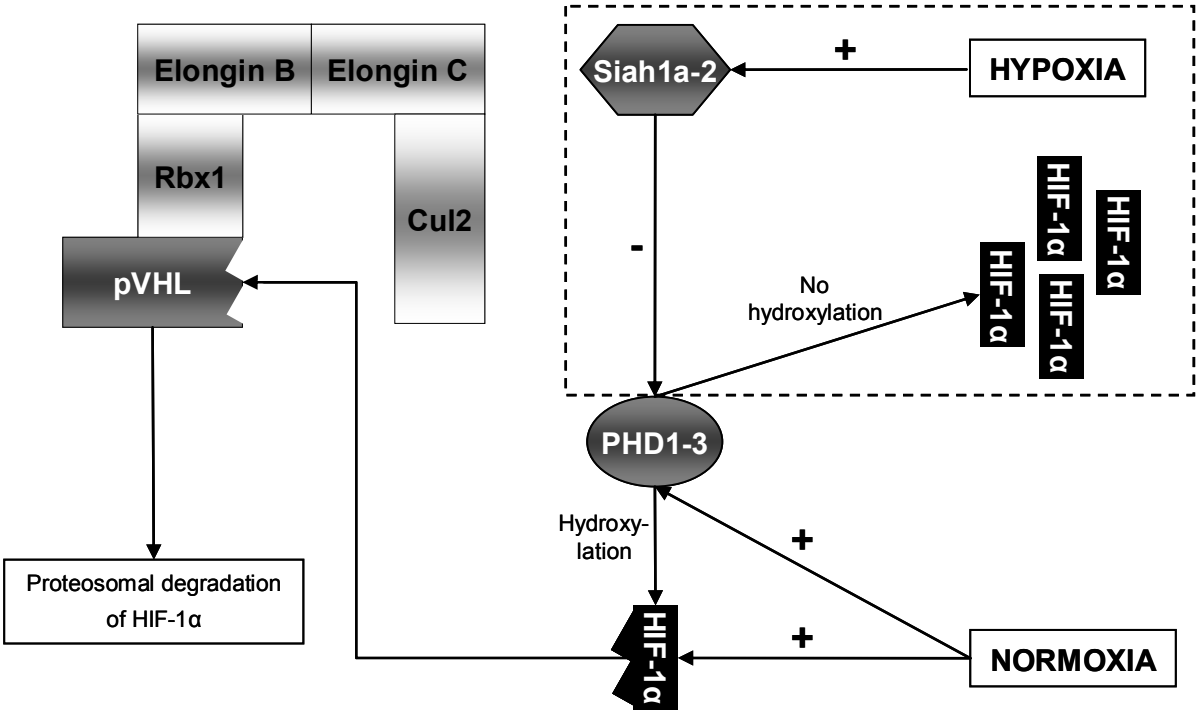


Postnatal lung development

Postnatally, pulmonary oxygen levels rapidly increase which sets off several physiological reactions in the cardiopulmonary system, such as closure of the ductus arteriosus/foramen ovale and structural changes of the pulmonary vessels. These reactions rapidly decrease pulmonary vascular resistance, thus adjusting the body to postnatal life. Their failure to occur may lead to serious neonatal adaptation problems and threatened hypoxia known as pulmonary hypertension of the neonate.[10]

Under normoxic conditions the HIF-α subunits are rapidly degraded through association with the von Hippel-Lindau E3 ubiquitin ligase, thereby annihilating HIF-1s downstream effects. A dysfunction of the VHL protein leads to VHL disease characterized by highly vascularized tumors.[11] Recognition of HIF-α by VHL only occurs when two conserved proline residues (Pro 402 and Pro 564) in the oxygen dependent degradation domain (ODD) of HIF-α are hydroxylated.[12,13] This hydroxylation is catalyzed by a family of prolyl hydroxylase domain enzymes, named PHD1, PHD2, and PHD3.[14,15] The PHDs are also degraded by E3-ubiquitin-ligases, namely Siah (seven in absentia homolog) 1 and 2. Degradation is enhanced by hypoxia and Siahs are transcriptionally upregulated during hypoxia.[16] Thus, various proteolytic mechanisms are responsible for control of HIF-α stability (*figure 2.2*).

Figure 2.2 Overview of the various mechanisms controlling HIF-1α stability



Within the dashed-lined box the hypoxic mechanism is depicted. Normoxic circumstances are depicted outside the dashed-lined box.

CLINICAL IMPLICATIONS

The rational development of new and innovative treatment strategies for neonatal lung disease requires full understanding of the molecular basis of lung development. These new treatment strategies should preferably be tested in therapeutic settings in the form of randomized controlled trials. This thesis describes the induction of the HIF pathway in murine lung development and also the presence of this pathway in normal early human lung development. Furthermore the role of this pathway in some forms of congenital pulmonary vascular disorders is described.

The role of the HIF pathway is described in a series of neonatal lung disease entities leading to PHN (pulmonary hypertension of the newborn). This is a debilitating disease characterized by severe hypoxemia and right-to-left shunting through a patent foramen ovale, and/or a patent ductus arteriosus, and through intrapulmonary channels.[17,18] Also there is abnormal vascular structure, growth, and/or reactivity. PHN can occur without identifiable cause the so-called idiopathic PHN or in association with various diseases. Regardless of its etiology, vascular morphology in the various diseases is similar consisting of medial hyperplasia, adventitial thickening, peripheral extension of muscularization in normally non-muscularized vessels, and an overall reduction in pulmonary vascular density.[19-21] This pathology is totally different from other forms of pulmonary hypertension secondary to increased flow to the pulmonary vascular bed due to left-to-right

shunting in case of congenital heart disease. Under these circumstances a so-called plexiform arteriopathy develops consisting of necrotizing arteritis, concentric-laminar intimal fibrosis, plexiform lesions and dilatation lesions.[22,23]

PHN arises when the pulmonary vasculature fails to adapt to postnatal life or when prenatal development is not appropriate. Alveolar capillary dysplasia (ACD) is another albeit rare cause of PHN and is caused by a malformation of the pulmonary vascular bed. Since this is such a rare anomaly, with obvious angiogenic aberrations, this disease is discussed separately. ACD is a lethal disease caused by an improperly formed vascular bed impeding proper gas exchange. Veins share an adventitial sheath with the arteries in the bronchovascular bundle instead of their normal intra-acinar course away from the arterial branches.[24,25] The alveolar epithelium is surrounded by mesenchyme with small blood vessels but no capillaries or capillaries that are not adjacent to the epithelium where gas exchange takes place.[24,25]

Taken together, the research described in this thesis brings together the role of HIF during basic murine lung development and the potential impact of the HIF pathway in the etiology of human diseases with vascular aberrations such as PH and ACD. Furthermore it gives new insights that may be of benefit in contemplating innovative treatment strategies.

THIS THESIS

An introduction to normal lung development is given in **chapter 1**, detailing the various stages in development and their specific transcription and growth factors. Furthermore, this chapter describes several developmental errors and the diseases that may result from these aberrations.

Chapter 2 briefly outlines the role of HIF in lung development and states what clinical implications the studies described in this thesis may have.

Chapter 3 describes a study investigating whether constant activation of the HIF/VEGF pathway in murine lung development results in rapid maturation and hypervascularization. To this aim an oxygen-insensitive HIF-1α was expressed in the distal epithelium of the lung. Both fetal and postnatal lung development was assessed.

In **chapter 4** the hypothesis is tested that upregulation of HIF-1α in 20% O_2 lung explant culture leads to a similar branching pattern as seen in hypoxic (2% O_2) lung cultures, i.e. increased vascularization and increased branching. Chemical induction of HIF-1α was accomplished by adding either cobalt chloride ($CoCl_2$), dimethyloxalylglycine (DMOG), or desferrioxamine (DFO) to culturing media.

HIF-1α plays an important role in mediating oxygen-regulated events of pulmonary vascular development. However, the components regulating HIF-1α stability in the developing lung have remained uninvestigated so far. In **chapter 5**, we determined the spatial expression of various components of the HIF system in the human lung during the pseudoglandular stage of lung development (8 - 14 wks), when the primitive pulmonary vascular system develops.

The pathways involved in the development of pulmonary hypertension in neonates are largely unknown. These pathways may be dependent on the etiology of the various disease entities associated with pulmonary hypertension. Remodeling of the vessels occurs in all cases of PH, however the question remains whether a final common pathway is present leading to this remodeling. Therefore, **chapter 6** addresses the involvement of the HIF pathway (pVHL, HIF-1α, VEGF, VEGFR2, eNOS, and iNOS) in PH with various underlying causes.

In **chapter 7** the role of the HIF-pathway in the development of the pulmonary vascular bed in alveolar capillary dysplasia (ACD) patients is assessed through immunohistochemical analysis of the molecules pVHL, HIF-1α, VEGF, VEGFR2, eNOS, and iNOS in lung samples of ACD patients and age-matched controls.

In **chapter 8** the results of our studies are incorporated into the discussion of the main remaining questions about lung development and neonatal lung disease. Furthermore new treatment strategies are contemplated and suggestions are given for future research.

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Studies in Murine Lung

part 1

chapter 3

EPITHELIAL EXPRESSION OF CONSTITUTIVE ACTIVE HIF-1α STIMULATES POSTNATAL LUNG DEVELOPMENT

ABSTRACT

Hypoxia-induced pathways are of importance for normal and abnormal lung development. The hypoxia response is mediated via the hypoxia-inducible factor (HIF) family of transcriptional regulators. In hypoxia HIF is stable and initiates many downstream pathways, including VEGF-induced angiogenesis. However, normoxic conditions cause HIF to be rapidly degraded. Since the lung undergoes critical changes in oxygen supply during its development, we investigated whether a constitutive active HIF-1α increases postnatal vascularization and alveolarization. Transgenic mice expressing an oxygen-insensitive HIF-1α in the distal lung epithelium were created using the surfactant protein C promoter. To study the early effects of HIF-1α overexpression on vascularization and branching HIF-1α ΔODD mice were bred with Tie2-LacZ (endothelial LacZ marker) mice to visualize the vessels. At E11.5 primitive lung buds were dissected and cultured in 2% and 20% O_2. Low oxygen stimulated vessel growth in controls and transgenic lungs, but stable HIF-1α expression significantly increased vessel growth in normoxic conditions. This increase in vasculature did not have an impact on epithelial branching branching. HIF-1α ΔODD mice exhibited a significant increase in small vasculature at term (E18.5) and postnatal gestation (days 2 - 21). Histological and morphometric analysis, using mean linear intercept, alveolar volume, and secondary crest density, of postnatal transgenic lungs showed an advancement of alveolarization compared to controls. Stable HIF-1α stimulated postnatal $VEGF_{188}$ and in lesser extent $VEGF_{120}$ expression, while $VEGF_{164}$ and VEGFR2 expression was not affected. Postnatal expression of Pecam-1, a vascular marker, and aquaporin 5, a marker for type I cells, was up-regulated in HIF-1α ΔODD lungs. Collectively, the data show that stable HIF-1α stimulates early and postnatal vessel growth via up-regulation of VEGF expression. The increase in postnatal vasculature is accompanied by an enhanced alveolarization.

INTRODUCTION

Interactions between airways and blood vessels are critical for lung development.[1,2] Although airway branching has been extensively studied,[3-5] investigations in pulmonary vascular development have been limited. Traditionally, it has been thought that pulmonary vascularization takes place through distal vasculogenesis (i.e. formation of vascular lakes in the mesenchyme) and proximal angiogenesis (i.e. sprouting of new vessels from existing ones).[6] However, recent observations in mice have shown that connections between proximal and distal vasculature are already made at the start of lung development and a new mechanism, distal angiogenesis, has been proposed.[7] The mechanisms and signaling pathways that regulate distal angiogenesis remain poorly understood. Early lung development occurs in a relatively hypoxic environment *in utero.*[8] This low oxygen environment is beneficial for pulmonary vascular development and is mediated via the hypoxia-inducible factor (HIF) family of transcriptional regulators.[1]

Postnatal growth and maturation of the capillary network is vital for creating a proper blood-air barrier and to make gas exchange possible. Alveoli are formed through the process of septation, whereby secondary septa lift off of existing primary septa.[4] The capillary layer from the existing primary septum is pulled into the newly formed secondary septum and, therefore, the new septum contains a double capillary layer. The double capillary layer of the immature alveolar septa is later reduced to a single capillary layer.[9] Impaired septation may lead to bronchopulmonary dysplasia (BPD), a chronic lung disease of infancy that likely is a consequence of ventilator and oxygen therapy for acute respiratory failure after premature birth.[10,11] Important features of BPD are decreased alveolarization and abnormal vessel growth.[12] Furthermore, patients suffering from BPD have been shown to have low levels of pulmonary vascular endothelial growth factor (VEGF).[13] Interestingly, pharmacological blockade of VEGF signaling has been shown to impair postnatal lung development in newborn rats.[2,14] In contrast, VEGF gene therapy improved vascular development and prevented hyperoxia-induced BPD in newborn rats.[2] VEGF is a target gene for HIF[15] and enhancing HIF stability has been shown to increase the expression of VEGF and to stimulate angiogenesis.[16,17] Furthermore, pharmacologic HIF stabilization in a preterm baboon model of BPD was shown to be associated with enhanced lung growth, improved oxygenation and lung compliance.[18]

Clearly oxygen via HIF plays a major role in lung vascular development. HIF is composed of an alpha subunit and the aryl hydrocarbon receptor nuclear translocator (ARNT).[19] Two alpha subunits have been shown to positively regulate the hypoxic response, HIF-1α and HIF-2α.[20-22] HIF-3α may actually be a suppressor of hypoxic gene induction.[23] The regulation of HIF by oxygen occurs through modifications of the alpha subunit, whereas the beta subunit is constitutively expressed and not affected by hypoxia. Hypoxic conditions allow the HIF-α subunit

to accumulate and translocate to the nucleus, where, upon binding to ARNT, it recognizes HIF-response elements within the promoter regions of hypoxia-responsive target genes. HIFs are known to control over 500 genes, many of these being involved in endothelial cell proliferation and survival, such as VEGF.[15] Under normoxic conditions proline residues 402 and 564 of the oxygen-sensitive (ODD) domain in the alpha subunit are rapidly hydroxylated by prolyl hydroxylase domain enzymes.[24,25] The hydroxylated HIF-α subunits are recognized by the von Hippel-Lindau E3 ubiquitin ligase complex and targeted for proteosomal degradation.[26,27]

Based on the interaction between airways and vessels during development and the potential role of oxygen in regulating these interactions, we investigated whether expression of an oxygen-insensitive HIF during fetal and postnatal lung development would result in hypervascularization and accelerated lung branching and alveolarization.

MATERIALS AND METHODS

Animal protocols were in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care and Use Committee of the Hospital for Sick Children, Toronto, ON, Canada.

Transgene construction

The human full-length HIF-1α cDNA construct (generous gift of Dr. Semenza, Johns Hopkins University, Baltimore, MD, USA) was used as template to generate the HIF-1α ΔODD mutant. The deletion mutant (HIF-1$\alpha_{\Delta 401\text{-}603}$) was constructed by overlap extension using PCR as previously described.[28] The deletion was confirmed by DNA sequencing. The 1.95 kb HIF-1α ΔODD cDNA was subcloned 3' of the 3.7 kb human SP-C promoter[29] and 5' of the SV40 small T intron and polyadenylation sequences. The expression cassette was excised with NdeI and NotI, purified using Glass Milk (Gene Clean Kit Bio 101, BioCan, Canada) and Elutip-D columns (Schleier and Schuell, NY), and ethanol precipitated.

Production of transgenic mice

Transgenic SPC- HIF-1α ΔODD embryos were generated according to Hogan et al.[30] DNA injections into pronuclei of (C57BL/6 X SJL) F2 embryos were carried out at a concentration of 3 ng/µl. The genotype was established by PCR analysis of genomic DNA extracted from the embryonic tail and confirmed by Southern blot analysis. The primers used were 5'-TCACCTCTGTCCCCTCTCCCTACG-3' and 5'-CATTTCTCTCATTTCCTCATGGTCACATGG-3'. Annealing temperature was 62°C and 35 cycles were used for amplification. Tie2-LacZ mice were obtained from Jackson Laboratory, Bar Harbor, MN, U.S.A.[31] In Tie2-LacZ transgenic mice, the 2.1 kb 5' flanking region of the murine Tie2 promoter drives the expression of the bacterial LacZ reporter gene exclusively to endothelial cells.[31] Cells transcribing the LacZ

gene can be viewed by staining for β–galactosidase activity. The Tie2-LacZ mice were bred either with C57BL/6 X SJL mice or with HIF-1α ΔODD mice. Fetuses were taken at E11.5 for whole lung organ culture experiments.

Whole lung organ culture and LacZ staining

Lung buds were dissected from E11.5 HIF-1α ΔODD;Tie2-LacZ and C57BL/6 X SJL;Tie2-LacZ embryos (day of vaginal plug is E0.5) and placed on a floating (8 μm Whatman Nuclepore polycarbonate) membrane (Integra Environmental Inc. Burlington, ON, Canada). Explants were grown in DMEM/10% FCS (Gibco, Grand Islands, NY, U.S.A.) and maintained in 20% O_2 or 2% O_2 and 5% CO_2 at 37°C. Lung explants were fixed, washed and stained in X-gal staining solution as previously described.[1] For imaging, explants were cleared in methyl salicylate. For sectioning, explants were fixed in 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS), dehydrated and embedded in paraplast. Sections (12 μm) were mounted onto Superfrost slides (Fisher Scientific, Unionville) and counterstained with Nuclear fast red. Digital images were taken with a Leica MZ12 stereomicroscope equipped with a digital imaging system. Vessel density (ratio vessel pixels/whole lung pixels) was determined using Openlab software (Improvision Inc, Lexington, MA).

Fluorescent angiography

The chest cavity of E18.5 mice was opened and a 27½ gauge needle was inserted into the right ventricle. Blood was flushed out of the lungs by injecting PBS with heparin (2 U/ml) in the right ventricle until free return of the solution was observed through a small incision in the left atrium. The fetal arterial pulmonary circulation was then perfused with a solution of 0.75% low melting point agarose (Sigma, St. Louis, MO) containing 0.2 μm yellow-green fluorescent Fluospheres® carboxylate-modified microspheres (1:10 [v/v]) (Molecular Probes, Eugene, OR). Fetuses were put on ice and the agarose was allowed to solidify. Lungs were dissected out and fixed in 4% paraformaldehyde at 4°C overnight. Thick sections (160 μm) were cut with a vibratome (Leica VT1000S), counterstained with α-smooth muscle actin antibodies (Neomarkers, Montreal, QC, Canada) in combination with a rhodamine-conjugated secondary antibody (Calbiochem, San Diego, CA, USA). Sections were mounted with Aquaperm (Shandon, Waltham, MA, USA) and digital images were taken with a confocal microscope (Leica TCS SP5).

Immunohistochemistry

Postnatal lungs were fixed under constant inflation of 10 cm H_2O. Immunohistochemical analyses were performed as previously described.[32] Rabbit polyclonal antibodies against HIF-1α (Cell Signaling Technologies, Danvers, MA) were used at 1:50 dilution. Biotinylated goat anti-rabbit IgG (1:300) was used as secondary antibody. Color detection was performed according to instruction in the Vectastain ABC and DAB kit (Vector Laboratories, Burlingname, CA, USA) and

counterstaining was with Carazzi's haematoxylin. Control experiments were carried out by replacing the primary antibody with normal goat serum.

RNA isolation and real-time quantitative RT-PCR (qPCR)

Lungs were dissected, immediately frozen in liquid nitrogen, and stored at -70°C. RNA was extracted with the RNA easy kit (Qiagen, Mississauga, ON, Canada). Total RNA was reverse transcribed (37°C) in a total volume of 20 µl using random hexamers (Invitrogen, Burlington, ON, Canada). The resulting templates were quantified by real-time PCR (ABI Prism 7900) using SYBR green I (Eurogentec North America, SanDiego, CA).

Primers for total HIF-1α mRNA (measuring both mouse HIF-1α mRNA and human HIF-1α ΔODD mRNA) were (F) 5'-ATCCATGTGACCATGAGGAAATGAGAG-3' and (R) 5'-CCACACTGAGGTTGGTTACTGTTGGTA-3' while primers for measuring specifically human HIF-1α ΔODD mRNA were (F) 5'-GTTGTGAGTGGTATTATTCAGCACGACTTG-3' and (R) 5'-AGTAGGTTCTTGTATTTGAGTCTGCAGCAAAG-3'. Primers and TaqMan probes for total VEGF, $Vegf_{120}$, $Vegf_{164}$, and $Vegf_{188}$ were similar to previously published sequences,[33] while primers and TaqMan probes for Pecam-1, SP-C and Aquaporin 5 were purchased from ABI as Assays-on-Demand™ for murine genes. Reactions were carried out in 96 well plates in triplicate. For each probe, a dilution series determined the efficiency of amplification of each primer-probe set. For the relative quantitation, PCR signals were compared between groups after normalization using 18S as an internal reference. Briefly, relative expression was calculated as $2^{-(\text{Ctgene of interest-Ct18S})}$. Fold change was calculated according to Livak et al.[34] Data are presented as mean ± s.e.m. A $P < 0.05$ was considered statistically significant.

Western blotting

Nuclei were isolated using a nuclear enrichment kit for tissue (Pierce, Rockford, IL). Western blot analyses of nuclear lysates were performed as previously described.[35] Primary antibodies were rabbit polyclonal anti-human HIF-1α (dilution of 1:1000, Cell Signalling Technology, Danvers, MA) and goat anti-rabbit horseradish peroxidase–conjugated IgG was used as secondary antibody (dilution of 1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA).

Morphometric analysis

As rates of lung maturation vary between regions of the lung, all morphometric assessments were performed on the right middle lobe. The lungs were embedded in paraffin wax, cut in 5 µm sections, and stained with haematoxylin and eosin. Morphometric assessments were performed on coded images to mask the control and transgenic lungs. Digital images were captured using a Leica digital imaging system at 20X magnification with random sampling of all tissue in an unbiased fashion. Images of 10 non-overlapping fields from each slide, with 3 slides/animal, and 5 animals per group were captured. Tissue fractions and secondary crests densities were measured as previously described.[36] Mean linear intercepts were

measured and calculated as described by Dunhill.[37] Alveolar surface areas per unit lung volume were calculated as described by Kawakami and coworkers.[38] Slides were stained for elastin, to enhance recognition of vessels and number of small vessels (diameter range 20 to 65 μm) per field was assessed. Average values were calculated for 10 images per slide, and the average value for the three slides was used to calculate an average value for each animal.

Data presentation

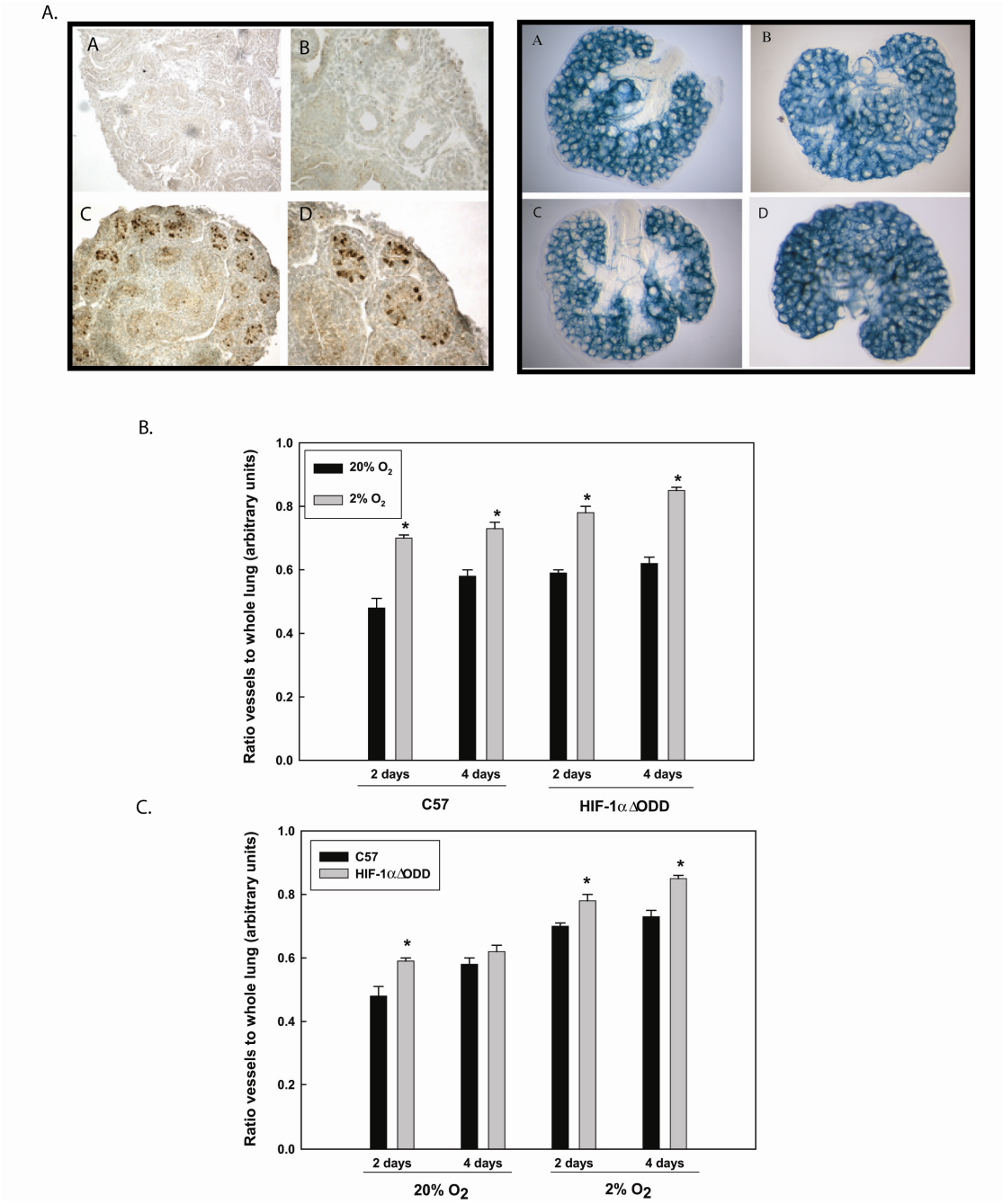
All morphometric values are mean ± SEM of 5 animals per time point. Statistical significance ($P < 0.05$) was determined by one way ANOVA, followed by *post hoc* analysis using Duncan's multiple range test where significant differences were found between groups (JMP software).

RESULTS

Over-expression of constitutive active HIF-1α on early lung development

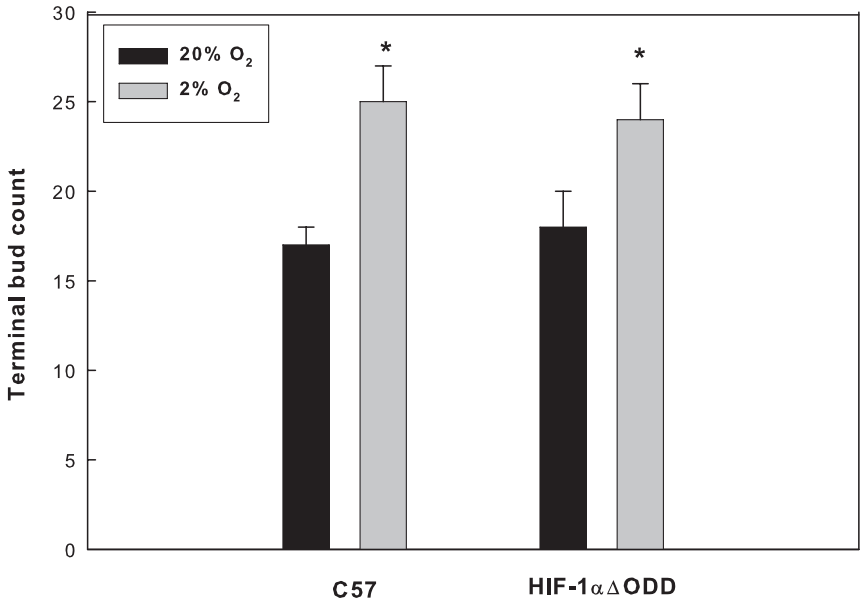
Tie2-LacZ mice were used to evaluate the effect of increased HIF-1α protein on vascularization and branching during early lung development (E11.5). The Tie2-LacZ mice were bred to HIF-1α ΔODD mice or to C57BL/6 X SJL (control) mice. Lungs were dissected at E11.5 and cultured either at 2% or 20% oxygen.[1] Immunostaining revealed expression of HIF-1α in the distal epithelium of lung explants of HIF-1α ΔODD, but not control, mice cultured for 4 days at 2% and 20% O_2 (*Figure 3.1A, left panel*). Whole mount LacZ staining revealed a complete overview of the vasculature in the lung.[1,7] In agreement with previous results[1] control C57 lungs cultured at 2% O_2 exhibited greater vessel densities than control lungs cultured at 20% O_2 (*Figure 3.1A; right panel-B vs. A; Figure 3.1B*). Low oxygen stimulated also vascularization in HIF-1α ΔODD lungs (*Figure 3.1A, right panel-D vs. C; Figure 3.1B*). HIF-1α ΔODD lungs cultured in 2% O_2 had greater vessel densities compared to control lungs cultured at 2% O_2 (*Figure 3.1C*). Similarly, vascularization in HIF-1α ΔODD lungs cultured at 20% O_2 was increased when compared to control lungs cultured at 20% O_2 (*Figure 3.1C*). These results confirm that low oxygen stimulates vessel growth in the developing lung and, more importantly, that stable HIF-1α can sustain vessel growth in normoxic conditions. To determine whether the increase in vascularization led to increase epithelial branching morphogenesis we counted the terminal buds of the E11.5 explants after 2 days in culture. Low oxygen increased terminal bud counts both in control and HIF-1α ΔODD lungs. However, no difference in early branching was noted between control and HIF-1α ΔODD lungs when cultured at 2 or 20% O_2 (*Figure 3.2*).

Figure 3.1 *(for color figures see page 154)*



*Effect of oxygen on vessel growth in lung explants (**A-left panel:** HIF-1α ΔODD was constitutively expressed independent of oxygen concentration (2 vs. 20%) as demonstrated by immunostaining using HIF-1α antibody (A-B: immunoreactivity in C57 lung explants kept at 20% O_2; C-D: immunoreactivity in HIF-1α ΔODD lung explants kept at 20% O_2. Same staining pattern was observed at 2% O_2. A-C: Bar = 100 μm; B-D: Bar = 25 μm). (**A-C**) Low oxygen enhanced vessel growth in C57 and HIF-1α ΔODD lungs, (**A-right panel**: (A) C57 20% O_2, (B) C57 2% O_2, (C) HIF-1α ΔODD 20% O_2, (D) HIF-1α ΔODD 2% O_2. All explants were cultured for 4 days). (**B**) Quantitative measurement of vascular density showed an increase in vascularization in both C57 and HIF-1α ΔODD lungs when kept at 2% O_2 compared to explants culture at 20% O_2. (**C**) Vessel density was greater in HIF-1α ΔODD lungs independent of O_2 concentration. Values are means ± SEM for 10 lungs in each group. *P < 0.05.*

Figure 3.2 Effect of oxygen on lung branching morphogenesis



*Terminal buds were counted from explants kept at either 2% or 20% O_2. Low oxygen stimulated branching in both C57 and HIF-1α ΔODD lung explants. Over-expression of constitutive HIF-1α did not increase lung branching morphogenesis above C57 controls. Values are means ± SEM for 25 lungs in each group. *P < 0.05.*

Overexpression of constitutive active HIF-1α on postnatal development

HIF-1α expression

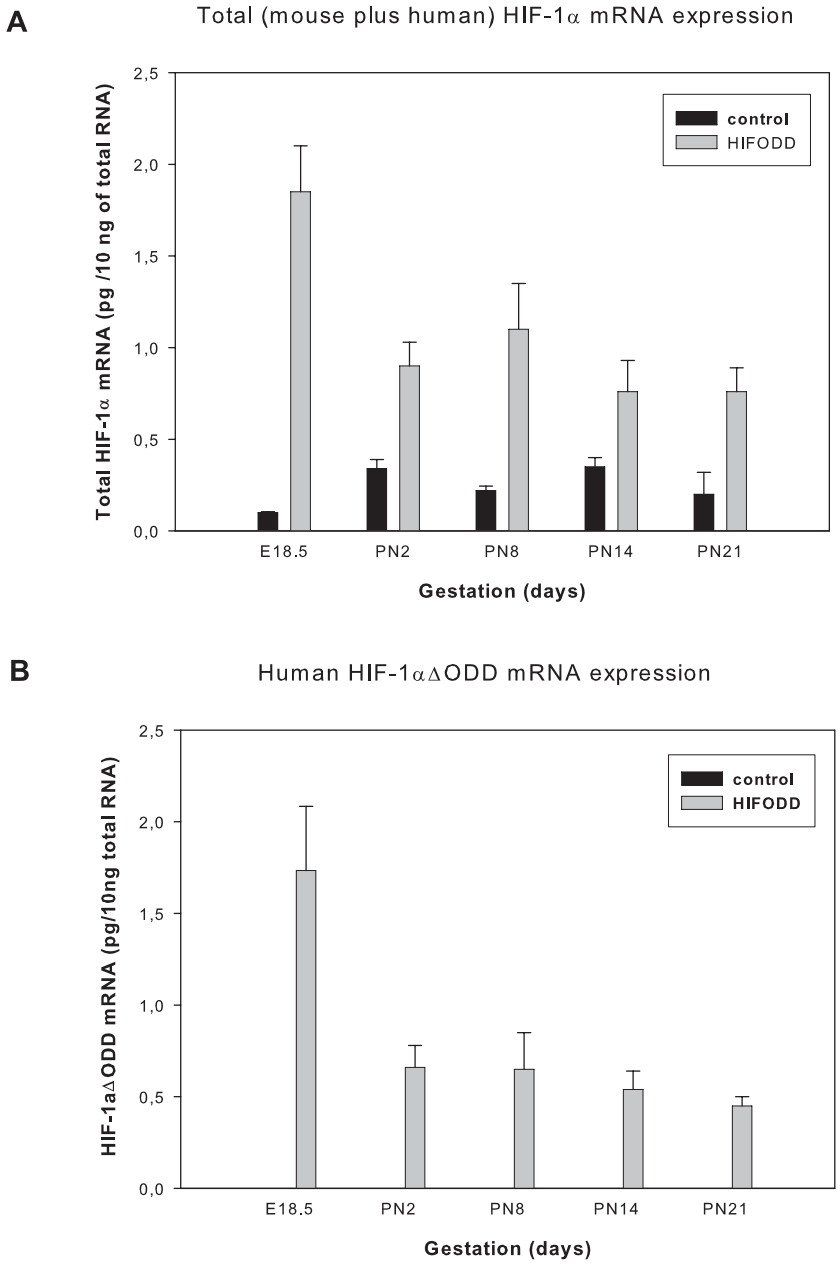
In control mice pulmonary expression of mouse HIF-1α mRNA remained relatively constant between E.18.5 and PN (postnatal day) 21 of gestation (*Figure 3.3A*). In contrast, pulmonary levels of total (mouse plus human) HIF-1α mRNA were significantly increased (> 15-fold) in E18.5 HIF-1α ΔODD mice. Total pulmonary HIF-1α mRNA amount decreased postnatally in the transgenic mice, but it remained significantly greater (> 3-fold) than that of C57 control lungs. The increase in total HIF-1α mRNA was due to the SPC promoter-driven expression of human HIF-1α ΔODD mRNA in the transgenes (*Figure 3.3B*). Immunohistochemical analysis revealed that HIF-1α protein was up-regulated in the distal epithelial type II cells of transgenic lungs when compared to control lungs during all postnatal ages (*Figure 3.4*). Western blot analysis corroborated these findings and further showed that the immuno-reactive product was the 80 kDa HIF-1α ΔODD recombinant protein (not shown).

VEGF expression

VEGF is a potent mitogen for endothelial cells, influencing angiogenesis and vasculogenesis.[39] Since its expression is regulated by HIF-1α we measured VEGF expression by real-time qPCR. Total VEGF expression in C57 control lungs was constant between E18.5 and postnatal day 21 (*Figure 3.5A*). However, the amount of total VEGF mRNA was significantly greater in HIF-1α ΔODD lungs at all gestational days compared to control lungs. VEGF exists in three different isoforms due to differential splicing and, therefore, we tested the possibility that VEGF

isoform expression could be different among the control and HIF-1α ΔODD lungs. We found that specifically the expression of $VEGF_{188}$ isoform was increased in the HIF-1α ΔODD lungs at all gestational days (*Figure 3.5B*). Message levels for the $VEGF_{120}$ isoform were slightly, but significantly, increased at postnatal day 2 and 8, while $VEGF_{164}$ mRNA expression was not altered in the HIF-1α ΔODD lungs. VEGFR2 mRNA expression was not affected by HIF-1α ΔODD over-expression when compared to controls (not shown).

Figure 3.3 Measurements of total HIF-1α and HIF-1α ΔODD gene expression in C57 control and HIF-1α ΔODD lungs during postnatal development



*Message levels of total (mouse and human) HIF-1α mRNA and (**B**) human HIF-1α ΔODD in murine postnatal lung, as assessed by real-time qPCR. The number of HIF-1α transcripts was significantly greater in HIF-1α ΔODD mice, which was due to human HIF-1α ΔODD mRNA expression. Values are mean ± SEM for 4 lungs in each group. *P < 0.05.*

Fluorescent angiography and vessel densities

In order to assess if vascularization is increased in HIF-1α ΔODD pups fluorescent angiography was employed. No obvious differences were observed in pulmonary vascularization between E18.5 HIF-1α ΔODD and control mice (*Figure 3.6A*). In contrast, when we counted the number of small vessels (diameter range 20 to 65 μm) we found a significant increase in vessel density in the HIF-1α ΔODD lungs compared to the control lungs (*Figure 3.6B*). Increased PECAM-1 expression in HIF-1α ΔODD transgenes at postnatal days 8 and 14 further corroborated enhanced pulmonary vessel formation (*Figure 3.9A*).

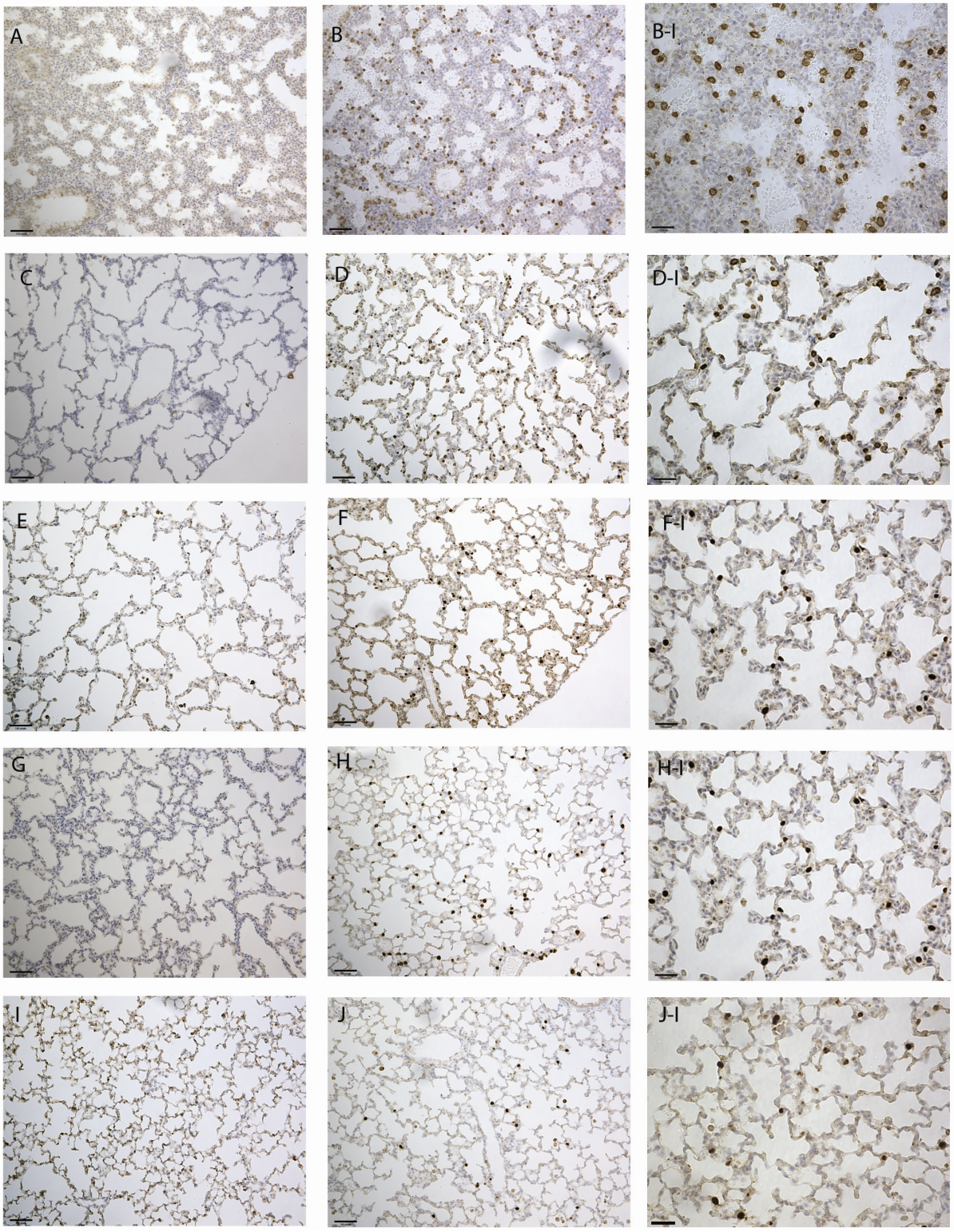
Lung morphometry

Histologically HIF-1α ΔODD lungs appeared to have an increased cellular complexity in comparison to C57 pups during days 2, 8, and 14 of the post gestational period. (*Figure 3.7A, B, C vs. E, F, G*). Tissue fraction analysis confirmed a significant increase in the tissue to air ratio in ODD pups vs. C57 control pups on postnatal days 2, 8, and 14 (*Figure 3.7*). The number of alveoli per unit area was higher in HIF-1α ΔODD pups vs. C57 control pups in general, but significantly higher in HIF-1α ΔODD pups on post gestational days 8 and 14 (*Figure 3.7*). To quantitatively assess alveolar development, we calculated the secondary crest number/field and the secondary crest/tissue ratio. Secondary crest counts per unit area were significantly increased in HIF-1α ΔODD pups *vs.* C57 control pups on post gestational days 2, 8, and 14, but when corrected per tissue fraction secondary crest formation was only increased during the early post (P2) gestational period (*Figure 3.7*). These data suggest increased secondary crest formation. To further evaluate alveolar development, we measured mean linear intercept and alveolar surface area per unit lung volume. The linear mean intercept (Lm) decreased in the HIF-1α ΔODD and C57 control pups with advancing postnatal gestation (*Figure 3.8A*). Interestingly, HIF-1α ΔODD pups had significantly lower Lm at post gestational days 8 and 14 when compared to C57 control pups, consistent with a reduction in airspace diameter and enhanced alveolar formation. In line with these findings, alveolar surface density (SvA) increased in both HIF-1α ΔODD and C57 control pups throughout postnatal gestation (*Figure 3.8B*) and a significant increase in SvA was observed in HIF-1α ΔODD pups on postnatal days 8 and 14 when compared to C57 control pups, consistent with enhanced alveolar formation.

Alveolar Type II and Type I cell marker expression

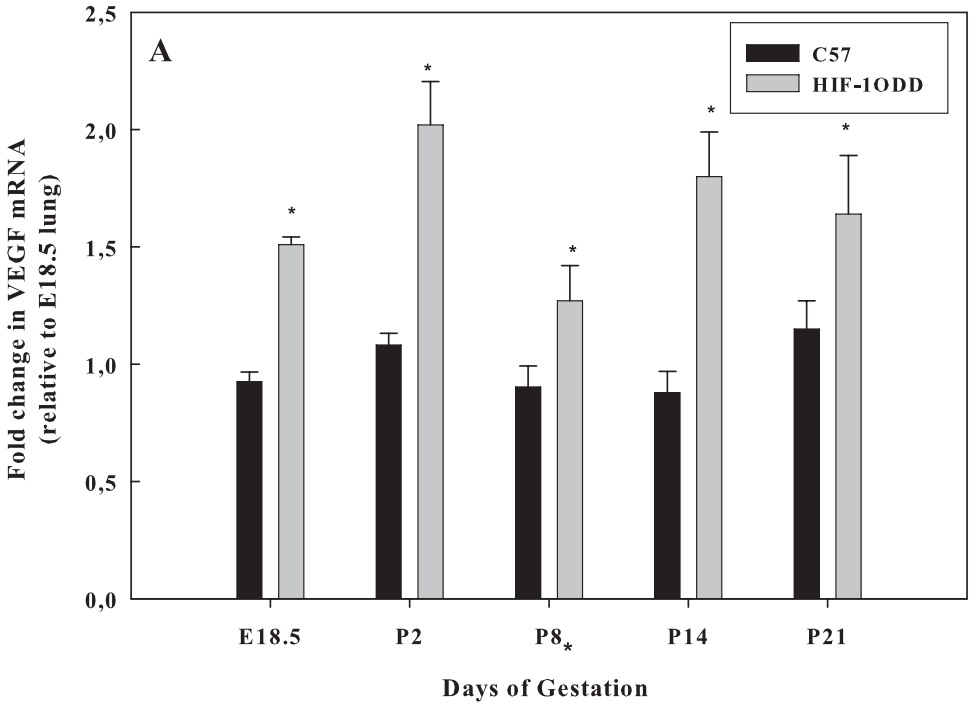
Surfactant protein C is a marker for alveolar type II cells,[40] while aquaporin 5 is an alveolar type I cell marker.[41] SP-C mRNA expression peaked around birth and fell with advancing postnatal gestation as previously described.[42,43] Expression of constitutive HIF-1α had no affect on its expression pattern (*Figure 3.9B*). In contrast, aquaporin 5 mRNA expression increased with advancing gestation and was significantly up-regulated at postnatal days 2, 8 and 14 (*Figure 3.9C*), consistent with enhanced alveolar type I cell formation.

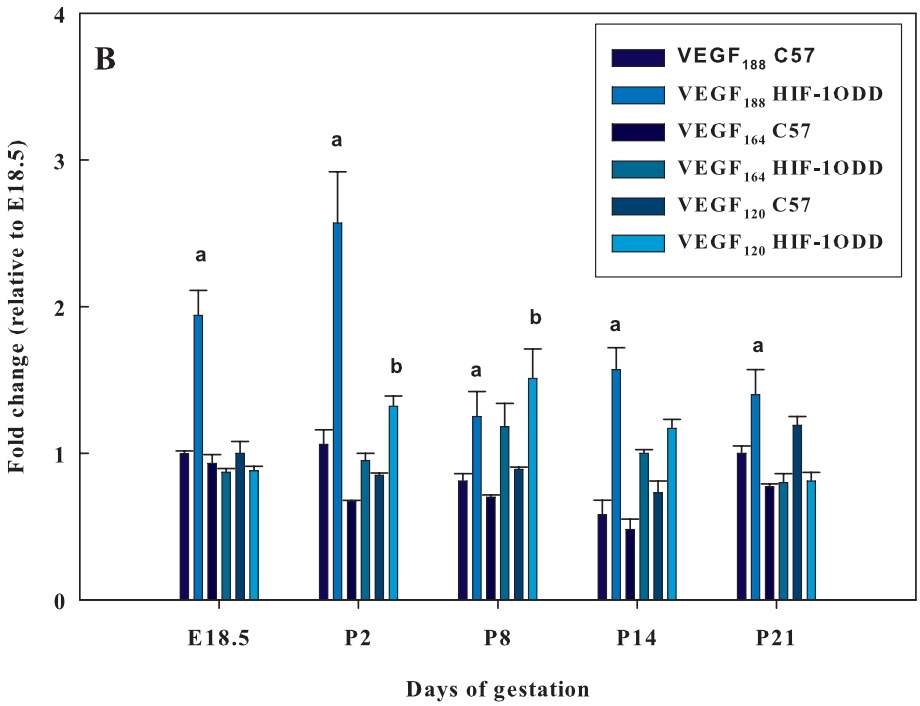
Figure 3.4 *(for color figures see page 155)* Immunohistochemical analysis of HIF-1α in C57 control and HIF-1α ΔODD lungs



Strong positive brownish staining for HIF-1α is noted in the nuclei of distal airway epithelial type II cells of HIF-1α ΔODD mice (B, B-I, D, D-I, F, F-I, H, H-I, J, J-I), but not C57 control mice (A,C,E,G,I) during all postnatal ages (E18.5: A,B,B-I; P2: C, D, D-I; P8: E, F, F-I; P14: G, H, H-I; P21: I, J, J-I. Bar: 200 μm (A-J); 50 μm (B-I, D-I, F-I, H-I, J-I).

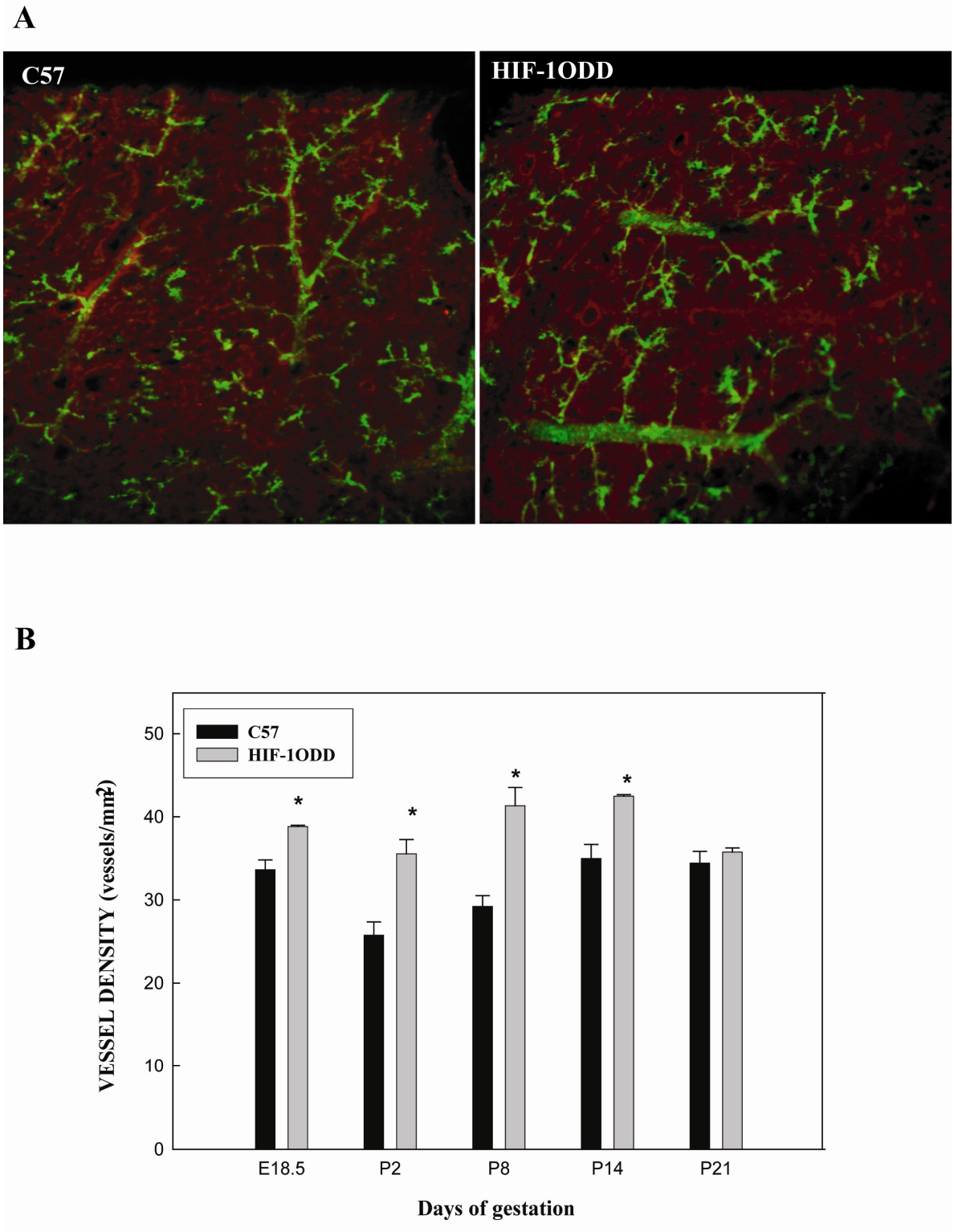
Figure 3.5 *(for color figures see page 156)* VEGF gene expression in C57 control and HIF-1α ΔODD lungs during postnatal development





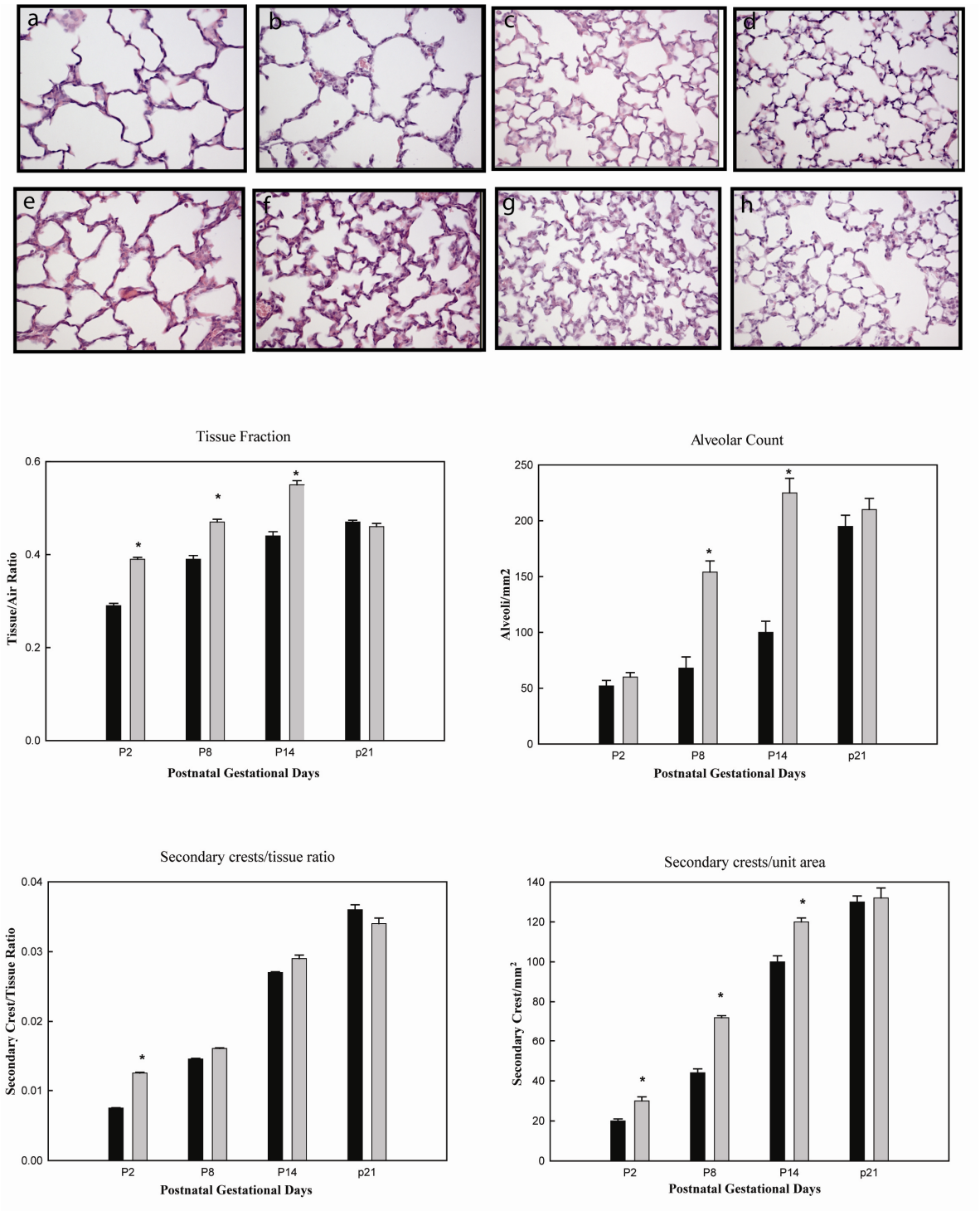
***A**) Expression of total VEGF mRNA and (**B**) expression of $VEGF_{188}$, $VEGF_{164}$ and $VEGF_{120}$ mRNA in murine lung, as assessed by real-time qPCR. Data are expressed as relative fold changes in expression when compared to E18.5 lungs. Values are mean ± SEM for 4 lungs in each group. *P < 0.05.*

Figure 3.6 *(for color figures see page 157)* Effect of constitutive active HIF-1α expression on vascular formation during postnatal development



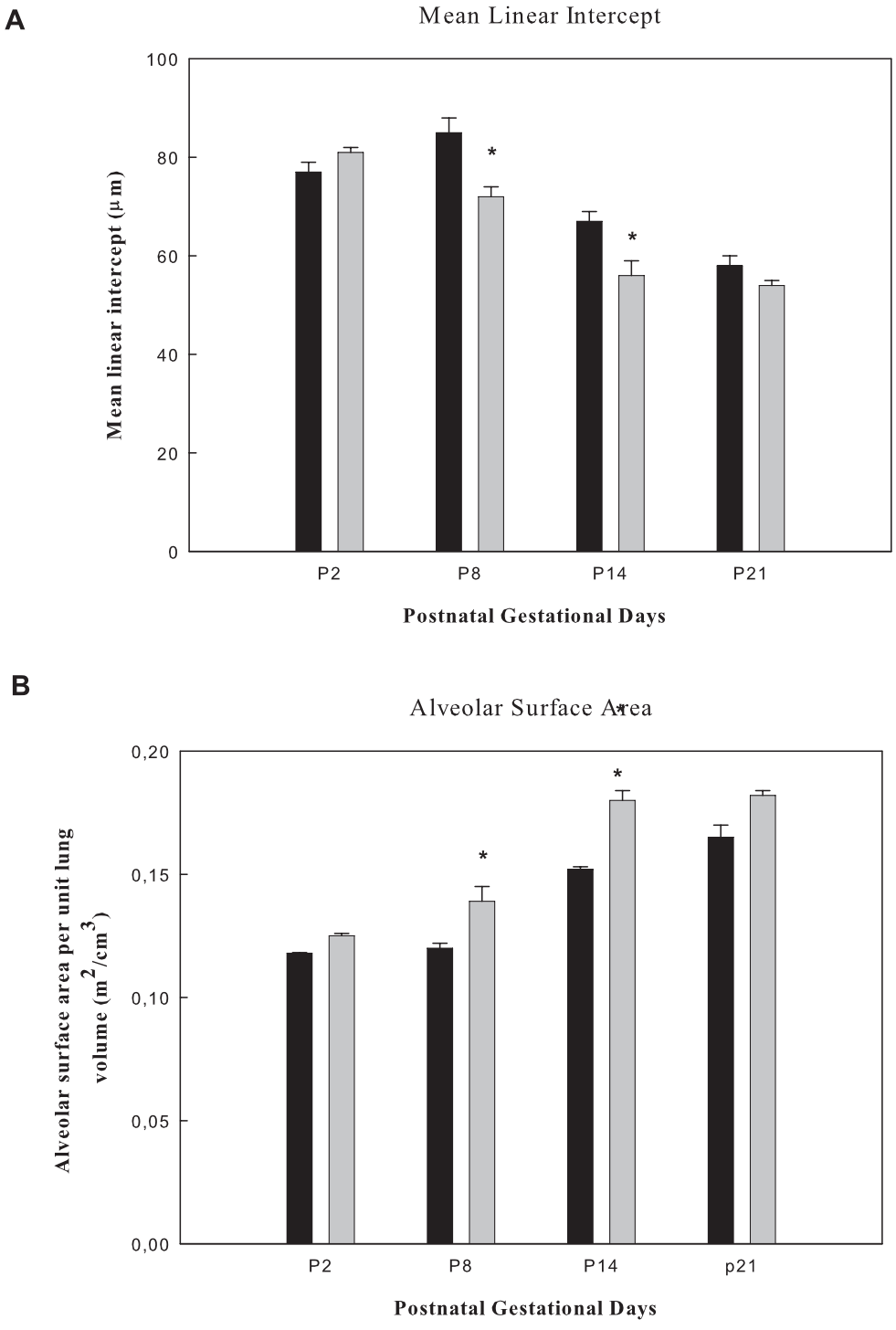
*(**A**) Fluorescent angiography of E18.5 lungs revealed no differences between C57 and HIF-1α ΔODD lungs. (**B**) Small vessel (diameter range 20 to 65 μm) number per unit area was increased in HIF-1α ΔODD lungs compared to C57 control lungs at all gestational ages.*

Figure 3.7 *(for color figures see page 158)* Lung histology and morphometry of neonatal C57 control and HIF-1α ΔODD mice



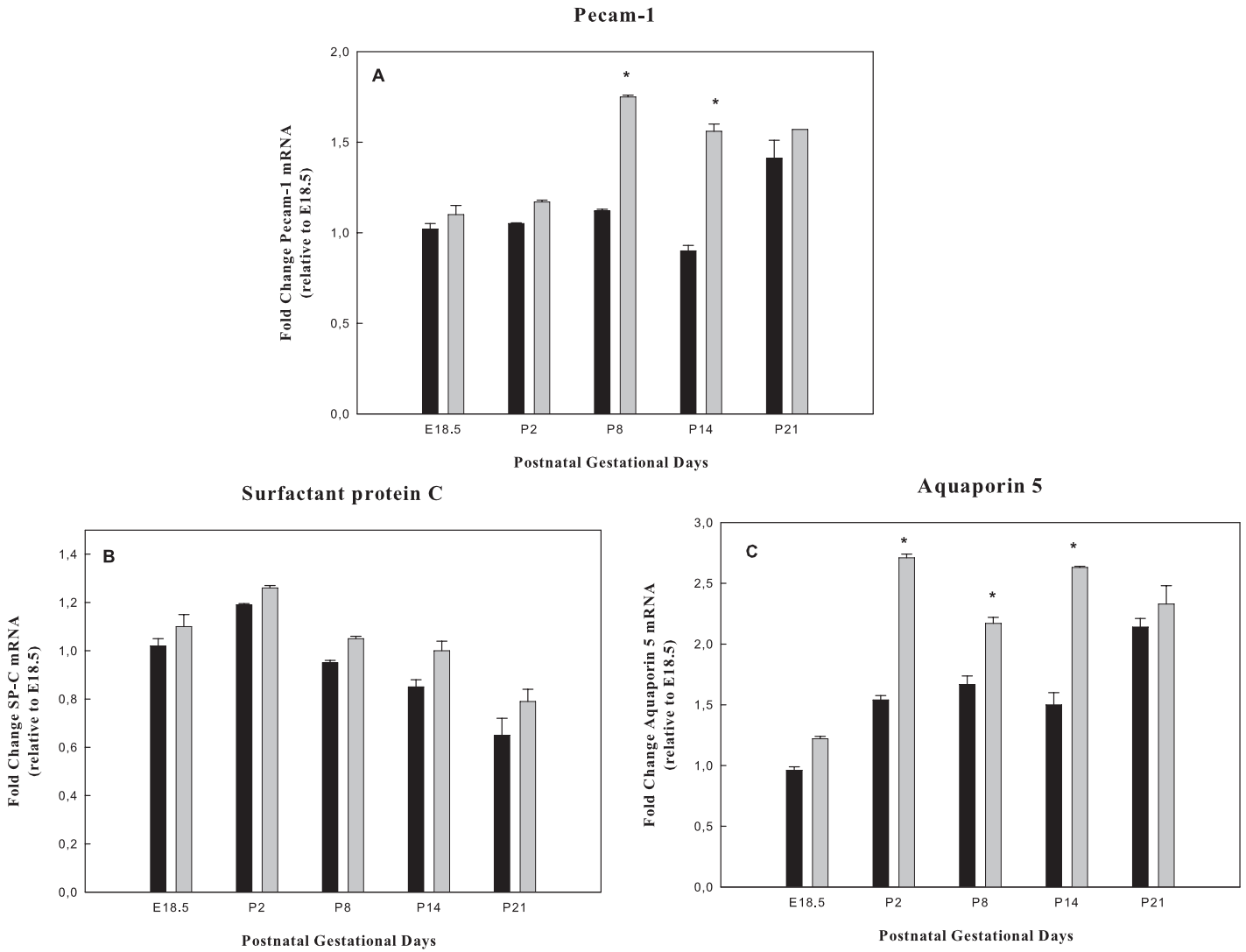
*(a-h) Haematoxylin and eosin staining of HIF-1α ΔODD lungs (e-h) showed increased parenchymal tissue per unit area of lung and smaller distal airspaces compared to C57 lungs (a-d) consistent with enhanced alveologenesis (Bar = 200 μm). Tissue fraction and number of alveoli per unit area were significantly increased in HIF-1α ΔODD lungs at postnatal day 2, 8 and 14. There was no significant difference in secondary crest/tissue ratio between HIF-1α ΔODD and C57 control pups except for postnatal day 2. Secondary crest number per unit area was significantly increased in HIF-1α ΔODD lungs at postnatal days 2, 8 and 14. Values are means ± SEM for five pups in each group. *P < 0.05*

Figure 3.8 Measurements of mean linear intercept and alveolar surface area per unit lung volume



*A significant decrease in mean linear intercept (A) and alveolar surface area per unit lung volume (B) was observed in HIF-1α ΔODD pups at postnatal days 8 and 14, consistent with enhanced alveolar formation. Values are means ± SEM for five pups in each group. *P < 0.05.*

Figure 3.9 Pecam-1, surfactant protein C and aquaporin expression in C57 control and HIF-1α ΔODD lungs



*Expression of Pecam-1 (**A**), surfactant protein C (**B**) and aquaporin 5 (**C**) expression mRNA in murine lung, as assessed by real-time qPCR. Data are expressed as relative fold changes in expression when compared to E18.5 lungs. Values are mean ± SEM for 4 lungs in each group. *P < 0.05.*

DISCUSSION

Herein we demonstrate that pulmonary expression of constitutive active HIF-1α enhances postnatal alveolar formation in mice. The enhanced alveolarization is accompanied by an increase in postnatal vascularization which is likely due to the up-regulated VEGF expression. Increasing evidence suggests that HIF plays an important role in early pulmonary organogenesis and postnatal alveolarization. Blocking HIF-1α expression in the early embryonic lung led to a decrease in vascularization and decreased branching morphogenesis.[1] Pharmacologic HIF stabilization in preterm baboons has been shown to enhance lung growth, improved oxygenation and lung compliance.[18] Furthermore, enhancing HIF stability has been shown to increase the expression of VEGF and to stimulate angiogenesis.[16,17] Moreover, VEGF gene therapy has been shown to prevent hyperoxia-induced BPD in newborn rats by stimulating vascular development.[2]

Our present data show that low oxygen stimulates vessel growth in the developing lung as previously reported.[1] A small, but not significant, increase in vascularization was noted between C57 and HIF-1α ΔODD lung explants cultured at low oxygen. Under normoxic conditions, vascularization of HIF-1α ΔODD lungs was significantly greater than controls, demonstrating that stable HIF-1α can sustain vessel growth even in normoxic conditions. Interestingly, no difference in branching was seen between control and HIF-1α ΔODD lungs in normoxic conditions. At 20% O_2 transgenic lung explants had increased vascularization, but branching was not altered compared to controls. In hypoxic conditions branching equally increased in both control and HIF-1α ΔODD lung explants. Thus, it appears that other factors than HIF-1α have to be held responsible for the increase in branching in hypoxia. Moreover, it is apparent that an increase in vascularization does not necessarily mean an increase in branching. Therefore, vascularization and branching in the early lung are not as tightly linked as previously thought.[1]

The mechanisms that regulate alveolar formation and development remain poorly understood, although experimental models that mimic diseases such as BPD and abnormal alveolar formation have given some insight in molecular regulation of alveolarization.[2,13,14,44-47] A multitude of studies show that in cases of neonatal pulmonary disease and/or injury there is loss of formation and development of the alveoli and pulmonary vasculature.[44-48] Herein, we show that expression of stable HIF-1α stimulates postnatal alveolarization, which was associated with an increase in peripheral small vessel formation. The continuous activation of the HIF-1 pathway and subsequent up-regulation of the VEGF pathway may account for this enhanced alveolar development. We discovered that a specific isoform of VEGF, namely $VEGF_{188}$, is up-regulated in the HIF-1α ΔODD mice at all gestational days. $VEGF_{188}$ is mostly a cell-surface- and extracellular-matrix-associated VEGF isoform.[49] Galambos and Ng[50,51] showed that a finely tuned balance of VEGF isoforms is necessary for proper blood vessel branching morphogenesis in the lung.

Even more, absence of $VEGF_{164}$ and $VEGF_{188}$ isoforms impairs lung microvascular development and delays airspace maturation.[50] Thus, it is likely that the stimulation of alveolarization seen in HIF-1α ΔODD mice is caused by the rise in $VEGF_{188}$ expression. Jakkula and colleagues[48] used a VEGF antagonist, Su-5416, to demonstrate that angiogenesis was essential to alveolarization in the developing rat lung. Other studies have shown that VEGF and its receptors are decreased in lungs of infants dying with bronchopulmonary dysplasia.[45] Increasing pulmonary VEGF in experimental BPD models by stabilizing HIF-1α[18] or VEGF gene therapy[2] have been shown to stimulate vessel and alveolar formation. These findings and our observations are compatible with the idea that distal lung vascularization is a driving force of alveolarization.

Taken together, the data show that stable HIF-1α was able to sustain vessel growth in early lung development, even under normoxic conditions. Also stable HIF-1α stimulates postnatal vascularization and alveolarization through $VEGF_{188}$ up-regulation and in lesser extent through $VEGF_{120}$. Clinical challenges, both developmental abnormalities and iatrogenic injuries, can be better treated if the basic physiological and molecular knowledge on the course of lung development is understood. Results from this study give insight in the importance of HIF-1 in lung development. The clinical application of such data is evident as VEGF gene therapy has been experimentally shown to promote lung angiogenesis and subsequently prevent alveolar damage in cases of hyperoxia induced pulmonary injuries.[2] However, VEGF therapy alone led to increased, but abnormal, vessel formation in the lung and other angiogenic factors such as angiopoietin 1 needed to be delivered to correct for the leaky vessels.[2] Whether stable HIF-1α would circumvent these complications remains to be seen, but our HIF-1α ΔODD transgenic mice appear normal and animal experiments using selective PHD inhibitors to stabilize HIF-1 are encouraging.[18]

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chapter 4

CHEMICAL STABILIZATION OF HYPOXIA INDUCIBLE FACTOR DOES NOT LEAD TO A HYPERVASCULAR LUNG DURING EARLY DEVELOPMENT

ABSTRACT

Low oxygen stimulates pulmonary vascular development and airway branching and involves Hypoxia Inducible Factor (HIF). HIF is stable and initiates expression of angiogenic factors under hypoxia, whereas normoxia triggers hydroxylation of the HIF-1α subunit by proline hydroxylases (PHDs) and subsequent degradation. Herein, we investigated whether chemical stabilization of HIF-1α under normoxic (20% O_2) conditions would stimulate vascular growth and branching morphogenesis in early lung explants. Tie2-LacZ mice were used for visualization of the vasculature. E11.5 lung buds were dissected and cultured in 20% O_2 in the absence or presence of cobalt chloride ($CoCl_2$; a hypoxia mimetic), dimethyloxalylglycine (DMOG, a non-specific inhibitor of PHDs) or desferrioxamine (DFO; an iron chelator). Vascularization was assessed by X-gal staining and terminal buds were counted. The fine vascular network surrounding the developing lung buds seen in control explants disappeared in $CoCl_2$ and DFO treated explants. Also, epithelial branching was reduced in the explants treated with $CoCl_2$ and DFO. Both treatments increased VEGF mRNA expression without affecting HIF-1α mRNA expression. DMOG had no clear visible effect on branching and vascularization. Since HIF-1α induces vascular growth factor (VEGF) expression, the effect of SU5416, a potent VEGF receptor blocker, on early lung development was also investigated. Inhibition of VEGFR2 signaling in explants maintained under hypoxic (2% O_2) conditions completely abolished vascularization and slightly decreased epithelial branching. Taken together, the data suggest that stabilization of HIF-1α during early development does not lead to a hypervascular lung and that airway branching proceeds without the vasculature, albeit at a slower rate.

INTRODUCTION

Early lung development occurs in a relatively hypoxic environment *in utero*.[1] This may be beneficial for vascular growth since several angiogenic factors are induced by low oxygen.[2,3] Indeed, culturing early embryonic lungs in a low oxygen environment stimulates vascular development[4] and enhances vascular endothelial growth factor gene expression.[5] Other reports have shown that hypoxia affects lung branching morphogenesis.[2,6] Recent studies have demonstrated that fetal oxygen tension also stimulates branching of the *Drosophila* tracheal system.[7] In most mammalian cells the response to hypoxia is mediated through hypoxia inducible factor-1 (HIF-1).[3,8] The HIF-1 complex consists of one of three alpha subunits (HIF-1α, HIF-2α or HIF-3α) and a beta subunit, known as aryl hydrocarbon nuclear translocator (ARNT).[9] The regulation of HIF by oxygen occurs through modifications of the alpha subunit, while the beta subunit is oxygen insensitive. Under normoxic conditions proline residues 402 and 564 of the alpha subunit are rapidly hydroxylated by prolyl hydroxylase domain enzymes (PHD1-3) allowing recognition of the alpha subunit by the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex and subsequent proteosomal degradation.[10,11] In addition, hydroxylation of the asparagine 803 residue in the transactivation domain of HIF-α by an asparagyl hydroxylase, called FIH-1, inhibits the recruitment of transcriptional co-activator proteins.[12,13] Under hypoxic conditions, PHDs are degraded by the SIAH (seven in absentia homologue) E3-ubiquitin-ligases.[14-16] The degradation of PHDs in hypoxia allows the alpha subunit of HIF-1 to escape hydroxylation and allow it to accumulate in the nucleus, where upon binding to ARNT it recognizes hypoxia response elements (HRE) within the promoter regions of hypoxia-responsive target genes. Numerous genes have HREs and many of them seem to be involved in endothelial cell proliferation and survival.[3]

Herein, we investigated whether chemical up-regulation of HIF-1α expression and stability under normoxic (20% O_2) conditions would increase vascularization and branching in early lung explants similar to that seen in explants cultured in low (3%) oxygen.[2,5,6] Either cobalt chloride ($CoCl_2$), dimethyloxalylglycine (DMOG), or desferrioxamine (DFO) were used.[17-19] $CoCl_2$, a transition metal, chemically mimics hypoxia which stabilizes HIF-1α.[20] It is a weak inhibitor of PHD1-3, and a strong inhibitor of FIH.[21] In addition, it inhibits VHL binding to HIF-1α even when HIF-1α is hydroxylated.[22] DMOG is a nonspecific 2-oxoglutarate(OG)-dependent dioxygenase inhibitor. The 2-OG dioxygenase family includes PHD1-3 and FIH-1.[18] Inhibition of the 2-OG dioxygenase family results in an upregulation of HIF-1α and an induction of oxygen-regulated gene expression.[18] Recent studies have shown that PHD inhibition with DMOG enhanced HIF-1α and VEGF expression in lung cells.[23] DFO, which removes intracellular iron, is known to stabilize HIF-1α.[24] The enzymatic actions of prolyl hydroxylases are dependent on both molecular oxygen and iron.[24] DFO inhibits FIH-1, partly inhibits PHD3 and is a weak inhibitor of PHD1.[21] It does not inhibit PHD2.[21] The chemical treatments with $CoCl_2$, DMOG and DFO to induce

vascularization and branching were compared with SU5416, a well known inhibitor of VEGFR2 (Flk-1/KDR) signaling and lung development.[25]

MATERIALS AND METHODS

Mice

CD1 and Tie2-LacZ mice were obtained from Jackson Laboratory, Bar Harbor, MN, U.S.A.[26] In Tie2-LacZ transgenic mice, the 2.1 kb 5' flanking region of the murine Tie2 promoter drives the expression of the bacterial LacZ reporter gene exclusively to endothelial cells.[26] Cells transcribing the LacZ gene can be viewed by staining for β-galactosidase activity. The transgenic line was maintained on a CD1 background. All mouse protocols were in accordance with Canadian Counsel of Animal Care guidelines and were approved by the Animal Care and Use Committee of the Hospital for Sick Children, Toronto, ON, Canada.

Whole lung organ culture

Lung buds were dissected from E11.5 CD1 and CD1;Tie2-LacZ mouse embryos (day of vaginal plug is E0.5) and placed on a floating (8 μm Whatman Nuclepore polycarbonate) membrane (Integra Environmental Inc. Burlington, ON, Canada). Explants were grown in DMEM plus 10% FCS (Gibco, Grand Islands, NY, USA) and maintained in an atmosphere of 20% O_2/75% N_2/5% CO_2 at 37°C. Hundred μM cobalt chloride ($CoCl_2$, Sigma-Aldrich, Oakville, ON, Canada), 25 μM dimethyloxalylglycine (DMOG; Frontier Scientific, Logan, UT, USA), 10 μM desferrioxamine (DFO, Sigma-Aldrich, Oakville, ON, Canada), or 20 μM SU5416 (VEGF receptor 2 kinase inhibitor III, 676487, Calbiochem, La Jolla, CA, USA) were added to the medium at day 0. The explant experiments with SU5416 were carried out in a hypoxic environment (2% O_2/93% N_2/5% CO_2). Medium was changed every other day, branching was assessed every day and vascular growth was assessed on days 2 and 4. Pictures were taken using a Leica microscope and a digital imaging system.

MTT assay

Cell proliferation was determined using the Vybrant® MTT assay (V-13154, Invitrogen, Burlington, ON, Canada). The MTT assay involves the conversion of water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan. Experiments were carried out according to the manufacturer's protocol. Briefly, at day 2 and day 4 of culture, medium of explants was replaced by phenol red-free medium containing 1.09 mM MTT and the conversion to formazan was allowed to continue for 4 hours. The formazan was then solubilized by adding SDS-HCl (0.35 M SDS in 0.01 M HCl) to the medium, and incubation continued for another 16 hours. The concentration of formazan was determined by measuring the optical density at 570 nm.

LacZ staining

LacZ lung explants were fixed (in 1% formaldehyde, 0.1% glutaraldehyde, 2 mM $MgCl_2$, and 5 mM EGTA in 0.1 M sodium phosphate buffer pH 7.8 - 8.0 for 45 minutes at 4 °C), washed (in 2 mM $MgCl_2$, 0.01% deoxycholate, and 0.02% NP-40 in 0.1 M sodium phosphate buffer pH 7.8 - 8.0) 4 times for 30 minutes at 4 °C, and stained overnight at 37 °C in X-gal staining solution (5mM $K4Fe(CN)_6$:$3H_2O$, 5mM $K_3Fe(CN)_6$ in wash buffer, mixed 40:1 with X-gal stock solution (40 mg/ml in dimethyl formamide). Explants were washed with PBS, fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4 °C, and stored in 70% ethanol. For imaging explants were dehydrated with graded alcohol, placed in 100% methanol, and cleared in methyl salicylate. For sectioning, explants were dehydrated in a graded series of ethanol, and embedded in paraffin. Twelve µm thick sections were mounted on Superfrost slides (Fisher Scientific, Unionville, ON, Canada) and counterstained with Nuclear fast red. Digital images were taken with a Leica digital imaging system.

RNA isolation and real-time RT-PCR

After 4 days culture, lung explants were rinsed in ice-cold PBS and immediately frozen in liquid nitrogen, and stored at -70 °C. RNA was extracted with the RNA easy kit (Qiagen, Mississauga, ON, Canada). Total RNA was reverse transcribed (37 °C) in a total volume of 50 µl using random hexamers (Invitrogen, Burlington, ON, Canada). The resulting templates were quantified by real-time PCR (ABI Prism 7700). Primers and TaqMan probes for HIF-1α, VEGF and VEGFR2 (KDR/Flk-1) were purchased from ABI as Assays-on-Demand™ for murine genes. Reactions were carried out in 96 well plates in triplicate. For each probe, a dilution series determined the efficiency of amplification of each primer-probe set, and the relative quantification method was employed.[27] For the relative quantitation, PCR signals were compared among groups after normalization using 18S as an internal reference. Relative expression was calculated according to Livak and Schmittgen.[27] $P < 0.05$ was considered statistically significant.

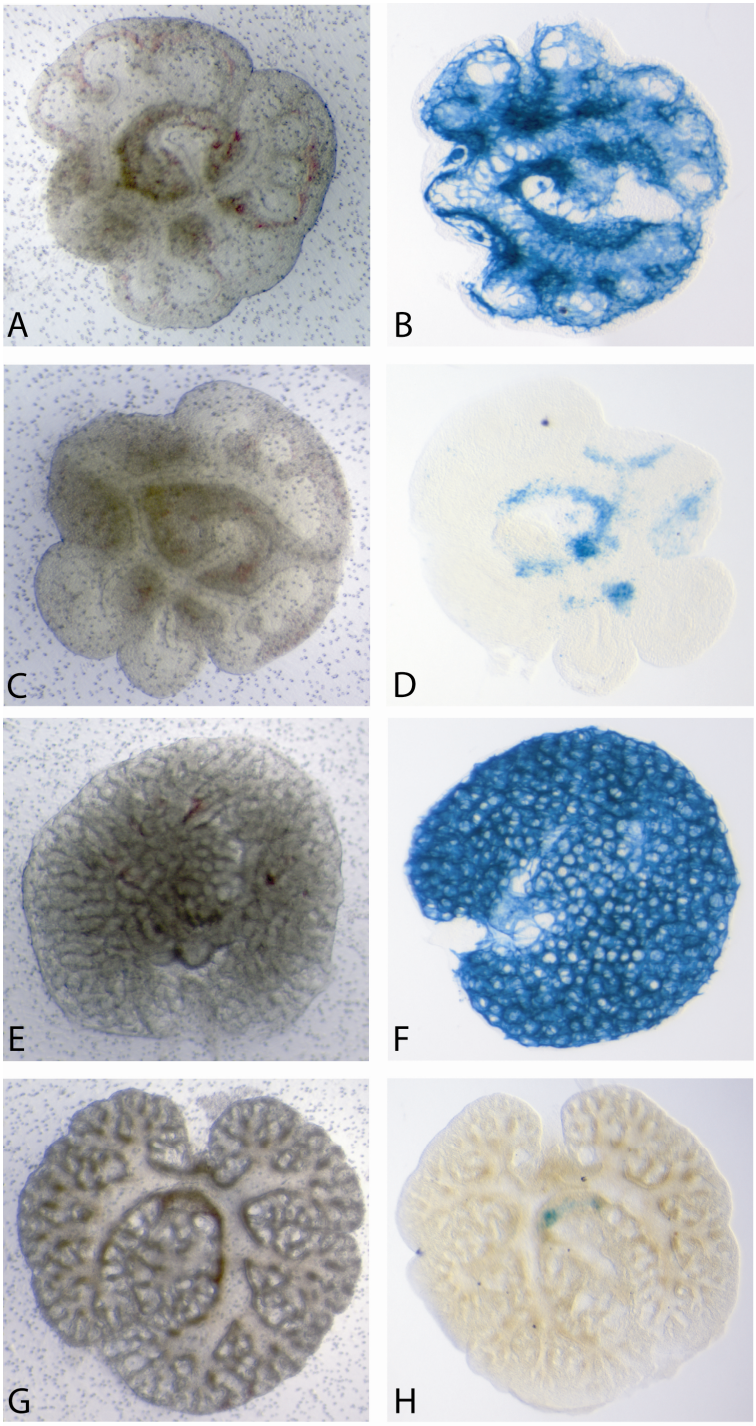
RESULTS

Inhibition of VEGFR2 signaling blocks vascularization and slightly decreases epithelial branching

We first investigated whether inhibition of VEGF/VEGFR2 signaling affected vascular growth and epithelial branching morphogenesis using CD1;Tie2-LacZ mice. Lungs were dissected at E11.5 and cultured in 2% oxygen to stimulate optimal vascular growth.[4] Whole mount LacZ staining revealed that incubation of E11.5 mouse lung explants with SU5416, a cell-permeable selective ATP-competitive inhibitor of VEGFR2 (Flk-1/KDR), completely abolished vascular growth. Inhibition of vascular development (absence of X-gal positive vessels) was already noticeable after 24 hrs of incubation (*Figure 4.1 B vs. D*) whereas epithelial branching appeared unaffected

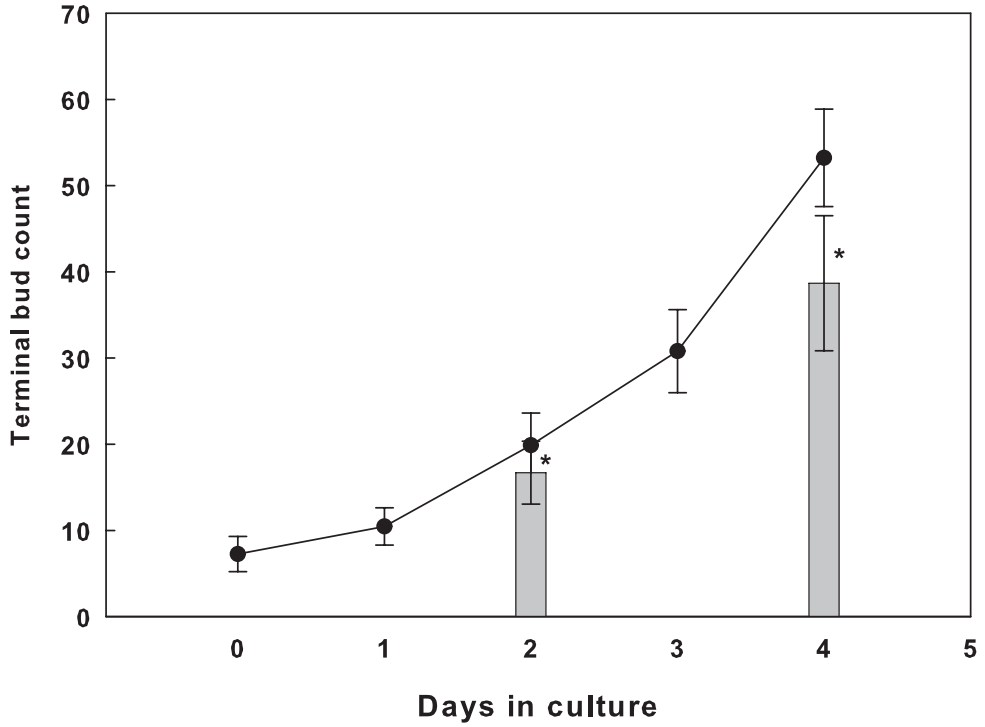
(*Figure 4.1 A, C*). After 48 and 96 hours of incubation of lung explants with SU5416 a small, but significant, decrease in epithelial branching was noticed compared to control explants (*Figure 4.2*). Thus, epithelial branching proceeded without vascular development, albeit at a lower rate (*Figures 4.1 and 4.2*).

Figure 4.1 *(for color figures see page 159)* SU5416 inhibits vascularization in early lung explants



E11.5 Tie-LacZ lung explants were maintained in 2% O_2 with (C, D, G, H) and without (A, B, E, F) SU5416. Vascularization was assessed after 24 (A-D) and 144 hours (E-H) by X-gal staining (B,D,F,H). Unstained control explants (A, C, E, G). Blue color represents positive X-gal staining in the vessels.

Figure 4.2 SU5416 reduces branching morphogenesis

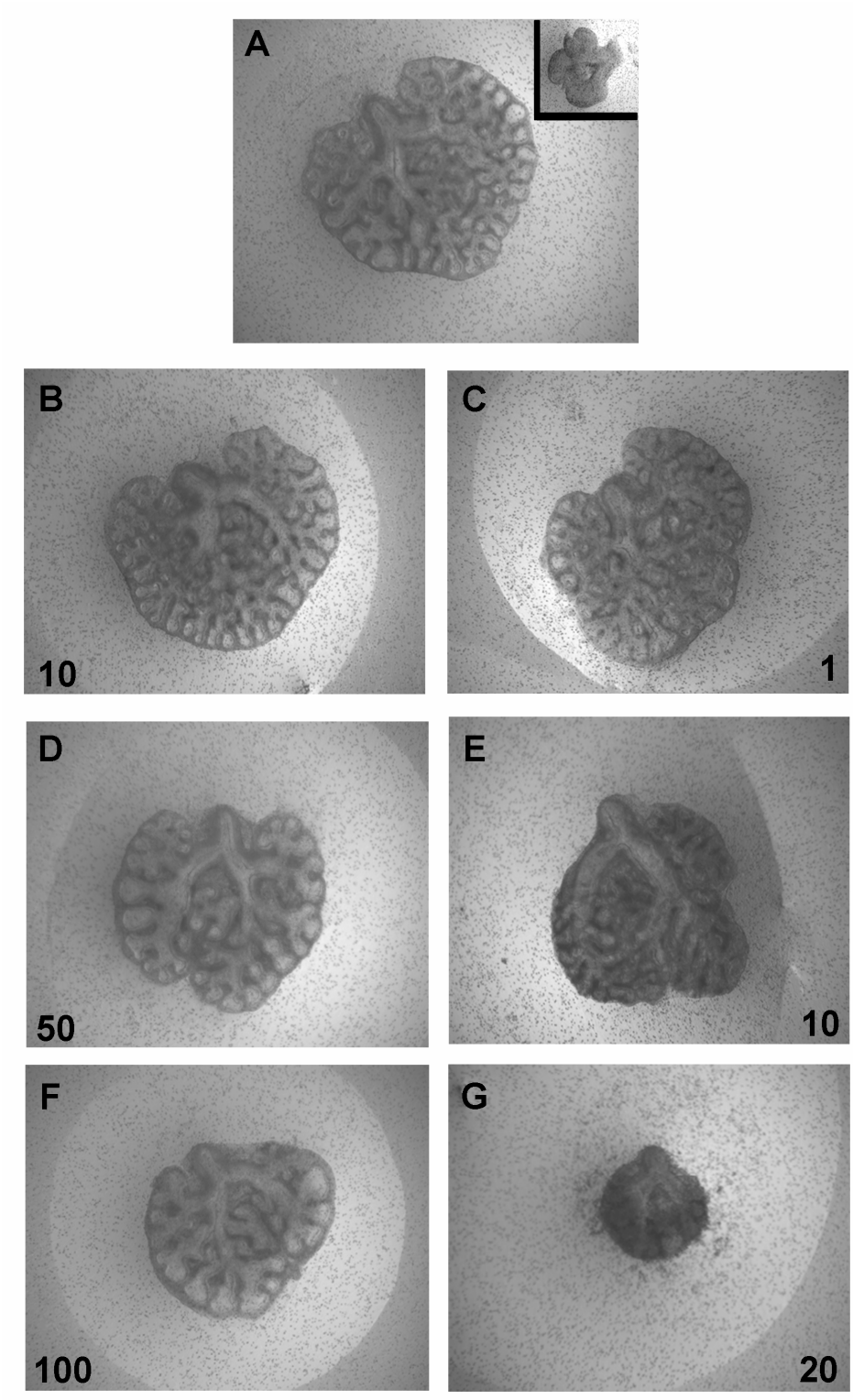


E11.5 Tie-LacZ lung explants were maintained in 2% O_2 with and without SU5416. Terminal lung buds were counted every day for controls (solid line) and at 48 and 96 hours for explants treated with SU5416 (filled bars). Data are mean ± s.e.m., n ≥ 35 explants per time point, n ≥ 15 per SU5416 treatment.

Chemical induction of HIF

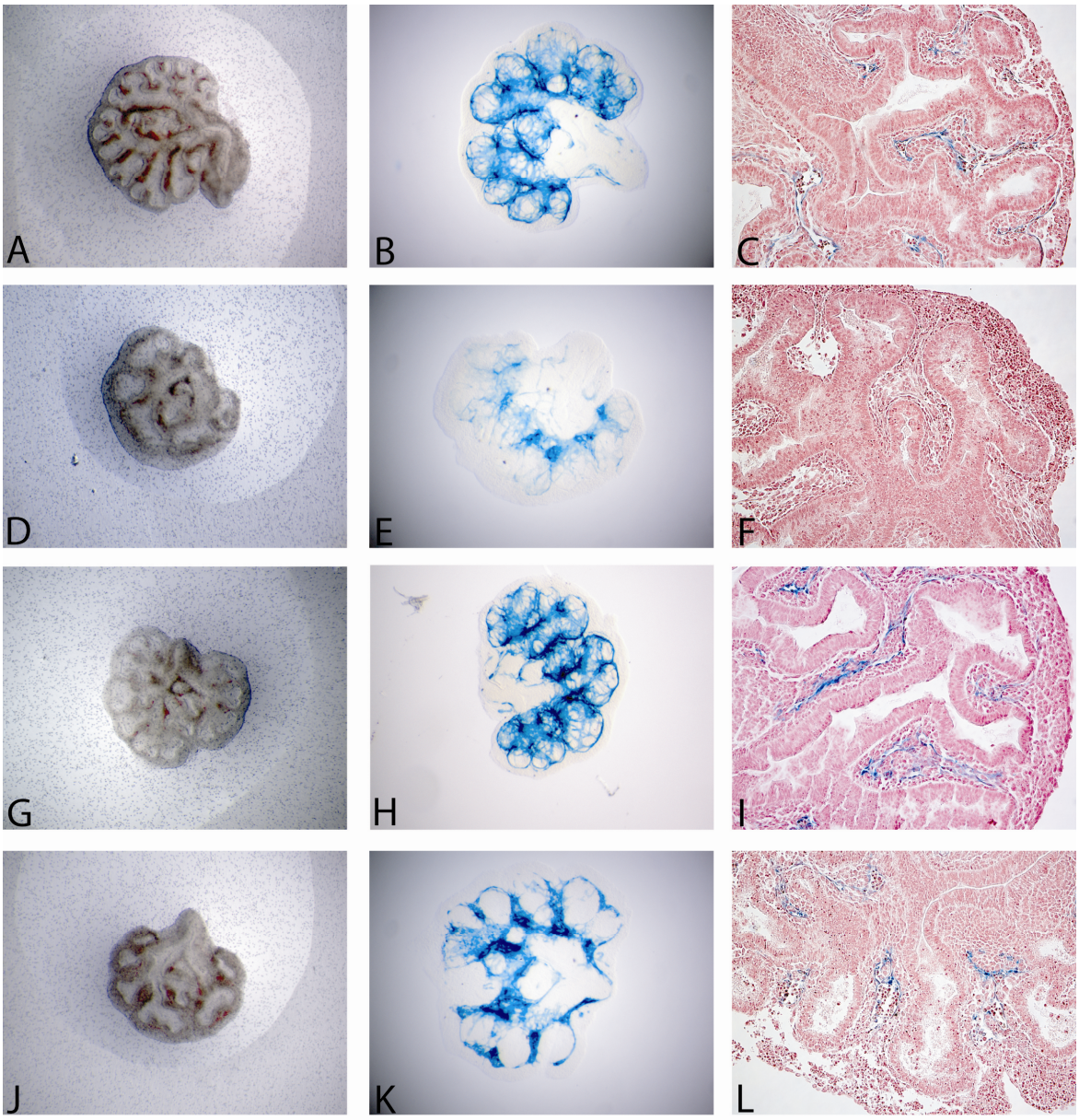
Since low oxygen has been shown to stimulate VEGF expression in murine lung explants, most likely via HIF-1,[4] we investigated whether enhancing HIF-1α stability under normoxic (20% O_2) conditions would promote vascular development and, thereby, epithelial branching. E11.5 lungs from CD1;Tie2-LacZ mice were incubated with either $CoCl_2$, DMOG or DFO to stabilize HIF-1α. First, we determined the dose-toxicity curves of the chemicals for lung explant growth and branching (*Figure 4.3*). Based on these pilot studies we used 100 µM $CoCl_2$ and 10 µM DFO in the subsequent explant experiments. These $CoCl_2$ and DFO concentrations have previously been shown to increase HIF-1α protein amounts in various cell[28-30] and tissue systems.[31] The concentration of DMOG (25 µM) was lower than reported previously,[32,33] since higher concentrations were toxic for the lung explants (not shown). Lungs cultured with $CoCl_2$ showed a significantly decreased vascular LacZ staining after 48 hours of culture when compared to control lungs (*Figure 4.4E, F vs. B, C*). Also, DFO treatment resulted in reduced vascular development (*Figure 4.4K, L vs. B, C*). The fine vascular network surrounding the developing lung buds seen in control explants had disappeared in $CoCl_2$ and DFO treated explants. The X-gal positive vessels along the trachea, main bronchi and larger airways appeared to be unaffected, especially in the DFO treatment group. After 96 hours of culture, vascular growth was further reduced in explants treated with $CoCl_2$ and DFO (*Figure 4.5B, C vs. A*).

Figure 4.3 Dose-toxicity curves of cobalt chloride and desferrioxamine for lung explant growth and branching



E11.5 Tie-lacZ lung explants were cultured for 3 days in 20% O_2 with and without increasing dosages of either $CoCl_2$ (B, D, F) or DFO (C, E, G). A: untreated control; inset: freshly isolated E11.5 lung. B, D, F: 10-100 μM $CoCl_2$. C, E, G: 1-20 μM DFO.

Figure 4.4 *(for color figures see page 160)* Cobalt chloride and desferrioxamine, but not dimethyloxalylglycine, reduce vascularization in early lung explants



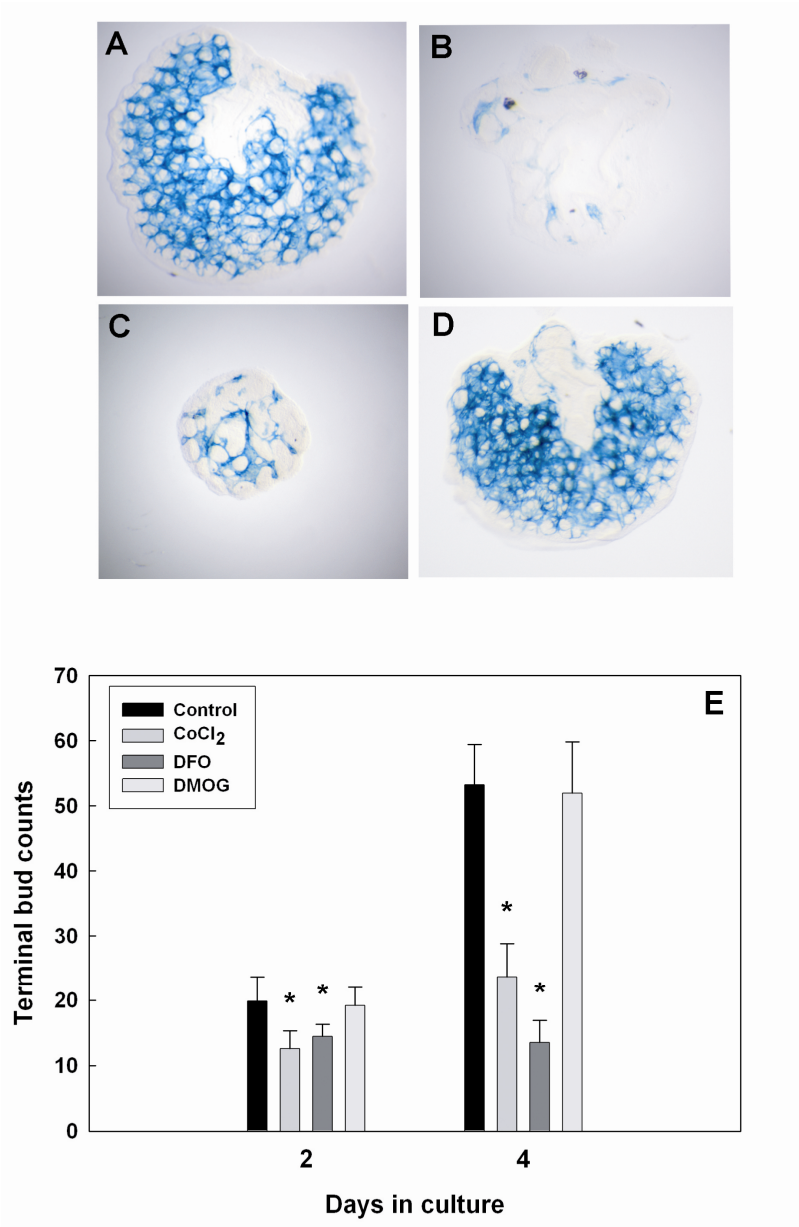
E11.5 Tie-lacZ lung explants were maintained in 20% O_2 with and without $CoCl_2$, DFO and DMOG. Vascularization was assessed after 48 hours by X-gal staining. A-C: control, D-F: $CoCl_2$, G-I: DMOG, and J-L: DFO. Blue color represents positive X-gal staining in the vessels (B, E, H, K) of the explants. Sections of Xgal stained explants (C, F, I, L) were counterstained with nuclear fast red.

Epithelial branching was significantly reduced in explants treated with $CoCl_2$ and DFO (*Figure 4.5E*). Addition of 50 µM $FeCl_2$ to DFO treated explants reversed the inhibitory effect of DFO on vascular growth and epithelial branching (*Figure 4.6*). Epithelial branching and vascular growth were not affected by DMOG treatment (*Figures 4.4 and 4.5*). The Vybrant® MTT assay was performed to determine the effect of $CoCl_2$, DMOG and DFO on explant cell growth. $CoCl_2$ and DFO treatment of lung explants markedly inhibited cell proliferation when compared to control

explants, while DMOG treatment had no effect (data not shown). These findings agree with the branching morphogenesis results.

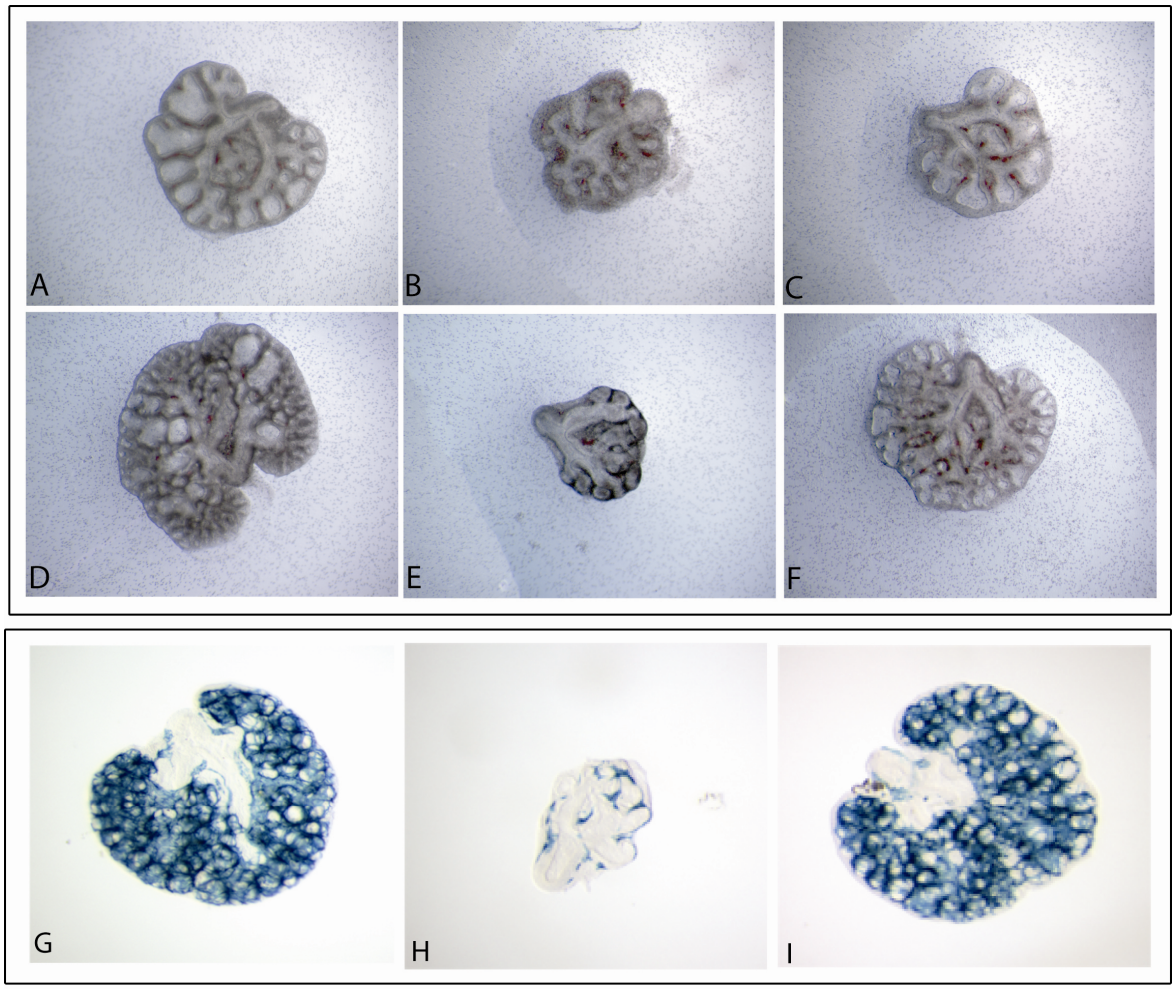
To determine the effect of the treatments on HIF-1α, VEGF and VEGFR2 mRNA expression, RNA was isolated from explants after 96 hours of culture and real-time PCR was performed for HIF-1α, VEGF and VEGFR2 (KDR/Flk-1). None of the treatments affected HIF-1α mRNA expression (*Figure 4.7*). Treatments with DFO and $CoCl_2$ increased VEGF mRNA expression, whereas incubation with DMOG had no affect. In contrast, all treatments reduced VEGFR2 mRNA expression.

Figure 4.5 *(for color figures see page 161)* Cobalt chloride and desferrioxamine, but not dimethyloxalylglycine, reduce branching morphogenesis



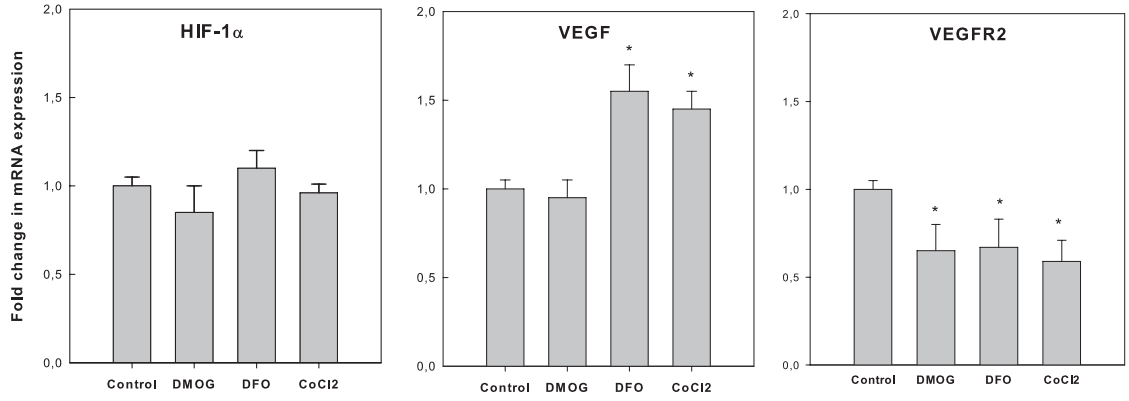
E11.5 Tie-lacZ lung explants maintained in 20% O_2 for 96 hrs with and without $CoCl_2$, DFO and DMOG. A-D: X-gal staining (A: control, B: $CoCl_2$, C: DFO, and D: DMOG). E: terminal buds counts, mean ± s.e.m., n ≥ 25 explants per treatment.

Figure 4.6 *(for color figures see page 162)* Addition of iron chloride rescues the desferrioxamine-induced decrease in vascularization and branching



E11.5 Tie-lacZ lung explants were cultured for 48 (A - C) and 96 hours (D -I) without (A, D, G) and with 10 μM DFO (B, E, H) or 10 μM DFO + 50 μM $FeCl_2$ (C, F, I). X-gal stained explants (G, H, I).

Figure 4.7 Effect of dimethyloxalylglycine, cobalt chloride and desferrioxamine on HIF-1α, VEGF and VEGFR2 mRNA expression



RNA was extracted from Tie-lacZ lung explants maintained in 20% O_2 for 96 hours with and without $CoCl_2$, DFO and DMOG and mRNA expression quantified by real-time RT-PCR. Data are mean ± s.e.m., n = 4.

DISCUSSION

Previously, we have reported that low oxygen levels enhance epithelial branching morphogenesis and vascular development in E11.5 murine lung explants (4). We hypothesized that HIF-related pathways were involved and, indeed, antisense knockdown of HIF-1α mRNA expression decreased epithelial branching morphogenesis and vascularization.[4] Herein, we show that inhibition of VEGF/VEGFR2 signaling with SU5416 completely abolished vascular growth. However, epithelial branching proceeded without vascular development, albeit at a slower rate. This indicates that HIF/VEGF signaling is important for vascularization, but that other downstream targets of the HIF pathway play a role in epithelial branching. Also, it suggests that epithelial branching is not as tightly linked to vascular development in the early phase of lung development as previously thought.[4] Galambos et al[34] showed that later in gestation (> E16) loss of one VEGF allele led to a decrease in lung development, suggesting that vascularization plays a greater role in epithelial branching at later gestation. This is supported by studies in which a VEGFR2 inhibitor was administered postnatally (days 1 - 13) to rat pups. Treated animals had enlarged airspaces, decreased alveolar number, and decreased arterial density.[25] EMAP II, another anti-angiogenic agent, decreased vessel density and arrested airway epithelial morphogenesis in E14.5 lung allografts.[35]

VEGF expression in murine lung explants is stimulated by low oxygen, most likely through a HIF mediated mechanism.[4] Stimulation of HIF protein via inhibition of PHDs has been reported to enhance expression of VEGF and PECAM (platelet-endothelial adhesion molecule) both *in vivo* and *in vitro*.[36,37] Furthermore, it stimulated *in vitro* angiogenesis.[23,37] Intravenous treatment of preterm baboons with a PHD inhibitor (FG-4095) was shown to be effective for HIF protein stabilization.[23,37] Another study showed that this treatment is associated with enhanced lung growth, improved oxygenation and lung compliance.[38] Thus, the increase of HIF proteins goes hand in hand with improved postnatal lung development. In contrast, we observed that chemical stabilization of HIF-1α in early lung explants did not lead to improved vascularization and increased branching as seen in explants cultured at low oxygen.[4]

In explants treated with $CoCl_2$ and DFO, the fine vascular network surrounding the developing lung buds disappeared, while the vessels along the trachea, main bronchi and larger airways remained intact. Although VEGF expression was up-regulated, VEGFR2 expression was downregulated by both treatments. It is possible that VEGFR2 downregulation is responsible for the lack of peripheral vascularization since VEGF-VEGFR2 signaling is important for the sprouting of new vessels from existing ones and VEGF is a potent mitogen for endothelial cells [for review, see 39]. However in explants treated with SU5416 not only the finer vasculature disappeared but also the larger more proximal vasculature. An explanation may be that SU5416 is not solely a VEGFR2 inhibitor, but also a platelet derived growth

factor (PDGF) β-receptor inhibitor. VEGFR2 accounts for most VEGF effects on endothelial cells, such as cell proliferation, NO and prostacyclin production, angiogenesis, and vascular permeability.[40,41] The PDGF β-receptor is activated by PDGF-B, which is a potent mitogen of vascular smooth muscle cells (VSMC) and is also involved in the migration of pulmonary VSMCs.[42,43] Inhibition of both VEGF and PDGF pathways has been shown to be more effective than blocking VEGF alone, causing vessel regression in multiple models of neovascular growth.[44] Thus, the blockade of both receptors may explain the complete absence of vascularization seen in explants incubated with SU5416.

Epithelial branching and vascularization were not affected by DMOG treatment. As mentioned earlier, we used a significantly lower dosage of DMOG compared to previous studies.[32,33] It is possible that this dosage had no inhibitory effect on PHDs and, therefore, HIF-1 stability as it did not increase VEGF mRNA expression. However, DMOG treatment also reduced VEGFR2 expression without affecting the peripheral vascularization, implying that VEGFR2 downregulation alone is not sufficient to explain the loss of peripheral vascularization in $CoCl_2$ and DFO treated explants.

The up-regulation of VEGF mRNA in the lung explants by both $CoCl_2$ and DFO treatments suggest stabilization of HIF-α, at least if we assume that VEGF gene expression is under HIF control.[4] As discussed above, however, the increase in VEGF expression did not lead to improved vascularization. Moreover, lung explant growth and branching was severely reduced. Both $CoCl_2$ and DFO interfere with binding of molecular oxygen to heme proteins, thereby mimicking hypoxia. It is possible that the hypoxia was too extensive and led to cell death. DFO and $CoCl_2$ have been shown to induce apoptosis in a variety of non-lung cells.[45-47] However, no increase in apoptotic or necrotic cell death of fetal alveolar type II cells was reported when the cells were exposed to 3% O_2.[48] In the latter study the fetal cells were exposed to 3% O_2 only for 24 hrs. To prevent oxygen depletion and cell death, cells exposed to very low oxygen levels downregulate ATP consuming pathways in order to decrease oxygen demand. The reduction in oxygen consumption in A549 cells was, however, not reversible after the cells were exposed to hypoxia for 24 h.[49] Thus, the prolonged $CoCl_2$- or DFO-induced hypoxia likely triggered cell death in the lung explants. Together with the reduction in cell proliferation, this may explain the negative effect of both treatments on lung growth and branching. Moreover, $CoCl_2$ and DFO will not only act as hypoxia mimetics, but interfere with all ferro-dependent reactions needed for proper vessel formation and airway branching. The importance of iron for proper lung development was demonstrated by the reversal of the inhibitory effect of DFO with excess iron.

ACKNOWLEDGMENTS

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Studies in Human Lung

part 2

chapter 5

HYPOXIA INDUCIBLE FACTORS IN THE FIRST TRIMESTER HUMAN LUNG

ABSTRACT

Lung development takes place in a relatively low oxygen environment, which is beneficial for lung organogenesis, including vascular development. Hypoxia Inducible Factor (HIF)-1 plays an important role in mediating oxygen-regulated events. HIF-1 is stable and initiates gene transcription under hypoxia whereas in normoxia interaction with the Von Hippel Lindau tumor suppressor protein (VHL) leads to rapid degradation of the HIF-1α subunit. The interaction with VHL requires hydroxylation of HIF-1α proline residues by prolyl hydroxylases (PHDs). Herein, we investigated the expression of the various components regulating HIF-1α stability in first trimester (8 - 14 wks) human lungs. Spatial expression was assessed by immunohistochemistry and temporal expression by quantitative PCR. Immunoreactivity for PHD1, PHD3, and seven in absentia homologue (SIAH)1 was noted in the pulmonary epithelium. PHD2 was not expressed in the airway epithelium, but in the lung parenchyma. HIF-1α and Vascular Endothelial Growth Factor (VEGF) immunoreactivity was primarily detected in the branching epithelium. HIF-2α and ARNT proteins localized to the developing epithelium as well as mesenchymal, most likely vascular, structures in the parenchyma. VEGF receptor 2 (VEGFR2) was found in the subepithelium as well as in vascular structures of the mesenchyme. All components of the VEC complex (VHL, NEDD8, and Cullin2) were found in the epithelium. Quantitative PCR analysis demonstrated that VEGF, VEGFR1, HIF-1α, HIF-2α, ARNT, PHD1, PHD2, PHD3 and SIAH1 gene expression was constant during early pulmonary organogenesis. Cumulatively, the data suggest that the lung develops in a low oxygen environment which allows for proper vascular development through HIF regulated pathways.

INTRODUCTION

Lung disease continues to be one of the most important challenges facing the neonatologist, despite modern treatment modalities. The origin of some of these disorders can be found in developmental aberrations *in utero*. These disorders include idiopathic persistent pulmonary hypertension of the neonate, congenital diaphragmatic hernia, congenital cystic adenomatoid malformation of the lung, pulmonary hypoplasia, and alveolar capillary dysplasia.[1] Patients with these diseases exhibit lung dysfunction characterized by arrested lung development and/or defective pulmonary angiogenesis. Although molecular lung development, especially lung endodermal branching, has been intensively studied,[2-5] pulmonary vascular development has been somewhat ignored. The vascular involvement in bronchopulmonary dysplasia[6-8] has, however, stimulated interest in understanding molecular pulmonary vascular development.

Normal pulmonary development takes place in the relative hypoxic environment of the uterus[9] and this low fetal oxygen environment is beneficial for lung organogenesis, including vascular development.[10,11] In most mammalian systems the cellular responses to oxygen alterations are mediated through the highly conserved hypoxia-inducible factor (HIF) family of transcriptional regulators. The HIF transcriptional complex is a heterodimer composed of one of three alpha subunits (HIF-1α, HIF-2α or HIF-3α) and a beta subunit (ARNT; also known as aryl hydrocarbon receptor nuclear translocator).[12] The regulation of HIF by oxygen occurs through modifications of the alpha subunit, whereas ARNT is a constitutive nuclear protein and is not affected by oxygen. Under hypoxic conditions, the alpha subunit is stable, allowing it to accumulate in the nucleus, where upon binding to ARNT it recognizes HIF-response elements within the promoter regions of hypoxia-responsive target genes. Under normoxic conditions, the alpha subunit is rapidly degraded by means of ubiquitination and proteosomal degradation (half-life less then 5 minutes in 21% O_2).[13] Ubiquitination and proteosomal degradation of the alpha subunits requires von Hippel-Lindau tumor suppressor protein (VHL).[14] VHL is the HIF-α recognition component of an ubiquitin E3 ligase complex, VEC,[15] which consists of elongins B and C, Rbx1, and Cullin2.[16] Recognition of HIF-α by VHL only occurs when two conserved proline residues (Pro 402 and Pro 564) in the oxygen dependent degradation domain (ODD) of HIF-α are hydroxylated.[17,18] This hydroxylation is catalyzed by a family of prolyl hydroxylase domain enzymes, termed PHD1, PHD2, and PHD3.[19,20] The PHDs are also degraded by E3-ubiquitin-ligases, namely SIAH (seven in absentia homologue) 1 and 2. The degradation is enhanced by hypoxia and SIAHs are transcriptionally upregulated during hypoxia.[13] Thus, various proteolytic mechanisms are responsible for control of stability of HIF-1α.

We[11] and others[21,22] have reported that HIF-1α plays an important role in mediating oxygen-regulated events of pulmonary vascular development. However, we did not investigate the components regulating HIF-1α stability in the developing lung.

Herein, we determined the spatial expression of various components of the HIF system in the human lung during the pseudoglandular stage of lung development (8 - 14 wks), when the primitive pulmonary vascular system develops.

MATERIALS AND METHODS

Human lung samples

All tissues were collected after informed consent in accordance with the Ethics Guidelines of the University of Toronto's Faculty of Medicine. Human lung samples were obtained from elective terminations of pregnancies. Samples were processed for immunohistochemistry and for quantitative RT-PCR. Gestational ages for the samples used for immunohistochemistry were 8 weeks (n = 3), 10 - 12 weeks (n = 3), and 13 weeks (n = 2). Age of the samples used for quantitative RT-PCR was 10 weeks (n = 4), 13 weeks (n = 3), and 16 weeks (n = 3). Gestational age of the embryos was assessed by foot length as described before by Mhaskar et al.[23]

Immunohistochemistry

Lung samples were fixed overnight in 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS), dehydrated, and embedded in paraplast. Immunostaining was performed using the avidin-biotin (ABC) immunoperoxidase method as described by Hsu and coworkers.[24] Seven micron thick sections were deparaffinized and rehydrated in a graded series of ethanol. Antigen retrieval was achieved with heating in 10 mM sodium citrate pH 6.0. Sections were washed in PBS and endogenous peroxidase was blocked in 3% (v/v) H_2O_2 in methanol. Blocking was done with 5% (w/v) NGS and 1% (w/v) BSA in PBS (for Siah staining NGS was substituted with donkey serum). Sections were then incubated overnight at 4°C with antibodies. Primary antibodies were rabbit polyclonal anti-human Cullin2 antibody (1:200; RB-046-p1, Neomarkers, Freemont, CA, USA), a rabbit polyclonal anti-human NEDD8 antibody (1:500; ALX-210-194-R200, Alexis Biochemicals, San Diego, CA, USA), a rabbit polyclonal anti-human PHD1 antibody (1:400; AB100-310, Novus Biologicals, Littleton, CO, USA), a rabbit polyclonal anti-human PHD2 antibody (1:400; AB4561, Abcam, Cambridge, MA, USA), a rabbit polyclonal anti-human PHD3 antibody (1:200; AB4562, Abcam, Cambridge, MA, USA), a goat polyclonal anti-human SIAH1 antibody (1:50; AB2237, Abcam, Cambridge, MA, USA), a mouse monoclonal anti-mouse HIF-1α antibody (1:200; NB 100-105, Novus Biologicals, Littleton, CO, USA), a rabbit polyclonal anti-mouse ARNT antibody (1: 500; NB 100-110, Novus Biologicals, Littleton, CO, USA), a rabbit polyclonal anti-mouse HIF-2α antibody (1:600; NB 100-122, Novus Biologicals, Littleton, CO, USA), a mouse monoclonal anti-human VHL antibody (1:60; OP102, Oncogene, Mississauga, ON, Canada), a rabbit polyclonal anti-mouse VEGFR2 (KDR/Flk-1) antibody (1:400; MAB1667, Upstate USA Inc., Charlottesville, VA, USA), or a rabbit polyclonal anti-mouse VEGF antibody (1:200; SC152, Santa Cruz Biotechnology, Santa Cruz, CA, USA). All were diluted in blocking solution (5% NGS

and 1% BSA in PBS, while donkey serum was used for Siah1). Sections were subsequently incubated with biotinylated secondary antibodies (1:300 rabbit anti-mouse for HIF-1α and VHL; 1:300 donkey anti-rabbit for Cullin2, ARNT, PHDs, VEGF, VEGFR2 and 1:300 donkey anti-goat for SIAH1 (Santa Cruz Biotechnology, Santa Cruz, CA)) and color detection was performed according to instruction in the Vectastain ABC and DAB kit (Vector Laboratories, Burlingname, CA, USA). Sections were counterstained with Carazzi's haematoxylin and mounted in Permount (Fisher Scientific, Unionville, ON, Canada). We have previously confirmed the specificity of all antibodies using human placental tissues.[25]

RNA isolation and quantitative PCR

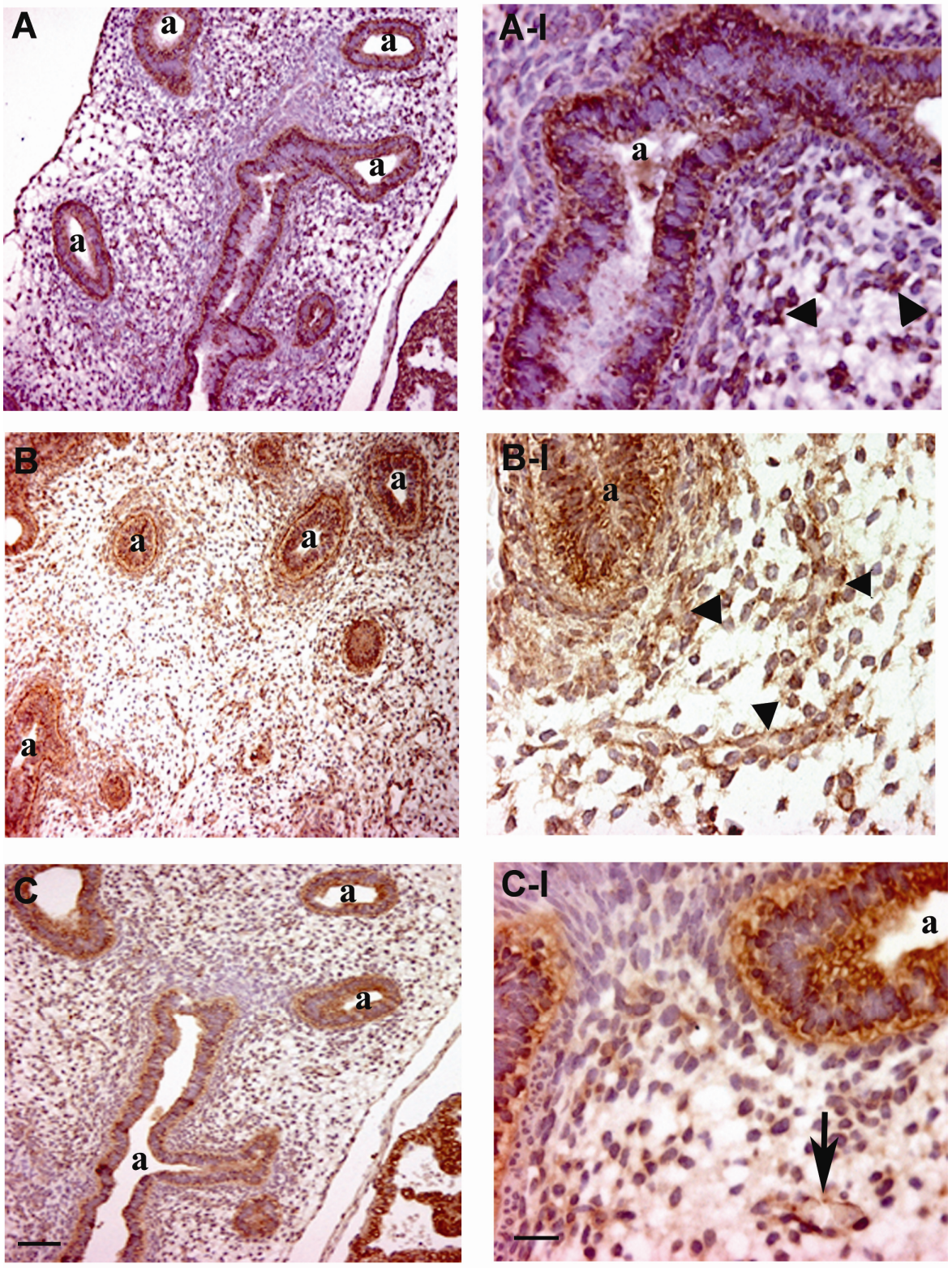
Human lungs were snap-frozen in liquid nitrogen, and stored at -70°C. Total RNA was extracted using the TRIzol® Reagent (Invitrogen, Burlington, ON, Canada) and total RNA was reversed transcribed (37°C) using random hexamers (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR (qPCR) was used to validate differential gene expression. Primers and Taqman probes for VEGF, VEGFR1 (Flt-1), HIF-1α, HIF-2α, ARNT, PHD1, PHD2, PHD3 and SIAH1 were purchased from Applied Biosystems (Foster City, CA) as Assays-on–Demand™ for human genes. For each probe, a dilution series determined the efficiency of amplification of each primer-probe set, allowing the relative quantification method to be employed.[26] For the relative quantitation, PCR signals were compared among groups after normalization using 18S as an internal reference. Briefly, relative fold change was calculated as $2^{-(\Delta Ctexp-\Delta Ctcontrol)}$ where ΔCt = average of (Ctgene of interest-Ct18S) of each sample within a group. Herein, the experimental groups (lungs of 10 and 13 weeks' gestation) were compared to the control group (lungs of 16 weeks' gestation).

RESULTS

HIF and VEGF

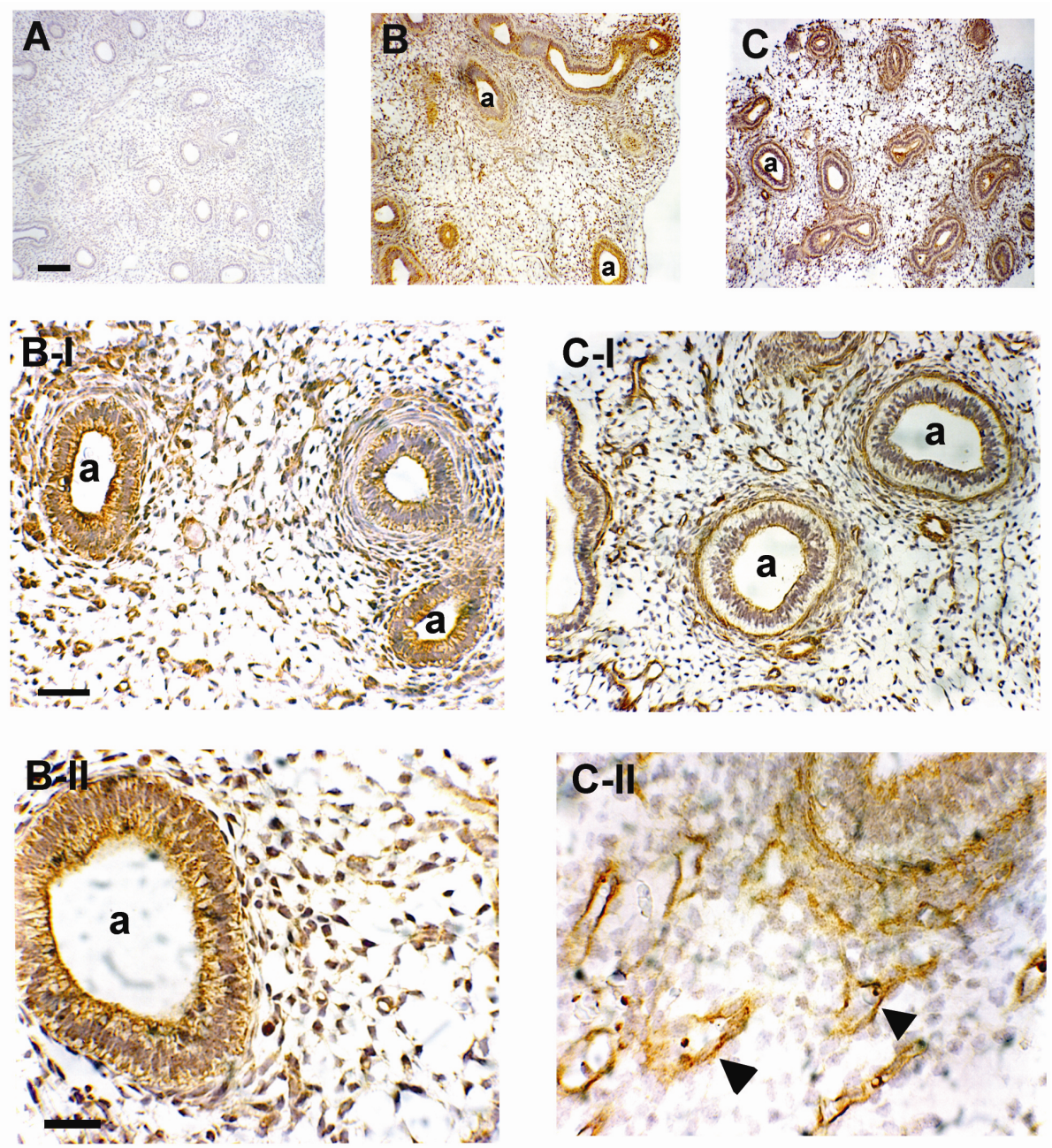
Immunohistochemical staining for HIF-1α, HIF-2α and ARNT revealed that all three HIF subunits were expressed in the pseudoglandular human lung of 7 - 13 wks gestation (*figure 5.1* shows 10 wks). HIF-1α immunoreactivity was primarily detected in the branching epithelium of the first trimester lung (*figure 5.1A, A-I*). Some mesenchymal cells stained positive for HIF-1α (arrow heads, *figure 5.1A-I*). Although HIF-2α protein was found in the airway epithelium (*figure 5.1B*), it was also detected in mesenchymal, most likely vascular, structures (arrow heads, *figure 5.1B-I*). ARNT was predominantly found in the epithelium (*figure 5.1C*), although some positive staining was noted in the lung parenchyma (arrow, *figure 5.1C-I*). Since VEGF (vascular endothelial growth factor) is a downstream target of HIF-1[27] we determined the spatial expression of VEGF in the first trimester human lungs and observed that VEGF was strongly expressed in the epithelium (*figure 5.2B, B-I and B-II*), in agreement with previous studies in humans[28] and mice.[29]

Figure 5.1 *(for color figures see page 163)* Immunohistochemical analysis of HIF subunits in first-trimester (10-weeks) human lung



Airway (a) epithelium shows positive brownish staining for HIF-1α (A, A-I), HIF-2α (B, B-I) and ARNT (C, C-I). Positive signals are also detected in the lung parenchyma (arrow heads and arrow). Bar: 100 μm (A,B,C); 25 μm (A-I, B-I, C-I).

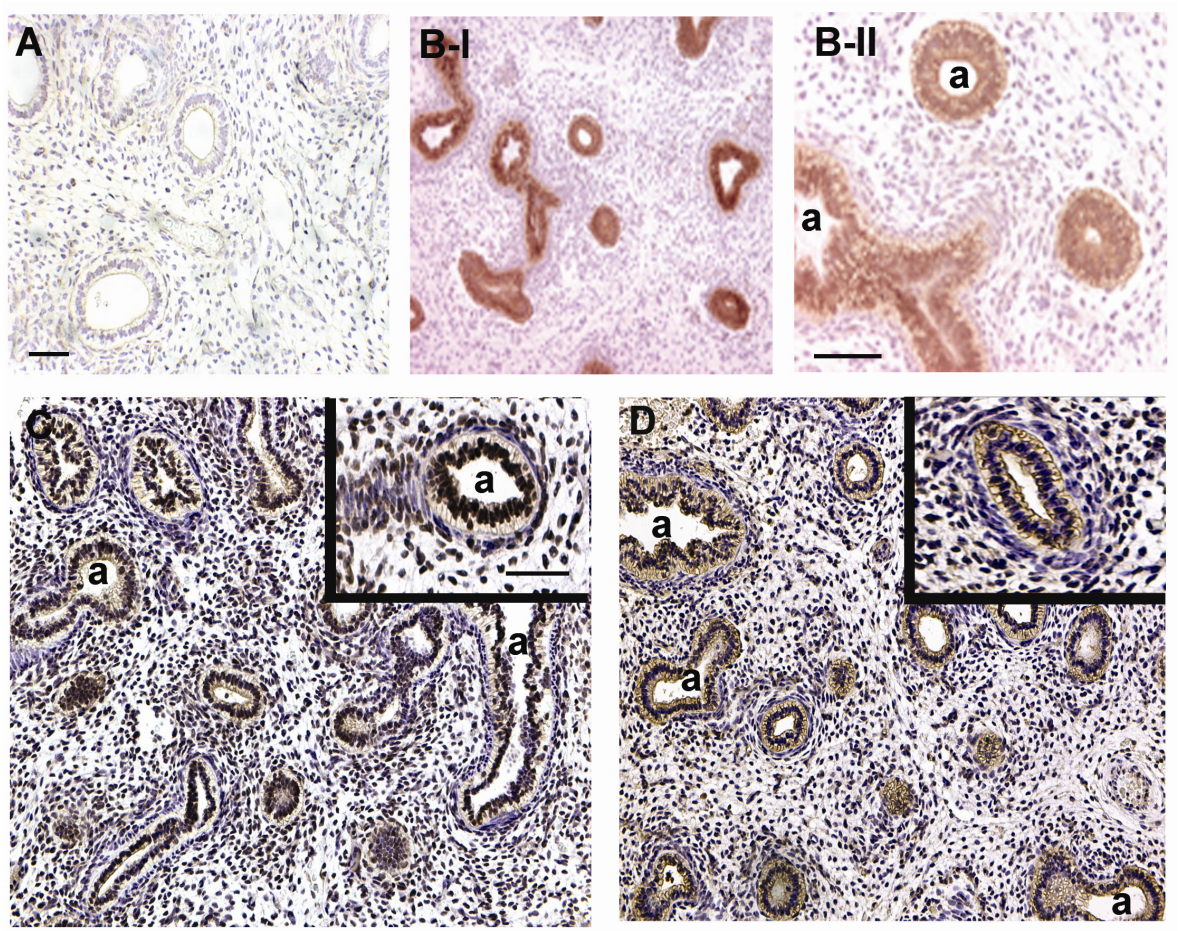
Figure 5.2 *(for color figures see page 164)* Immunohistochemical analysis of VEGF and VEGFR2 in first-trimester (10-weeks) human lung



Strong positive brownish staining for VEGF (B, B-I, B-II) is noted in the airway (a) epithelium, but VEGF is also detectable in some mesenchymal structures. The VEGFR2 signal (C, C-I, C-II) is localized to the mesenchymal cells immediately underlying the epithelium and vascular structures (arrow heads) in the parenchyma. Weaker VEGFR2 staining is detectable in the luminal airway epithelium. Negative control staining is shown in panel A. Bar: 100 μm (A, B, C); 100 μm (B-I, C-I); 50 μm (B-II, C-II).

As reported for mice,[29] positive VEGF staining was also noted in mesenchymal, most likely vascular, structures of the first trimester human lung (*figure 5.2B-I*). Positive immunoreactivity for VEGFR2, an endothelial specific tyrosine kinase receptor for VEGF,[30,31] was found in mesenchymal cells immediately adjacent to the epithelium as well as in vascular (arrow heads) structures of the mesenchyme (*figure 5.2C, C-I and C-II*). The VEGFR2 antibody was also reactive with the luminal airway epithelium. No immunoreactivity was observed in control sections in which primary HIF-1α monoclonal antibodies (*figure 5.3A*) or HIF-2α, ARNT, VEGF and VEGFR2 polyclonal antibodies (*figure 5.2A*) were omitted.

Figure 5.3 *(for color figures see page 165)* Immunolocalization of components of the VEC complex in first-trimester (10-weeks) human lung



Positive brownish staining for VHL is evident in the airway (a) epithelium (B-I, B-II), while a monoclonal antibody control is negative (A). Airways (a) show positive staining for Cullin2 (C) and NEDD8 (D). Some positive brownish staining is also detected in the lung parenchyma, but the mesenchymal cells immediately subjacent to the epithelium are negative (insets in C and D). Bar: 100 μm (A, B-I, C, D); 50 μm (B-II, inset C and D).

VEC complex

At 10 weeks' gestation strong positive immunoreactivity for VHL was noted in the lung epithelium of developing airways (*figure 5.3B-I, B-II*). No mesenchymal staining was observed. VHL-associated ubiquitination activity requires Cullin2, which was primarily detected in the branching epithelium of the first trimester human lung (*figure 5.3C*). However, some mesenchymal staining was apparent. Since ligation of NEDD8 (neural precursor cell expressed, developmentally down-regulated) to Cullin2 may be important for VHL-mediated HIF-1α degradation,[32,33] we also analyzed the first trimester human lung for NEDD8 protein expression. NEDD8 exhibited a similar cellular distribution as Cullin2 (*figure 5.3D*). Interestingly, both Cullin2 and NEDD8 were absent from mesenchymal cells immediately adjacent to the epithelium (see inserts in *figures 5.3C, D*). The staining patterns for all three VEC proteins were similar for all age groups, i.e. 8 weeks, 10 - 12 weeks and 13 weeks (not shown). No immunoreactivity was observed in control sections in which primary VHL (*figure 5.3A*), Cullin2 and NEDD8 (*figure 5.2A*) antibodies were omitted.

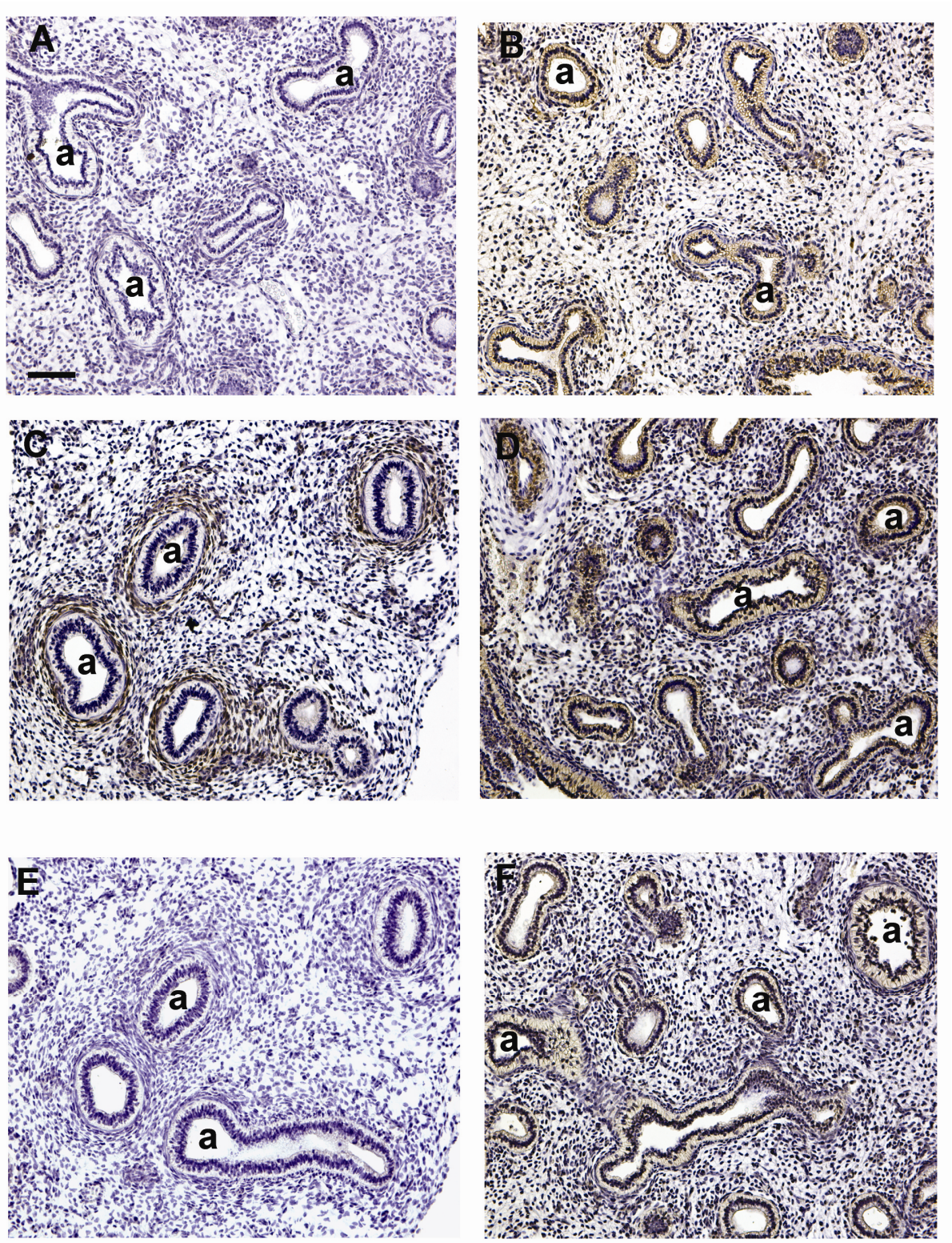
PHDs and SIAH1

Strong positive immunoreactivity for PHD1 and PHD3 was noted in the branching epithelium of the first trimester lung (*figure 5.4 B, D*). In contrast, PHD2 was not expressed in the airway epithelium (*figure 5.4C*). Positive staining for PHD2 was detected in mesenchymal cells subjacent to the newly forming airways as well as in mesenchymal, most likely vascular, structures in the parenchyma. SIAH1 immunoreactivity was only found in the airway epithelium (*figure 5.4F*). No differences between the age groups were observed. Again, no immunoreactivity was observed in control sections in which primary PHDs (*figure 5.4A*) and SIAH1 (*figure 5.4E*) antibodies were omitted.

Quantitative RT-PCR

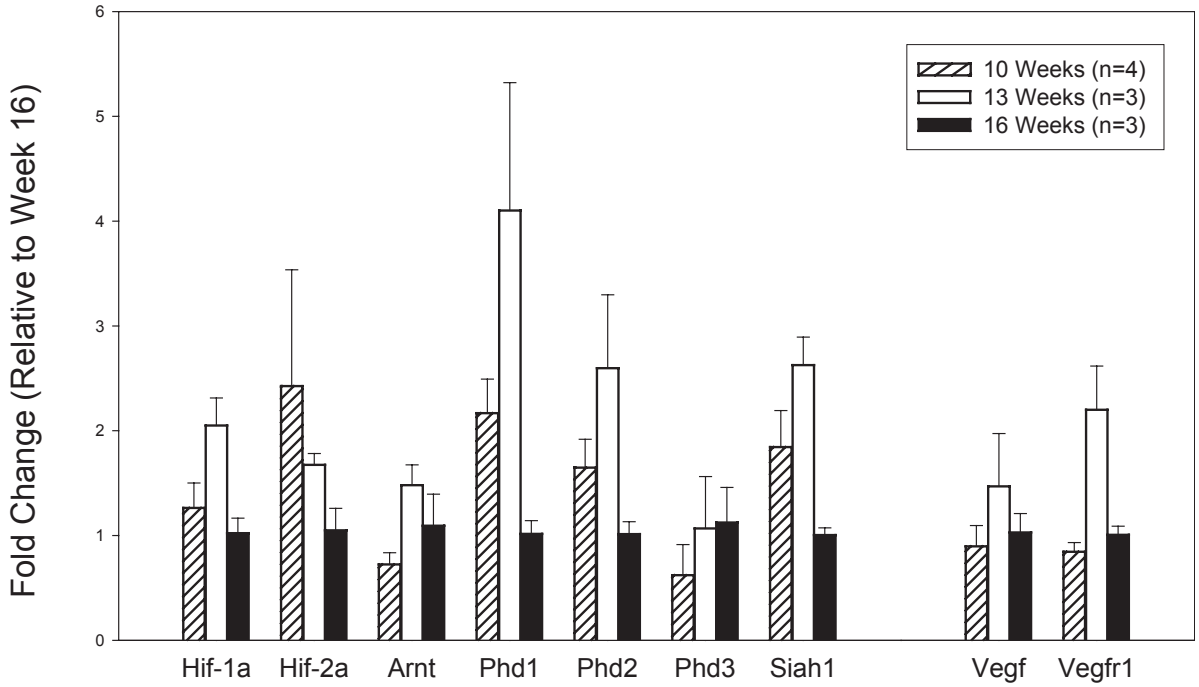
In order to quantify the mRNA expression of HIF-pathway related molecules in the different age groups, we performed real-time PCR. The PCR analysis showed that VEGF, VEGFR1 (Flt-1), HIF-1α, HIF-2α, ARNT, PHD1, PHD2, PHD3 and SIAH1 were all expressed during early pulmonary organogenesis (*figure 5.5*). No changes in expression were noted between the early age groups (10 wks vs. 13 wks), corroborating the immunohistochemical findings. Most HIF related genes decreased with advancing gestation (16 wks).

Figure 5.4 *(for color figures see page 166)* Immunolocalization of PHDs and SIAH1 in first-trimester (10-weeks) human lung



Airway (a) epithelium stains positive for PHD1 (B), PHD3 (D) and SIAH1 (F), while PHD2 protein localized to the lung parenchyma, especially to the mesenchymal cell layer subjacent to the epithelium (C). Negative control staining for primary rabbit and goat polyclonal antibodies are shown in panels A and E, respectively. Bar: 100 μm (A-F).

Figure 5.5 Gene expression of components of HIF/VEGF pathway in first trimester human lung



Expression of VEGF, VEGFR1, HIF-1α, HIF-2α, ARNT, PHD1, PHD2, PHD3 and SIAH1 mRNA in human lung, as assessed by real-time PCR Data are expressed as relative fold changes in expression when compared to human lungs of 16 weeks' gestation (n = 3 - 4 samples for each gestational group; values are mean ± s.e.m.).

DISCUSSION

During the early phase of pregnancy oxygen supply to the fetus changes dramatically. The intervillous space in the placenta opens to maternal blood after 10 - 12 weeks of gestation and oxygen saturation of blood supplied to the fetus rises from 2 - 3% O_2 to 8 - 10% O_2.[34,35] It has been shown that placental degradation of HIF-1α takes place after 10 weeks of gestation at the time when placental oxygenation increases.[25] This also implies a changed oxygen concentration in the blood going to the fetus. Fetal blood oxygen rises and subsequently more oxygen is delivered to the organs. Angiogenesis and branching morphogenesis are very important during this phase of development in the fetal lung. Therefore, we investigated the spatial and temporal expression of HIF/VEGF pathway related molecules in the lung. All molecules examined (HIF-1α, HIF-2α, ARNT, VHL, Cullin2, NEDD8, VEGF, VEGFR1-2, PHD1, PHD2, PHD3, and SIAH1) were expressed simultaneously, suggesting that HIF regulatory mechanisms are already in effect from week 8 of gestation.

Expression of HIF-1α did not decrease after week 10, and expression of molecules involved in degradation of HIF-1α did not increase. HIF-1α expression is mainly restricted to branching epithelium whereas HIF-2α appeared also to be present in the vascular structures of the lung parenchyma. Their constitutive counterpart ARNT

was present in both mesenchymal, most likely vascular, structures and epithelial structures, providing a basis for dimerization and further downstream activation of both HIF-1α and HIF-2α. The different spatial expression of HIF-1α and HIF-2α may reflect their different roles in pulmonary development. HIF-1α knockout mice suffer from severe cardiovascular defects and die in utero,[36,37] while HIF-2α knockout mice suffer from postnatal respiratory distress due to insufficient surfactant production.[38] A heterozygous deficiency in HIF-2α protects mice from developing pulmonary hypertension.[39] It seems that HIF-1α is more important for organogenesis and HIF-2α's function is the fine tuning of pulmonary vascularization and remodeling. The fact that HIF-1α is found in the branching epithelium supports this as pulmonary branching morphogenesis depends on vascularization.[11]

Components of the VEC complex (VHL, NEDD8, and Cullin2) are all present in the epithelium. VHL expression is restricted to epithelial structures and is not present in the mesenchyme. Nedd8 and Cullin2 were also detected in the branching epithelium but not in the immediate subepithelium. This means that all components for the assembly of the VEC complex are present in the epithelium, but not in the mesenchyme. The presence of this complex in the epithelium suggests a necessity for fine tuning of HIF/VEGF expression and vascularization in this specific area. The absence of VEC complex in the mesenchyme allows HIF-2α to accumulate in the vascular cells in the mesenchyme. Our immunohistochemical data show that angiogenic factors such as VEGF are strongly expressed in the early pseudoglandular lung. There was no obvious decrease in VEGF expression after the rise in fetal oxygen saturation. It could be that relatively oxygen rich blood is shunted away from the lung, through the open foramen ovale and the ductus arteriosus, due to the high pulmonary vascular resistance. This mechanism ensures a low oxygen environment in the developing lung, providing a good basis for angiogenesis.

Of the three PHD's the main regulator of HIF-α hydroxylation (and thereby degradation) appears to be PHD2.[19,20] In the developing human lung, PHD2 was differently expressed from PHD1 and 3; i.e. staining was only positive in the mesenchyme directly adjacent to developing airways, while the other two PHD's were expressed in the branching epithelium. This suggests that in the areas with high HIF-1α expression no PHD2 was present, making sure that HIF-1α is degraded at a slow rate. The mesenchymal expression of HIF-2α is in concordance with PHD2 suggesting that vascular development induced by HIF-2α is controlled by PHD2. PHD1 and PHD3 are under the control of their E3 ubiquitin ligase SIAH1 as their expression is restricted to airway epithelium. The fact that all these mechanisms are already present in the developing epithelium suggests that the development of the vascular bed surrounding the branching epithelium is a delicate and finely tuned process.

The continuous activation of HIF pathway even after opening of the intervillous space in the placenta suggests that the lung remains in a relatively low oxygen environment during its development. This allows for vascular growth in the mesenchyme through downstream HIF effectors such as VEGF and VEGFRs.

ACKNOWLEDGEMENTS

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chapter 6

Pulmonary Hypertension of the Newborn: Different Causes of Onset, Same Final Common Pathway?

ABSTRACT

Aims: Persistent pulmonary hypertension of the newborn (PPHN) is a life-threatening disease, characterized by severe hypoxemia. Abnormal vascular structure, growth and/or reactivity are observed in these patients. Therefore we evaluated the expression of several angiogenic factors in diseases associated with PPHN. These factors are von Hippel-Lindau protein (pVHL), Hypoxia Inducible Factor-1α (HIF-1α, Vascular Endothelial Growth Factor (VEGF), Vascular Endothelial Growth Factor receptor 2 (VEGFR2), inducible Nitric Oxide Synthase (iNOS), endothelial Nitric Oxide Synthase (eNOS).

Methods: Control lungs (n = 14) and lungs of patients with idiopathic PPHN (n = 9), meconium aspiration syndrome (MAS n = 12) and pulmonary hypoplasia (n = 8) were obtained from autopsies. Immunohistochemistry for the abovementioned proteins was performed on these lungs.

Results: Compared to normal (control) lungs idiopathic PPHN lungs had low pVHL expression in arterial endothelium and media, whereas MAS lungs displayed high levels. HIF-1α staining in veins and capillaries was high in control, moderate in MAS and hypoplasia and low in idiopatic PPHN. iNOS expression was moderate in MAS capillary endothelium, whereas in other groups staining was strong.

Conclusion: Despite similar pulmonary morphology in PPHN with various underlying causes there appears to be no final common pathway concerning HIF-related angiogenesis, since differences were found in expression pattern of HIF-related molecules.

INTRODUCTION

Persistent pulmonary hypertension of the newborn (PPHN) is a potentially life-threatening disease with an estimated incidence of 0.2% of live-born term infants.[1] PPHN is characterized by severe hypoxemia and right-to-left shunting through a patent foramen ovale, and/or a patent ductus arteriosus, and through intrapulmonary channels.[2,3] PPHN is characterized by abnormal vascular structure, growth, and/or reactivity. It arises when the pulmonary vasculature fails to adapt to postnatal life or when prenatal development is not appropriate. PPHN can occur without identifiable cause (idiopathic PPHN), or in association with various different diseases e.g. meconium aspiration syndrome (MAS), pulmonary hypoplasia, and congenital diaphragmatic hernia (CDH).

Therapeutic modalities for treatment of pulmonary hypertension consist of administration of oxygen through artificial ventilation (either conventional or high frequency oscillation ventilation [HFO]), inhaled nitric oxide (iNO), intra-tracheally administered prostacyclin (PGI_2), extracorporeal membrane oxygenation (ECMO), and most recently phosphodiesterase inhibitors (for review see ref. 4).

It is currently thought that treatment should not only be directed to result in acute vasodilation but potentially should also be directed at preventing the remodeling that takes place in the pulmonary vessels of patients with PPHN.[5] This remodeling or the failure of remodeling in the fetus results in a vessel morphology consisting of medial hyperplasia, adventitial thickening, peripheral extension of muscularization in normally non-muscularized vessels, and an overall reduction in pulmonary vascular density.[6-8]

The pathways involved in the development of pulmonary hypertension in neonates are largely unknown. These pathways may be dependent on the etiology of the various disease entities associated with pulmonary hypertension. The process of vascular remodeling that is present in PPHN can arise as a primary response to injury, or through stimuli within the resistance vessels of the lung, such as hypoxia.[9] In this paper, we focus on the effect that these hypoxic circumstances have on the vasculature in the pulmonary hypertensive lung. The pulmonary circulation responds to hypoxia by guiding its bloodflow to better ventilated areas. This is done by constriction of the vessels that are in a hypoxic area. If the entire lung is hypoxic this will result in constriction of all lung vessels and increased pressure in the pulmonary circulation eventually leading to pulmonary hypertension with the abovementioned vessel remodeling.[10]

A number of processes occur at the cellular level in hypoxia, including transcription of genes controlling glycolysis, glucose transport, cell survival and death, angiogenesis and erythropoiesis.[11] HIF-1 (Hypoxia Inducible Factor) is a key intracellular oxygen-sensor regulating these hypoxia-induced pathways.[11] HIF-1 is a

heterodimeric transcription factor composed of ARNT (aryl-hydrocarbon receptor nuclear translocator also known as HIF-1β) and one of three HIF-α subunits.[12] In normoxia, HIF-1α is rapidly ubiquitinated and degraded through a proteasomal pathway involving an E3 ubiquitin ligase complex containing von Hippel-Lindau protein (pVHL). A dysfunction of the VHL protein leads to VHL disease characterized by highly vascularized tumors.[13] Under hypoxic circumstances HIF-1α accumulates and translocates to the cell nucleus where it dimerizes with its constitutively expressed and stable counterpart ARNT. Activation of hypoxia inducible genes now takes place through binding of the HIF-1 dimer to a hypoxia response element (HRE) in these genes. It is well known that hypoxia stimulates angiogenesis and indeed HIF-1 induces vascular endothelial growth factor (VEGF) which is a potent mitogen for endothelial cells thus influencing angiogenesis.[14,15] It does so by activating its receptor VEGFR2 (also known as Flk-1).[16] It is currently believed that VEGFR1 regulates excessive endothelial cell proliferation by antagonizing VEGFR2.[16] The intracellular transduction pathways activated by VEGF receptors are likely to be regulated by nitric oxide (NO).[16] VEGF is also an important mediator of NO production and this is believed to occur through VEGFR2.[17] NO is an important molecule for many physiological processes and is produced by three different nitric oxide synthases (NOS) of which inducible NOS (iNOS)[18] and endothelial NOS (eNOS) are the most important for VEGF inducible angiogenesis.[19]

The importance of the HIF pathway in pulmonary development is underlined by the fact that treatment of mouse lung explants with antisense oligonucleotides for HIF-1α and VEGF resulted in an almost completely abrogated vascular development and epithelial branching morphogenesis.[20] Previously we found that expression of iNOS in pulmonary arterial endothelium of newborns with CDH is decreased.[21] ECMO therapy normalized these values and we described the beneficial effect ECMO has on vascular abnormalities seen in PPHN.[22]

Against the background of the developmental biological aspects of the HIF-1 pathway we evaluated the expression of HIF-1α, VHL, VEGF, VEGFR2, eNOS, and iNOS in lung samples of patients suffering from PPHN with variable underlying etiology. We hypothesize that aberrations in the HIF-1 pathway may lead to abnormal vascular morphology, growth and reactivity seen in these patients.

MATERIALS AND METHODS

Lung samples

Lung tissue was obtained from autopsies performed in the Departments of Pathology of the Erasmus MC, Rotterdam, and the Radboud University Nijmegen Medical Centre, Nijmegen. All procedures were performed after approval of experimental design and protocols by the medical ethical committee of the Erasmus MC, Rotterdam. Autopsy was performed within 24 hours after death.

A total of 43 lung samples was used; 29 lung samples of patients with PPHN and 14 age-matched controls. The patient group with PPHN was subdivided according to 3 different underlying causes: idiopathic PPHN (n = 9), meconium aspiration syndrome (MAS; n = 12), and pulmonary hypoplasia (n = 8). The controls (n = 14) used were the same as used previously by our group.[23] The cause of death in control patients was unrelated to pulmonary problems. None of the controls received supplemental oxygen or mechanical ventilation. Control lung samples did not show signs of pulmonary hypoplasia. For patient characteristics see *table 6.1*.

The samples were processed by overnight fixation in 10% buffered formalin, dehydration and embedding in paraffin. Five micron tissue sections were mounted on 3-amino-propyl-trioxysilane coated glass slides (Sigma, St. Louis, MO) and processed for immunohistochemistry.

Table 6.1 Patient characteristics

	Control	iPPHN	Hypoplasia	MAS
Number of cases	14	9	8	12
Mean gestational age (weeks)	36	40	33	40
Mean weight (grams)	2440	2835	1925	3193
Mean age at death (hours)	22	72	24	72
% treated with ECMO	0	22	0	17
% treated with HFO	0	22	13	58
% treated with inhaled NO	0	44	0	33

Immunohistochemistry

Immunohistochemistry was performed using a standard avidin-biotin complex method. In brief, after deparaffinization for 10 minutes in xylene and rinsing in 100% alcohol, slides were treated with 3% H_2O_2 in methanol for 20 minutes to block the endogenous peroxidase activity. Antigen retrieval was accomplished by boiling the slides for 15 minutes in citric acid buffer, pH 6.0. No antigen retrieval was necessary for VEGF staining.

Slides were preincubated with blocking solution (Labvision Corporation, Fremont, CA) containing bovine serum albumin (BSA) for 7 minutes. Slides were then incubated with a primary antibody either against VHL, HIF-1α, VEGF-A (all isoforms), iNOS, eNOS, or VEGFR2 (*table 6.2*). After the primary antibody incubation slides were treated for 10 minutes with a biotinylated secondary antibody (Labvision Corporation). Now slides were incubated for 10 minutes with undiluted peroxidase-conjugated streptavidin (Labvision Corporation). Peroxidase was detected by diamino-benzidine tetrahydrochloride (Fluka, Buchs, Switzerland) with 0.03% H_2O_2 for 7 minutes. Finally slides were rinsed with running tap water and counterstained with haematoxylin, dehydrated through a graded alcohol series and xylene, and mounted. Negative controls were processed by omitting the primary antibodies.

Table 6.2 Primary antibodies

Antibody	Dilution	Incubation	Company
VHL	1/100	ON/4°C	Oncogene Research products, San Diego, CA
HIF-1α	1/10	ON/4°	A generous gift of Dr. H. Turley, Oxford, UK
VEGF-A	1/400	1 hr/RT	Santa Cruz Biotechnology, Santa Cruz, CA
eNOS	1/200	ON/4°	Santa Cruz Biotechnology, Santa Cruz, CA
iNOS	1/100	ON/4°	Transduction Laboratories, San Diego, CA
VEGFR2	1/200	1 hr/RT	Transduction Laboratories, San Diego, CA

Lung samples were evaluated by two independent observers (FG, JdR) as described before.[23] The expression of all markers was scored as positive, weakly positive, or negative for each of the following structures: endothelium of the pulmonary arteries, veins, and capillaries; vascular smooth muscle cells of the arterial medial layer; fibroblasts of the arterial adventitial layer; and epithelium of the proximal and distal airways. Weakly positive was defined as "clearly positive compared to the negative controls, but not as strong as the positive staining."

RESULTS

The expression pattern of VEGF, VEGFR2, and eNOS did not differ between patient groups and controls. VEGF, VEGFR2 and eNOS expression was strong in all epithelial structures, arterial endothelium, arterial media and venous endothelium. Expression was weak in capillaries and absent in arterial adventitia.
Differences between groups were seen in the expression pattern of pVHL, HIF-1α, and iNOS.
No differences were observed in staining patterns between patients who died within 1 hour after birth and children who lived longer.

pVHL (*figures 6.1, 6.4A,B,C*)
The arterial endothelium and medial smooth muscle cells of MAS lungs showed high expression of pVHL, while control and hypoplastic lungs showed moderate expression. Expression of pVHL in lungs of patients with idiopathic PPHN was low.
Veins, capillaries, arterial adventitia were negative and epithelium was positive in all patient groups (veins, capillaries, adventitia negative and epithelium positive).

HIF-1α (*figures 6.2, 6.4D,E,F*)
Veins were positive in control, MAS and hypoplastic lungs, whereas in idiopathic PPHN staining was negative. Expression of HIF-1α was high in normal capillaries, moderate in MAS capillaries and low in idiopathic PPHN, and hypoplastic lungs.
The expression of HIF-1α was weak in epithelial structures in all patient groups. The arterial endothelium and medial smooth muscle cells showed high expression in all groups. The adventitia was negative.

Figure 6.1 pVHL expression

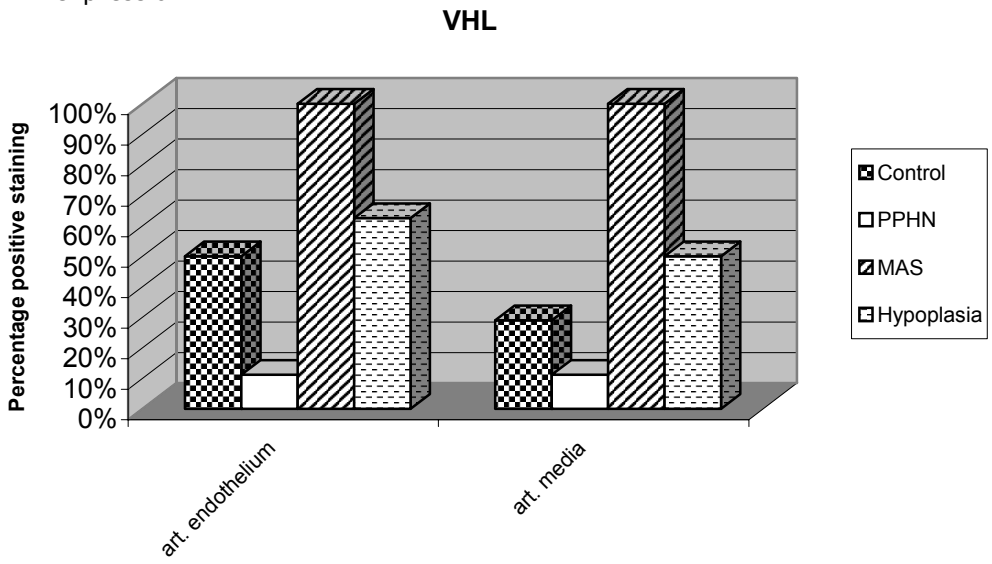


Figure 6.2 HIF-1α expression

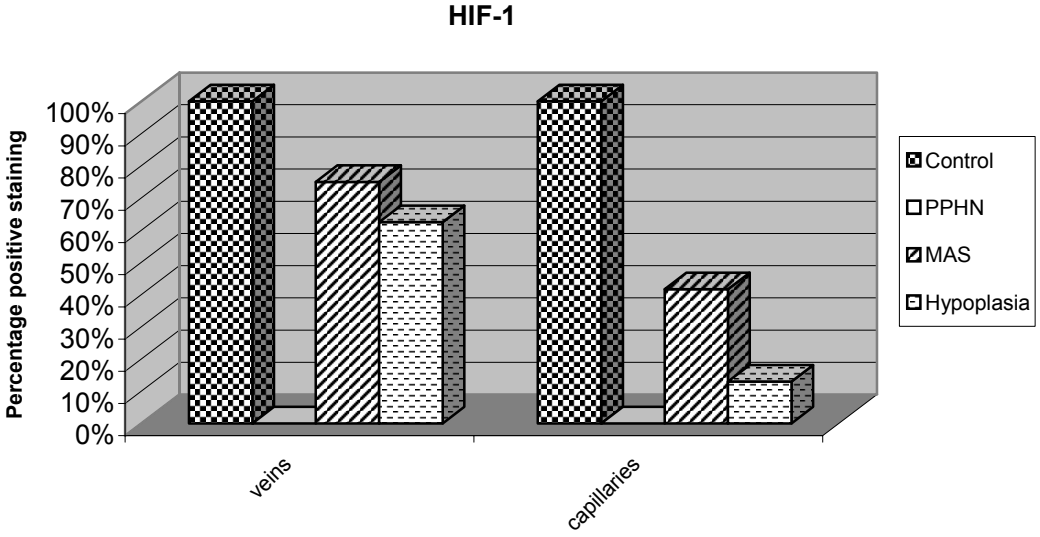
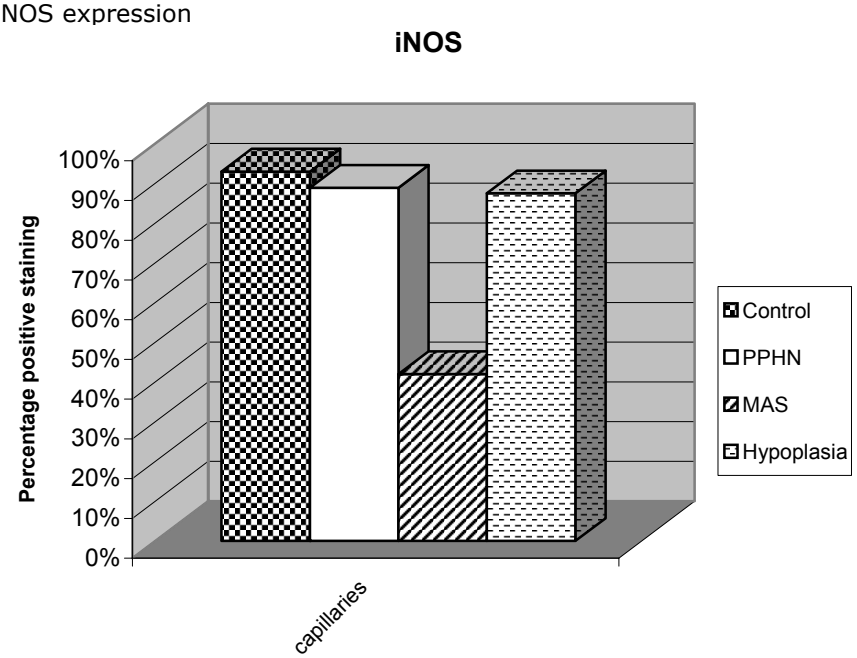
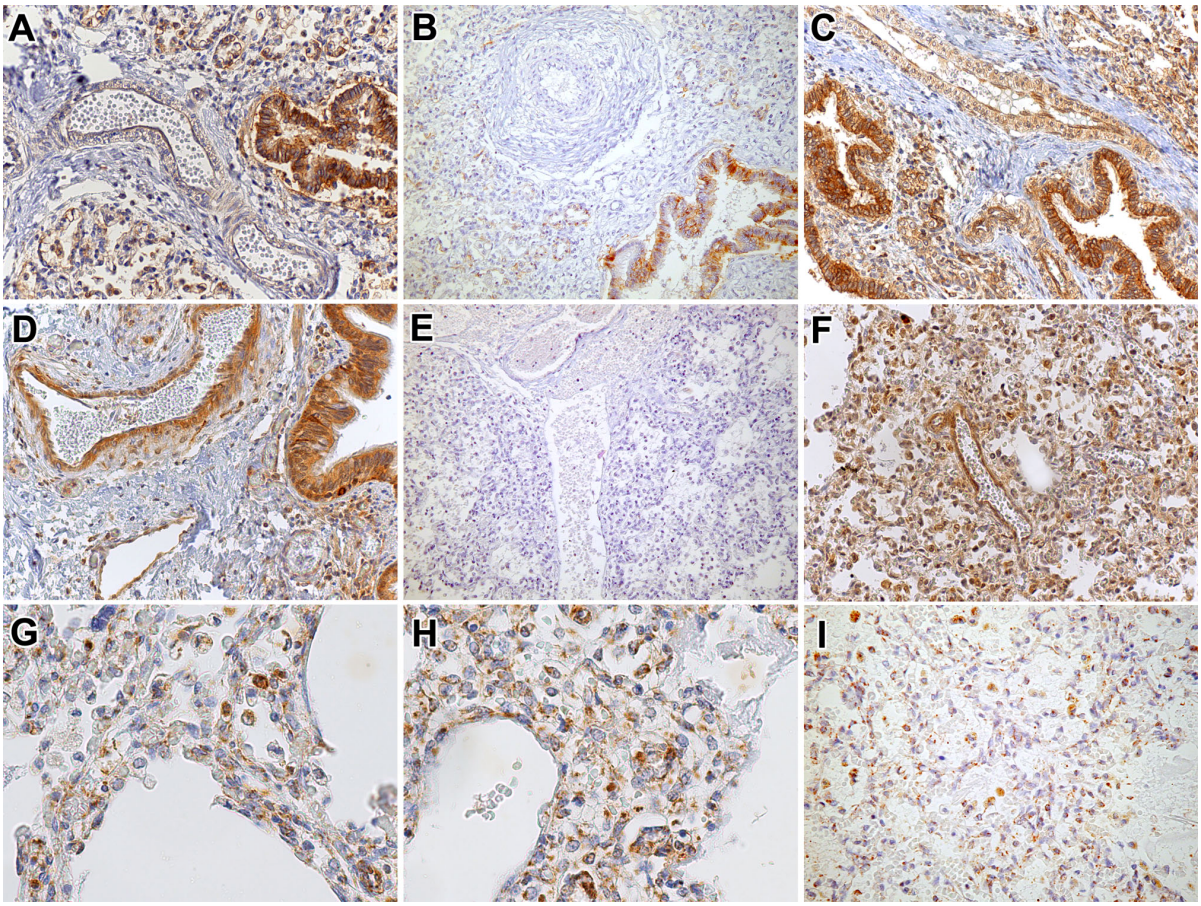


Figure 6.3 iNOS expression



iNOS (*figures 6.3, 6.4G,H,I*)
Capillaries showed differences in staining pattern between groups. Strong expression was seen in all patient groups except for the MAS patients, in which group only 42% scored positive. Expression of iNOS was high in epithelial structures, arterial endothelium, arterial media and veins. There was no expression in adventitia.

Figure 6.4 *(for color figures see page 167)*



A-C: VHL staining of normal control lungs (A), lungs from idiopathic PPHN (B) and from hypoplastic lungs (C), showing decreased expression of VHL protein in the arterial endothelium and medial smooth muscle cells in idiopathic PPHN as compared to controls, whereas hypoplastic lungs have increased expression in these cell types.
D-F: HIF-1α staining of normal control lungs (D), lungs from idiopathic PPHN (E) and from MAS (F), showing no staining at all in veins and capillaries of idiopathic PPHN whereas MAS showed a decrease in staining compared to controls.
G-I: iNOS staining of normal control lungs (G), lungs from idiopathic PPHN (H) and from MAS (I), showing capillary staining in MAS lungs compared to controls and idiopathic PPHN.

DISCUSSION

Staining patterns for VEGF, VEGFR2 and eNOS were similar in all patient groups compared to controls. Differences were seen in pVHL (arterial endothelium and media), HIF-1α (veins and capillaries), and iNOS (capillaries). Expression of pVHL in control lungs and hypoplastic lungs was moderate, but strong staining was observed in MAS lungs and weak staining in idiopathic PPHN lungs. HIF-1α expression was weaker in patient groups compared to controls, especially in idiopathic PPHN lungs. Staining for iNOS was lower in MAS lungs compared to control lungs and the other patient groups.

The different patient groups did not show the same aberrations in staining pattern for pVHL, HIF-1α and iNOS, suggesting that the underlying cause for pulmonary hypertension is of importance for the expression of these proteins.

Idiopathic PPHN

HIF-1α expression in pulmonary veins and capillaries of these patients was absent. Apparently rapid degradation of HIF-1α has occurred in these lungs, in spite of low VHL levels in veins and capillaries. Therapy for these patients consisted of oxygen administration which ensures HIF-1α degradation. This means that downstream activation of VEGF is not achieved in these patients and that induction of angiogenesis and vasodilation via eNOS activation is not accomplished. The resistance vessels of the lung (the small pulmonary arteries) showed a normal HIF-1α expression pattern, which may be explained by a decrease in expression of pVHL. This could be the cause of an upregulation of HIF-1α to normal expression (comparable with controls) in these vessels.

MAS

Patients who aspirated meconium at birth had decreased levels of HIF-1α in pulmonary veins and capillaries; however as opposed to the patients with idiopathic PPHN they had a marked increase in pVHL in the pulmonary resistance vessels, while HIF-1α levels in these vessels are normal (i.e. high expression). An increase in pVHL ensures degradation of HIF-1α in normoxic circumstances. Hypoxia in these vessels however disables degradation of HIF-1α.

An explanation for the development of pulmonary hypertension could be that fibronectin matrix assembly is upregulated in these resistance vessels. Davie and colleagues showed that fibronectin is increased in the vessel wall of the hypoxic pulmonary hypertension calf model.[24] Fibronectin deposition is among others regulated by pVHL.[25] The fibronectin and HIF-1α pathways are regulated independently; the modification of pVHL (covalent conjugation with NEDD8) needed for fibronectin synthesis does not interfere with the E3 activity of the VHL E3 ubiquitin ligase complex (VEC) to target HIF-1α.[25] Therefore elevated pVHL levels

may increase fibronectin deposition causing rigidity of the resistance vessels in this patient group.

In addition to the abovementioned findings there was less iNOS staining in the capillaries of this patient group. Lu and colleagues showed that meconium induces iNOS expression in a rat model of MAS and that treating these rats with inhaled NO decreases protein levels of iNOS.[26] Patients treated with inhaled NO indeed showed a decrease in capillary iNOS staining. However treatment with inhaled NO and HFO did not show the same results.

Hypoplasia

HIF-1α expression in veins and especially in capillaries was low, with a normal pVHL expression throughout the lung. It seems that the HIF pathway in this lung is completely normal except in veins and capillaries. We postulate that this phenomenon is caused by the hyperoxic therapy given to this patient group ensuring there is enough oxygen for the degradation of HIF-1α. In other patients with pulmonary hypoplasia not associated with pulmonary hypertension and not subjected to these forms of therapy a decrease in HIF-1α expression in veins and capillaries was seen.[23] However in hypoplasia patients with pulmonary hypertension arterial pVHL expression is not decreased, demonstrating similarities with CDH.[23] In pulmonary hypoplasia not associated with pulmonary hypertension pVHL was absent,[23] suggesting similar vascular pathology in CDH and lung hypoplasia associated with pulmonary hypertension. In this study we found levels of both iNOS and eNOS comparable to controls, while our group recently demonstrated an increase of iNOS in arterial endothelial cells in CDH that is normalized by ECMO.[21] Furthermore in humans normal eNOS levels were found.[27] These findings are in contrast with previously reported reduced eNOS levels in a CDH rat model.[28]

Grover et al found that blocking the action of VEGF causes pulmonary hypertension in fetal lambs and administration of rhVEGF (recombinant human VEGF) in pulmonary hypertensive lambs causes a decrease in medial thickness and an increase in eNOS expression.[29] In contrast to their findings we did not find differences in the staining pattern in any of our patient groups for VEGF and VEGFR2. Also in a porcine model of hypoxic pulmonary hypertension VEGF expression is upregulated, and eNOS levels stayed normal.[30] The fact that we did not see an upregulation of VEGF in our studies may be explained by the fact that all our patients were treated with 100% oxygen thereby lowering their HIF-1α levels and subsequently decreasing the amount of VEGF.

NO administration was one of the treatment modalities in our patients. NO has an important effect on HIF-1α; in normoxic conditions it stabilizes HIF-1α, but in hypoxic circumstances it results in the exact opposite i.e. degradation of HIF-1α.[31] Due to the limited number of patients in our study it is unrealistic to make any statements on the effect of inhaled NO on HIF-1α expression.

In this study we found that despite similar pulmonary morphology in PPHN with various underlying causes there does not seem to be a final common pathway concerning HIF-related angiogenesis, since differences were found in expression pattern of HIF pathway related molecules between the various diseases.
Expression of pVHL was comparable to controls in arterial medial smooth muscle cells and endothelial cells of hypoplastic lungs, while levels in idiopathic PPHN were decreased and levels in MAS were increased. HIF-1α expression was decreased in MAS and pulmonary hypoplasia, whereas a complete absence was observed in idiopathic PPHN.

The expression pattern of HIF-1α in CDH has similarities with idiopathic PPHN. Absent HIF-1α in veins and capillaries was observed in both diseases. However a decrease in pVHL in arterial medial smooth muscle cells was seen in idiopathic PPHN but an increase was observed in CDH. This resembles pVHL expression pattern in MAS and pulmonary hypoplasia.

We conclude that the differences between the various diseases suggest that these diseases are completely different entities, although vascular morphology in the end remains the same. Most likely the cause of the differences between the various diseases in HIF-pathway related molecules lies in the different moments of onset. It is generally accepted that CDH is an early developmental aberration, while idiopathic pulmonary hypertension can be the result of an alteration in development which occurs later preventing the development of a vascular system with low resistance. Severe pulmonary hypertension associated with MAS occurs perinatal.

Especially pVHL and HIF-1α seem to play a major role. Our data support the notion that the HIF pathway is of importance for pulmonary angiogenesis and vascular remodeling in both normal pulmonary growth and in pulmonary hypertension.

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chapter 7

Alveolar Capillary Dysplasia; Angiogenesis Gone Awry

ABSTRACT

Alveolar capillary dysplasia (ACD) is a rare lethal lung disease characterized by pulmonary hypertension of the newborn. Histology shows abnormal location of pulmonary veins, and paucity of capillaries in thick alveolar walls. Because the etiology of this disorder is unknown the role of angiogenesis-related proteins was investigated, including Hypoxia Inducible Factor-1α (HIF-1α), von Hippel-Lindau protein (pVHL), Vascular Endothelial Growth Factor (VEGF), Vascular Endothelial Growth Factor receptor 2 (VEGFR2), inducible Nitric Oxide Synthase (iNOS), and endothelial Nitric Oxide Synthase (eNOS). We performed immunohistochemistry for the abovementioned proteins on human ACD lungs (n = 11) and age-matched control lungs (n = 14). The frequency of pVHL expression was significantly lower in endothelial cells of arteries, veins, and capillaries of control lungs, compared with ACD lungs. In contrast, ACD lungs had a significantly lower expression of HIF-1α in capillary and venous endothelial cells than normal control lungs. ACD cases more frequently had eNOS staining in their capillaries than controls. No differences were seen for VEGF, VEGFR2, and iNOS. Our results show that HIF-pathway related angiogenesis appears to be disturbed in ACD. The increase in pVHL expression in the ACD group may lead to a decrease in angiogenic pathway induction through degradation of HIF-1α.

INTRODUCTION

Alveolar Capillary Dysplasia (ACD) is a rare fatal lung disease that clinically presents as persistent pulmonary hypertension of the neonate (PPHN). These infants usually die in the neonatal phase despite intensive medical support such as inhaled nitric oxide and extracorporeal membrane oxygenation.[1-3] The initial response to inhaled nitric oxide is usually good, but often PPHN recurs/persists.[4] In some cases the diagnosis is suspected clinically and proven later by lung biopsy or autopsy.[3] ACD is frequently accompanied by other congenital malformations of cardiac, genito-urinary, or intestinal origin.[3] Association with at least one major anomaly has been reported in up to 82% (n = 22) of patients with alveolar capillary dysplasia.[5] Several familial cases of ACD have been described suggesting that genetic factors may play a role in its etiology.[6-9]

Characteristic pulmonary histological findings in ACD include muscularization of small pulmonary arteries and a decrease in the number of alveoli and capillaries, which often are abnormally dilated.[3] The alveolar epithelium is surrounded by mesenchyme with small blood vessels but no capillaries or capillaries that are not adjacent to the epithelium where gas exchange takes place.[2] It seems that the vessels fail to grow towards and fuse with the type I cells to form a thin blood-air barrier making an efficient gas exchange virtually impossible.

Various names such as primary alveolar capillary dysplasia, misalignment of pulmonary veins, and ACD with misalignment of pulmonary veins have been used for this disease.[10] The misalignment of pulmonary veins means that veins share an adventitial sheath with the arteries in the bronchovascular bundle instead of their normal intra-acinar course away from the arterial branches; ACD can occur with or without misalignment of pulmonary veins.[2]

Rabah et al assume that in their patient group with ACD, associated with hypoplastic left heart syndrome (n = 3), pulmonary vasoconstriction occurs in utero.[11] The deficient capillary bed is bypassed and the arteries can only drain in the anomalous bronchial veins. This indeed seems to be the case as pulmonary angiography in an ACD patient showed that contrast does not pass the capillary bed but immediately drains in the veins.[12]

There are numerous factors important for lung development (for review see Groenman et al).[13] In patients with ACD the pulmonary vascular bed is malformed; therefore we focused on the factors important for angiogenesis and potentially relevant for our understanding of the etiology of ACD. Factors involved in angiogenesis include HIF-1 (Hypoxia Inducible Factor), VEGF (Vascular Endothelial Growth Factor), and eNOS (endothelial Nitric Oxide Synthase).[13]

HIF-1 is a dimer composed of a stable subunit, HIF-1β (or ARNT [Aryl Hydrocarbon Nuclear Translocator]), and one of three oxygen sensitive subunits, HIF-1α, HIF-2α or HIF-3α.[14,15] The HIF-α subunits are degraded under normoxic circumstances and are stabilized under hypoxia, whereas HIF-1β is oxygen independent. Since the lung develops in a relative hypoxic environment these factors are considered to be important for lung development. This is supported by the fact that antisense HIF-1α knock out in mice shows a decrease in lung vessel formation and branching morphogenesis.[16]

In normoxic circumstances HIF-1α is hydroxylated by oxygen-sensitive prolyl hydroxylases and subsequently recognized by an E3 ubiquitin ligase complex containing pVHL (von Hippel Lindau protein) and targeted for proteolytic degradation.[17] However, under hypoxic conditions HIF-1α accumulates, transfers from the cytosol to the nucleus and dimerizes with HIF-1β.[15] This heterodimer now binds to HREs (hypoxia response elements) which are present in the regulatory elements of hypoxia responsive genes.

Hypoxia-induced pathways include those for erythropoiesis, iron metabolism, vessel formation (both vasculogenesis and angiogenesis), glucose uptake, and glycolysis.[15] One of the downstream genes in the vascular pathway is VEGF, a potent growth factor for endothelial cells. VEGF can bind to four receptors, of which the one responsible for its vasoactive role is VEGFR2 (also know as Flk-1 [Fetal liver kinase]).[18-21] VEGFR2 ensures the release of NO through stimulation of eNOS (endothelial Nitric Oxide Synthase, which synthesizes NO from L-arginine).[19] Another producer of NO directly induced by HIF-1α is iNOS (inducible Nitric Oxide Synthase).[15] NO plays an essential role in angiogenesis through induction of endothelial cell proliferation, migration, and differentiation, culminating in the sprouting of new capillaries from existing vasculature.[22]

The HIF-pathway may be of critical importance in the developing fetal pulmonary circulation as the fetus develops in a relative hypoxic environment. We hypothesize that a derailment of this pathway is present in patients with ACD. This is supported by the fact that eNOS deficient mice have an ACD-like phenotype.[23] In order to examine the role of the HIF-pathway in the development of the pulmonary vascular bed in ACD patients we performed an immunohistochemical analysis of these molecules (pVHL, HIF-1α, VEGF, VEGFR2, eNOS, and iNOS) in lung samples of ACD patients and age matched controls.

MATERIALS AND METHODS

Patients

Archived lung material from the Departments of Pathology of the Erasmus MC-University Medical Centre, Rotterdam, the Radboud University Nijmegen Medical

Centre, Nijmegen, and the Children's Hospital, The Ohio State University, Columbus, OH, USA was used for this study. All materials were either derived from open lung biopsy or autopsy within 24 hours after death. Design and protocols were approved by local medical ethical committees and/or institutional review boards.

All lung samples were processed for immunohistochemistry as follows: after fixation in 10% buffered formalin overnight and, dehydration, samples were embedded in paraffin. Five micron tissue sections were cut and mounted on 3-amino-propyl-trioxysilane coated glass slides (Sigma, St. Louis, MO).

A total of 25 lung samples was used; 11 lung samples of patients with ACD and 14 age matched controls. For patient characteristics see *table 7.1 and table 7.2*. These were the same controls as used previously by our group.[24] Cause of death in control patients was unrelated to pulmonary problems. None of the controls received supplemental oxygen or mechanical ventilation. Control lung samples did not show signs of pulmonary hypoplasia.

Table 7.1 Patient characteristics

	Control	**ACD**
Number of cases	14	11
Mean gestational age (weeks)	36	39^{+5}
Mean weight (grams)	2440	3128
Mean age at death (days)	1	30
% treated with ECMO	0	64
% treated with HFO	0	55
% treated with inhaled NO	0	73

Table 7.2 ACD patient characteristics

Patient nr.	**Sex**	**Gestational age (weeks)**	**Birth weight (grams)**	**Age at death (days)**	**Treatment**		
					ECMO	**HFO**	**NO**
1	M	38	3350	16	Yes	Yes	Yes
2	F	42	2420	16	Yes	Yes	Yes
3	M	40	3650	75	Yes	No	Yes
4	F	40	3449	13	Yes	Yes	Yes
5	M	38	2700	16	No	No	Yes
6	F	36	2125	6	No	Yes	No
7	M	42	3277	16	No	Yes	Yes
8	M	41	3640	49	No	No	Yes
9	F	41	3600	17	Yes	Yes	No
10	M	34	2610	30	Yes	No	Yes
11	F	40	4200	28	Yes	No	No

Immunohistochemistry

A standard avidin-biotin complex method was utilized for immunohistochemistry. In brief, after deparaffinization for 10 minutes in xylene and rinsing in 100% alcohol, slides were treated with 3% H_2O_2 in methanol for 20 minutes to block the endogenous peroxidase activity. Antigen was retrieved by boiling the slides for 15 minutes in citric acid buffer, pH 6.0. For VEGF staining antigen retrieval was not necessary.

Non-specific binding sites were blocked by using a blocking solution (Labvision Corporation, Fremont, CA) containing bovine serum albumin (BSA) for 7 minutes. Slides were then subjected to a primary antibody either against VHL (Oncogene Research products, San Diego, CA, 1:100, 4 ºC, overnight), HIF-1α (A generous gift of Dr. H. Turley, Oxford, UK, 1:10, 4 ºC, overnight), VEGF-A (all isoforms, Santa Cruz Biotechnology, Santa Cruz, CA, 1:400, RT, 1 hr), iNOS (Transduction Laboratories, San Diego, CA, 1:100, 4 ºC, overnight), eNOS (Santa Cruz Biotechnology, Santa Cruz, CA, 1:200, 4 ºC, overnight), or VEGFR2 (Transduction Laboratories, San Diego, CA, 1:200, RT, 1 hr).

Hereafter slides were treated for 10 minutes with a biotinylated secondary antibody (Labvision Corporation). Now slides were incubated for 10 minutes with undiluted peroxidase-conjugated streptavidin (Labvision Corporation). Peroxidase was detected by diamino-benzidine tetrahydrochloride (Fluka, Buchs, Switzerland) with 0.03% H_2O_2 for 7 minutes. Finally slides were rinsed with running tap water and counterstained with haematoxylin, dehydrated through a graded alcohol series and xylene, and mounted.

No primary antibodies were used for the negative controls. To maintain consistency, all immunohistochemical stainings were performed under equal circumstances, and lung samples of both groups were processed at the same time with each antibody.

Lung samples were evaluated by two independent observers (FG, RdK) as described before by our group.[24] Per case (control and ACD) 3 sections were examined, in which at least 20 veins, 20 arteries and 50 capillaries were analyzed. In the entire section, the expression of all markers was scored as positive, weakly positive, or negative for each of the following structures: endothelium of the pulmonary arteries, veins, and capillaries; vascular smooth muscle cells of the arterial medial layer; fibroblasts of the arterial adventitial layer; and epithelium of the proximal and distal airways. Weakly positive was defined as "clearly positive compared to the negative controls, but not as strong as the positive staining." Statistical analyses were performed using SPSS 12.0.1 for windows. For comparison between the 2 groups we used Fishers exact test. *P* values < 0.05 were considered significant.

RESULTS

Staining pattern of VEGF, VEGFR2, and iNOS did not differ between controls and ACD cases. VEGF, VEGFR2 and iNOS expression was strong in all epithelial structures, arterial endothelium, arterial media and venous endothelium. Expression was weak in capillaries and absent in arterial adventitia. Significant differences in expression patterns between control samples and ACD patient material were observed for pVHL, HIF-1α, and eNOS.

The expression of pVHL did not differ in epithelium and adventitia. Epithelium showed strong staining and adventitial fibroblasts showed no staining.

All endothelial cells (arteries (not significant), veins $P < 0.001$, and capillaries $P < 0.05$) were positive in ACD cases, whereas expression in control cases was decreased in arterial and capillary endothelial cells and absent in veins. In addition, more ACD patients had positive staining in the arterial medial smooth muscle cells (for schematic representation see *figure 7.1* and for pictures see *figure 7.4 A, B*).

For HIF-1α no obvious differences were seen in epithelium and arteries (endothelium, media, and adventitia). Expression of HIF-1α was weak in epithelial structures, high in arterial endothelium and media, and absent in arterial adventitia (*figures 7.2 and 7.4 C, D*).

There were less ACD patients with positive staining of HIF-1α in veins and capillaries than control patients, which all had positive reactivity for HIF-1α in veins and capillaries ($P < 0.01$).

Figure 7.1 ACD lungs show a high expression of pVHL in all endothelial cells and arterial medial smooth muscle cells (* $P < 0.05$)

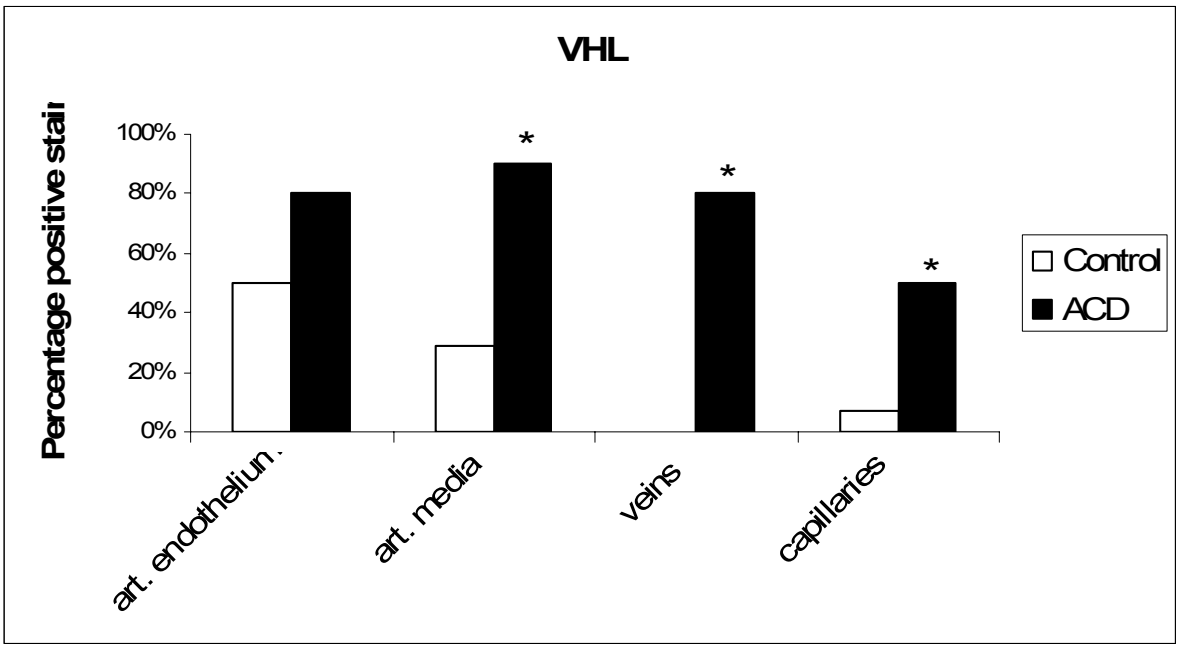


Figure 7.2 Controls have a higher HIF-1α expression then ACD lungs in pulmonary veins and capillaries (* $P < 0.05$)

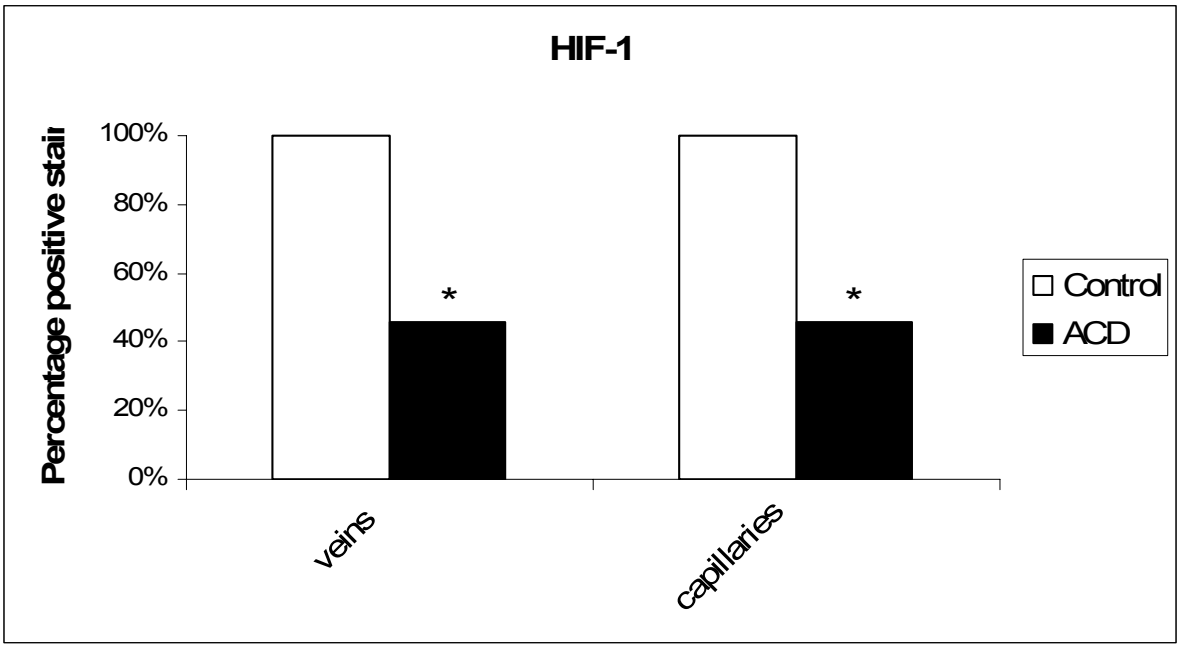
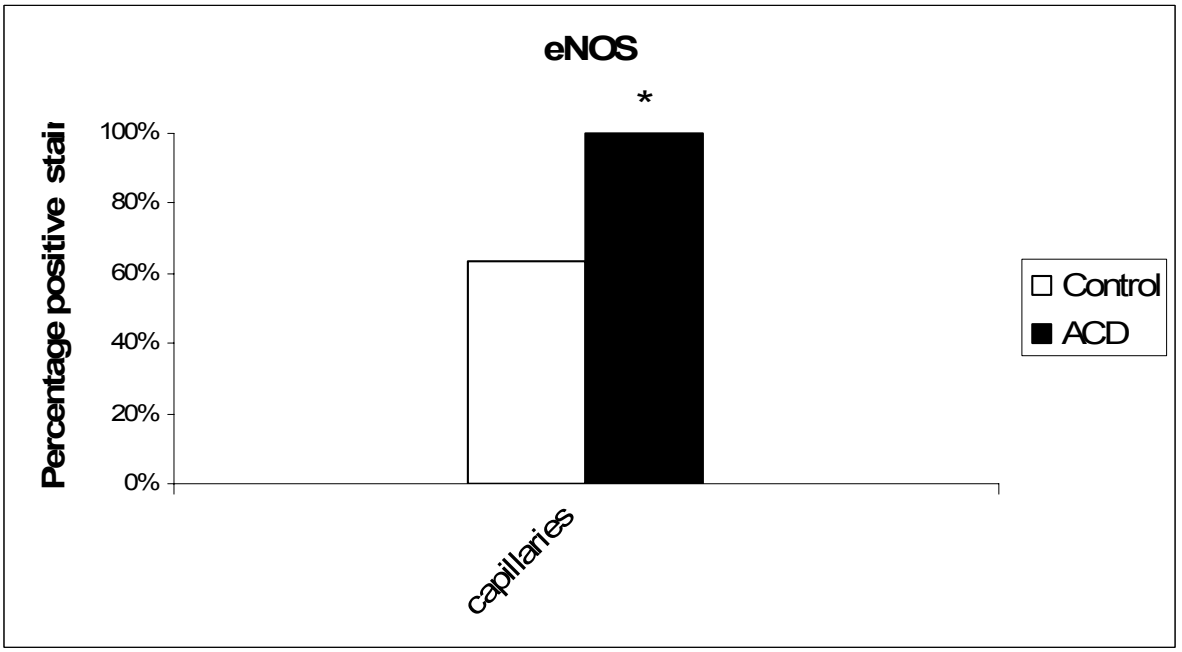


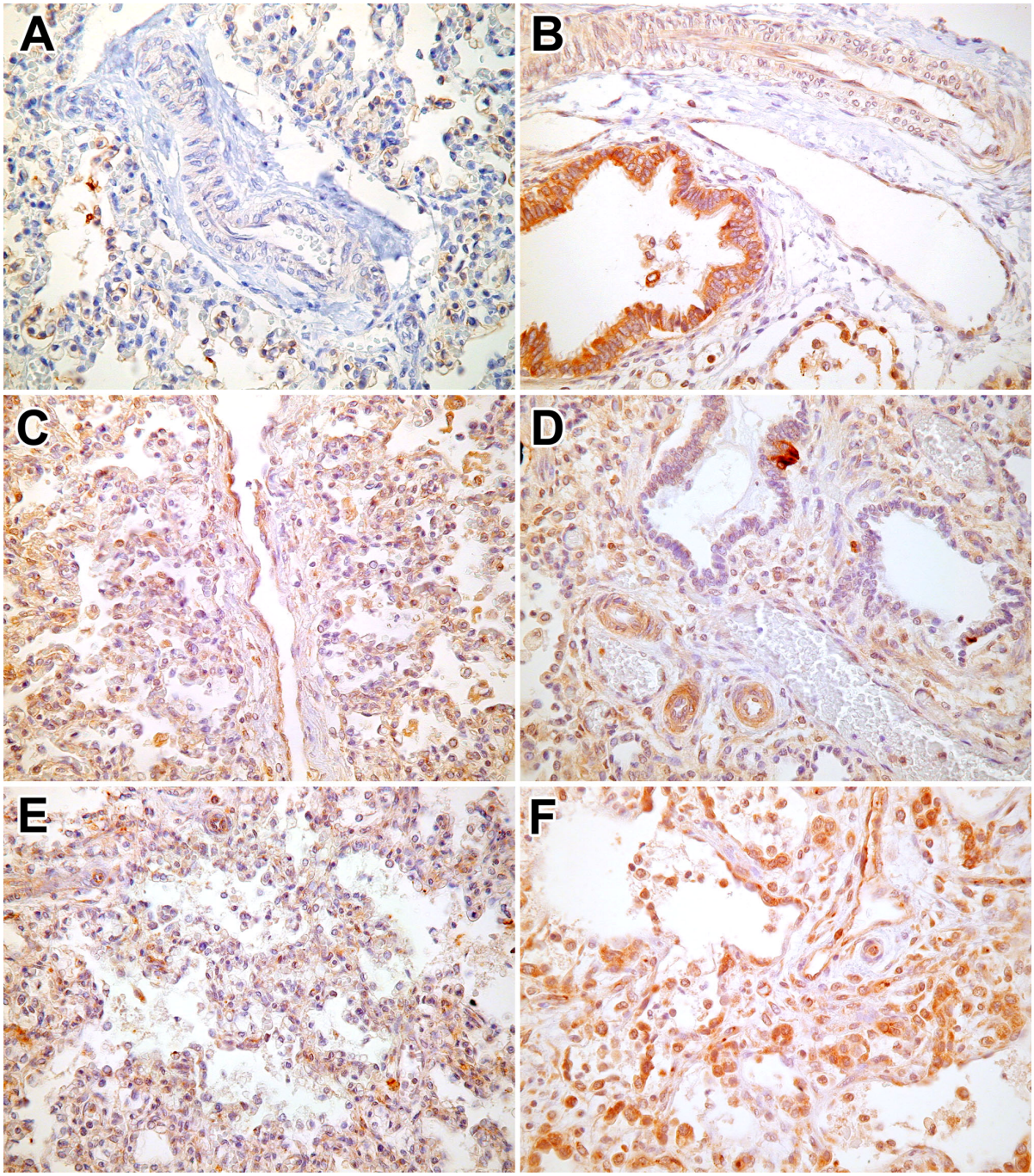
Figure 7.3 eNOS expression in ACD capillaries is slightly elevated compared to controls (* $P < 0.05$)



Expression of eNOS was similar between groups in epithelium, arteries (endothelium, media, and adventitia) and veins. Expression was strong in all epithelial structures, arterial endothelium, arterial media and venous endothelium. Expression was absent in arterial adventitia.

Differences were observed in capillary eNOS expression ($P < 0.05$). ACD cases had more frequently positive staining in their capillaries (*figures 7.3 and 7.4 E, F*).

Figure 7.4 *(for color figures see page 168)*



A, B: pVHL expression is higher in endothelium, medial smooth muscle cells, venous endothelium and capillaries of ACD lungs (B) compared to controls (A).
C, D: HIF-1α expression is higher in veins and capillaries of controls (C) than ACD (D).
E, F: eNOS expression in capillaries is increased in ACD (F) compared to controls (E).

DISCUSSION

ACD with misalignment of pulmonary veins is considered to be a disorder caused by an early disturbance of vascular assembly and possibly of mesenchymal-epithelial interaction.[25] The insufficient blood-air barrier caused by a lack of normal thinning of the alveolar walls and a deficient capillary bed results in hypoxemia that triggers pulmonary hypertension in these patients.[6] Furthermore, because of the striking decrease in acinar capillaries, the blood entering an acinus only drains via the anomalous veins without passing through the capillary bed.[6,12]

In this study we have tried to address the question whether alterations in the HIF-pathway are related to ACD. A striking aspect of the HIF-pathway in ACD is the increase in pVHL expression compared to controls. An increase in pVHL may cause a decrease in HIF-1α, which subsequently leads to a decrease in angiogenic pathway induction. Indeed HIF-1α levels are decreased in veins and capillaries. Thus, the lack of capillaries in the ACD phenotype may result through this decrease in HIF-1α. Alternatively, pVHL is known to have an effect on fibronectin deposition in the interstitium.[26] Therefore elevated pVHL levels could reduce the migration of vascular sprouts, thereby contributing to the growth failure of capillaries.

However the low levels of HIF-1α do not seem to affect VEGF, VEGFR2 and iNOS levels, since these are normal in these lungs. In contrast, eNOS levels are increased in the capillaries of these patients. Endothelial NOS knock out mice have an ACD-like phenotype, since misalignment of pulmonary veins was found in these mice and the capillary bed was not adjacent to the alveoli.[23] The origin of the vascular malformation may lie in a shortage of eNOS during early pulmonary organogenesis. In our patient group however we were unable to find a decrease in eNOS. On the contrary we found an increase in capillary eNOS expression. Expression of eNOS could be upregulated in capillaries in order to dilate the vessels as an attempt to obtain normal oxygen saturation in the blood. It seems that eNOS itself is not deficient in our patients, but that more upstream in the HIF-pathway a cause for ACD may be found, as our patients have low HIF-1α levels and high pVHL levels.

A possible explanation for the normal levels of downstream HIF-pathway molecules could be that the therapy these infants have received (inhaled NO) induced their HIF-pathway through NO mediated VEGF production.[27] Inhaled NO may have normalized VEGF, VEGFR2 and iNOS levels, and it may even have increased eNOS. In our patient group however there were not enough patients to draw conclusions with respect to the influence of inhaled NO on the HIF-pathway. It seems that the effect of inhaled NO in this particular disease is often transient,[4,28] which holds true in our patient group.

In addition, treatment with extracorporeal membrane oxygenation (ECMO) could have an effect on HIF-pathway alterations. ECMO associated pulmonary changes

include non-specific architectural changes such as septal widening with increased reticulin deposition, type II pneumocyte hyperplasia, and intra-alveolar haemorrhage.[29] Reversal of vascular changes to a certain extent is seen in patients with PPHN after ECMO treatment.[30] Unfortunately, it is impossible to exclude a role of ECMO therapy on the amount and origin of the immunohistochemical changes in HIF-pathway molecules observed in our patients, since most of our ACD patients (78%) received this therapy.

We realize that all patients with ACD died under hypoxic circumstances, whereas not all the controls did. The HIF-pathway is greatly influenced by hypoxia and therefore it is of importance to compare the data in our ACD patients with other patients dying under hypoxic circumstances to ensure the differences we found were not simply caused by hypoxia but are characteristic for ACD. We reported earlier that autopsy cases of congenital diaphragmatic hernia (CDH) had an increase in pVHL and a decrease in HIF-1α compared to hypoplastic lungs and control lungs.[24] It seems that CDH and ACD share similarities in HIF-pathway aberrations. Furthermore a difference in age of death between controls and ACD was present. ACD cases lived longer compared to controls. This could have an impact on VEGF expression, since in mice its expression peaks during the canalicular stage, when most of the vessel growth occurs in the lung, then decreases until day 10 postnatal (P10), when it plateaus at adult levels.[31] So in many of our ACD cases a lower level of VEGF is to be expected. However we did not find any differences in VEGF expression.

There is a scarcity of hypotheses on the etiology of ACD. Recently Pasutto et al suggest that etiology may lie in a mutation of STRA6 which is a member of a large group of genes stimulated by retinoic acid.[32] An open lung biopsy in an infant with such a mutation showed a reduced number of alveolar units and pulmonary capillary vessels, however no misalignment of lung vessels was present.[32] Han et al suggested that an eNOS defect may be the cause,[23] but based on our findings we hypothesize that the etiology of ACD lies upstream in the same pathway (pVHL and HIF-1α). Future studies should be directed at acquiring more quantitative data on HIF-pathway related molecules.

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chapter 8

GENERAL DISCUSSION

INTRODUCTION

Pediatricians in particular neonatologists and to a lesser extent pediatric surgeons and cardiologists are faced daily with extremely ill neonates suffering from respiratory failure due to extreme prematurity of the infant, as well as developmental defects. Respiratory insufficiency is an important cause for neonatal death and often leads to long-term morbidity.[1-3]

Pulmonary hypertension of the neonate (PHN) is a common cause for insufficient oxygen supply and occurs in as many as 6.8 of 1000 live births.[4] This "disease" may arise when an abnormal pulmonary vascular response occurs such as delayed vasodilatation at exposure in room air post partum (compared to the fetal environment). PHN can also arise when lungs are exposed to abnormal stimuli such as meconium aspiration durante partum. Other important underlying etiologies include a poorly developed pulmonary vascular bed as seen in congenital diaphragmatic hernia, alveolar capillary dysplasia or hypoplastic lungs due to PROM or a part of the Potter sequence. With regards to the clinical symptomatology PH is characterized by severe hypoxemia and right-to-left shunting through a patent foramen ovale, and/or a patent ductus arteriosus, and through intrapulmonary shunts.[5,6] PHN is characterized by abnormal vascular structure, growth, and/or reactivity with a hitherto unexplained similar morphology consisting of medial hyperplasia and adventitial thickening.

These critically ill children are often treated with a variety of therapeutic modalities all aimed at relieving their hypoxemia. These therapies consist of administration of oxygen through artificial ventilation (either conventional, high frequency oscillation ventilation [HFO] or experimental liquid ventilation), inhaled nitric oxide (iNO), intra-tracheally administered prostacyclin (PGI_2), extracorporeal membrane oxygenation (ECMO), and most recently phosphodiesterase inhibitors [for a review see 7]. In many cases the treatment remains trial and error.

Oxygen therapy combined with mechanical ventilation is one of the main risk factors for the development of so-called bronchopulmonary dysplasia (BPD).[8] The clinical definition of (severe) BPD is oxygen requirement for the first 28 days of life and a need for more than 30% oxygen and continuous positive airway pressure or mechanical ventilation at 36 weeks' postmenstrual age.[9] The histology of BPD lungs shows large, simplified alveolar structures, a dysmorphic capillary configuration, and variable interstitial cellularity and/or fibroproliferation.[10] Even when these children are discharged, their lungs remain their "weak spot". BPD follow-up studies show that readmission to hospitals is high and that lung function before the age of 2 shows abnormalities such as air trapping and high airway resistance.[11] As only the most severe critically ill children are sometimes subjected to ECMO it is not surprising that infants treated with extracorporeal membrane oxygenation (ECMO) for severe respiratory insufficiency had reduced expiratory flow during the first year

of life.[12] These neonatal ECMO survivors experience lung injury lasting into later childhood.[1] Lung dysfunction correlates with the extent and duration of volutrauma and oxygen exposure as neonates.[13] Also diminished lung function prior to neonatal unit discharge was associated with subsequent symptomatic respiratory syncytial virus (RSV) lower respiratory tract infection and respiratory morbidity in prematurely born infants.[2] Even in young adults with a history of premature birth there is evidence of abnormal gas trapping and airway hyper reactivity.[3] This shows that BPD often is accompanied by long term morbidity.

In this thesis the focus lies on the effect hypoxic circumstances have on the developing lung. In the fetal developing lung (*in utero*) the hypoxic circumstances are physiological. Postnatally, hypoxia is rapidly replaced by normal oxygen levels. Therefore it seems that the fluctuation of oxygen is an important contributor to normal and abnormal lung development. Attention in this thesis is specifically directed at the hypoxia-inducible factor (HIF) pathway, its regulators and downstream molecules. HIF-1α is a prominent oxygen-regulated molecule with a major impact on many downstream pathways, therefore studies with the following hypotheses were performed:

1. Expression of an oxygen insensitive HIF-1α *in vivo* leads to accelerated postnatal alveolar development (**chapter 3**)
2. Chemical stabilization of HIF-1α *in vitro* increases embryonic branching morphogenesis and vascular development (**chapter 4**)
3. Components regulating HIF-1α stability are present in the pseudoglandular phase in human lung development, when the primitive pulmonary vascular system develops (**chapter 5**)
4. A final common HIF/VEGF pathway-related problem is present in neonates suffering from PH with different underlying causes (**chapter 6**)
5. HIF/VEGF balance is disturbed in children with alveolar capillary dysplasia (**chapter 7**)

INTERPRETATIONS AND IMPLICATIONS

Studies in murine lung

The effect of expression of an oxygen-insensitive HIF-1α in the embryonal murine lung was tested *in vivo*. The HIF-1α ΔODD construct was placed under the control of the lung-specific SP-C promotor. Culture of transgenic embryonic lungs at E11.5 (early phase of lung development) showed that HIF-1α ΔODD effect was greatest in hypoxic (2% O_2) culture conditions. Vascularization increased, on the other hand branching did not increase. HIF-1α ΔODD lungs cultured in 2% O_2 had the highest density of vessels and a decrease in branching morphogenesis compared to C57 control lungs cultured in 2% O_2. Earlier studies have suggested that early lung branching is stimulated by proper angiogenesis.[14] However, our findings

demonstrate that in this early phase of lung development excess vascularization is not necessarily beneficial for branching morphogenesis.

At E18.5 (just before birth) fluorescent angiography showed no obvious increase in vascularization in the transgenic mice compared to controls. This suggests that also in control mice the HIF/VEGF axis is active and that a state of hypoxia during development is present. Postnatal lung development was also assessed in these transgenic mice. Morphometric analyses, using mean linear intercept, alveolar volume, and secondary crest density of postnatal mice at various stages in alveolar development (postnatal days 2, 8, 14, 21) show an advancement of alveolarization in the HIF-1α ΔODD pups. Moreover, small vessel density was increased in lungs of HIF-1α ΔODD pups at E18.5 and postnatal days 2, 8 and 14. The HIF-1α ΔODD pups had significantly higher VEGF mRNA expression than C57 pups on postnatal days 2, 8, and 14. The continuous activation of the HIF pathway and subsequent up-regulation of VEGF may account for this enhanced vascularization and subsequent alveolar formation. The fact that we did not find any differences in vascularization using fluorescent angiography at E18.5 is likely due to the insensitivity of the technique. On the other hand we were somewhat surprised to see an increase in small vessel density at E18.5 as there is still generalized hypoxia in the lung at that moment, causing the HIF/VEGF pathway to be active even in the control mice. After birth oxygen levels in the lung rapidly rise and vascular differences between our transgenic HIF-1α ΔODD pups and controls becomes apparent. Our results show that HIF-1α over-expression *in vivo* leads to an increase in pulmonary vascularization and enhanced postnatal alveolar development. The clinical application of such data is evident as VEGF gene therapy has been experimentally shown to promote lung angiogenesis and subsequently prevent alveolar damage in cases of hyperoxia induced pulmonary injuries.[15] New questions also arise as to whether alveolarization governs vascularization, or visa versa.

To contemplate new treatment strategies for neonates with respiratory distress it is important to know if chemical stabilization of HIF-1α *in vitro* has a beneficial effect on pulmonary vascular growth and epithelial branching morphogenesis. We first investigated whether inhibition of VEGF/VEGFR2 signalling affected vascular growth and epithelial branching morphogenesis. Incubation of E11.5 mouse lung explants with a VEGFR2 inhibitor (SU5416) completely abolished vascular growth. However, epithelial branching proceeded without vascular development, albeit at a lower rate. This indicates that HIF/VEGF signalling is important for vascularization, but that other downstream targets of the HIF pathway play a role in epithelial branching. Also, it suggests that epithelial branching is not as tightly linked to vascular development in the early phase of lung development as previously thought.[14]

Since low oxygen has been shown to stimulate VEGF expression in murine lung explants, most likely via HIF-1,[14] we investigated whether enhancing HIF-1α stability under normoxic (20% O_2) conditions would promote vascular development

and, thereby, epithelial branching. Chemical stabilization of HIF-1α can be accomplished by adding either cobalt chloride ($CoCl_2$), dimethyloxalylglycine (DMOG), or desferrioxamine (DFO) to culturing media.[16-18] $CoCl_2$ is a chemical hypoxia mimic which induces high expression of HIF-1α.[19] DMOG is a nonspecific PHD inhibitor, it upregulates HIF-α and induces oxygen-regulated gene expression.[17] DFO removes intracellular iron and is known to stabilize HIF-1α.[20] In explants treated with $CoCl_2$ and DFO, the fine vascular network surrounding the developing lung buds disappeared, while the vessels along the trachea, main bronchi and larger airways remained intact. Although VEGF expression was up-regulated, VEGFR2 expression was downregulated by both treatments. It is possible that VEGFR2 downregulation is responsible for the lack of peripheral vascularization since VEGF-VEGFR2 signalling is important for the sprouting of new vessels from existing ones and VEGF is a potent mitogen for endothelial cells [for a review see 21]. Epithelial branching and vascularization were not affected by DMOG treatment. However, DMOG treatment reduced VEGFR2 expression without affecting the peripheral vascularization, implying that VEGFR2 downregulation alone is not sufficient to explain the loss of peripheral vascularization in $CoCl_2$ and DFO treated explants.

None of the treatments affected HIF-1α mRNA expression. Treatments with DFO and $CoCl_2$ increased VEGF mRNA expression, whereas incubation with DMOG had no effect. In contrast, all treatments reduced VEGFR2 mRNA expression.

One can extrapolate from these data that it is not useful to try to enhance HIF-1α expression during early human pulmonary development in a fetus with a known loss in pulmonary development. However it seems that later in development upregulation of the HIF pathway is beneficial for both angiogenesis and alveolarization. More research is needed to determine the best method for therapy (e.g. VEGF gene therapy or chemical stabilization of HIF-1α) in the neonate with pulmonary disease.

Studies in embryonic human lung

We[14] and others[22,23] have reported that HIF-1α plays an important role in mediating oxygen-regulated events of pulmonary vascular development. Therefore, we determined the spatial expression of various components of the HIF system in the human lung during the pseudoglandular stage of lung development (8 - 14 wks), when the primitive pulmonary vascular system develops. During the early phase of pregnancy oxygen supply to the fetus changes dramatically. The intervillous space in the placenta opens to maternal blood after 10 - 12 weeks of gestation and oxygen saturation of blood supplied to the fetus rises from 2 - 3% O_2 to 8 - 10% O_2.[24,25] It has been shown that placental degradation of HIF-1α takes place after 10 weeks of gestation at the time when placental oxygenation increases.[26] This also implies a changed oxygen concentration in the blood going to the fetus. We found a continuous activation of HIF pathway even after opening of the intervillous space in

the placenta. This suggests that the lung remains in a relatively low oxygen environment during its development. This allows for vascular growth in the mesenchyme through downstream HIF effectors such as VEGF and VEGFRs.

Other data from our group show that VHL, HIF-1α, HIF-2α, HIF-3α, VEGF-A and VEGFR2 are expressed in human lung tissue from 13.5 weeks of gestation till birth.[27] Moreover, HIF-2α and VEGF mRNA expression increases with advancing gestation.[27] It seems that the role of HIF-2α increases with gestation and plays an important role in the last phases of pregnancy.

Pulmonary vascular pathology in the newborn period

PHN can occur without identifiable cause (idiopathic or primary PHN) or in association with various diseases e.g. meconium aspiration syndrome (MAS), pulmonary hypoplasia of different causes (premature rupture of membranes (PROM), oligohydramnios sequence, skeletal deformities and congenital diaphragmatic hernia (CDH)). We found differences in expression pattern of HIF-pathway related molecules between the various underlying causes. Despite similar pulmonary morphology there does not seem to be a final common pathway concerning HIF-related angiogenesis. Most likely differences between the various diseases in HIF-pathway related molecules are caused by variability in the moment of onset of the disease. It is generally accepted that CDH is an early developmental aberration,[28] while idiopathic pulmonary hypertension can be the result of an alteration in development which occurs later in gestation preventing the development of a vascular system with low resistance. Severe pulmonary hypertension associated with MAS occurs perinatal.

The major confounders for the interpretation of these results are:

- all material is autopsy based
- all patients died under hypoxic condition due to therapy resistant pulmonary hypertension.
- in some patients ECMO was applied which on its own has a known effect on lung morphology.
- the time between death and autopsy was variable while no open lung biopsies immediately following death were available.
- the effect of hyperoxic stress (inspiratory oxygen fractions were 1.0 in all patients) may have seriously influenced the morphology.

ACD with misalignment of pulmonary veins is considered to be a disorder caused by an early disturbance of vascular assembly and possibly of mesenchymal-epithelial interaction.[29] A striking aspect of the HIF-pathway in ACD is the increase in VHL expression compared to controls. An increase in VHL may cause a decrease in HIF-1α, which subsequently leads to a decrease in angiogenic pathway induction. Alternatively, pVHL is known to have an effect on fibronectin deposition in the interstitium. Therefore elevated pVHL levels could reduce the migration of vascular sprouts, thereby contributing to the growth failure of capillaries. Indeed HIF-1α

levels are decreased in veins and capillaries. However this does not seem to affect VEGF, VEGFR2 and iNOS levels, since these are normal in these lungs.

Our data support the notion that the HIF pathway is important for pulmonary angiogenesis, branching morphogenesis, alveolarization and vascular remodeling in normal pulmonary growth, and also in pulmonary hypertension and alveolar capillary dysplasia.

CLINICAL PERSPECTIVES

Vascular response and induction of angiogenesis is nowadays a hot topic in lung research. This is caused by the fact that aberrations in these systems may lead to devastating lung disease with high mortality and morbidity. Also a growing body of knowledge about lung vascular development, coupled with the fact that alveolarization is strongly associated with pulmonary microvascular development, has shifted scientific focus to the role of vascular development during fetal lung growth and postnatal lung growth.[30]

Oxygen fluctuation clearly plays a major role in pulmonary vascular development and vascular response.[14,31] Coordinated development of the distal epithelium and capillary network is essential to normal lung morphogenesis, which also entails the development of a low resistance pulmonary vascular bed post partum.

In this thesis we demonstrate that in murine lung development HIF plays a pivotal role in both fetal and postnatal lung development. Chemical stabilization of HIF-1α in lung explant cultures does not lead to the same phenotype.

Low oxygen levels during pulmonary organogenesis ensure high levels of HIF-1. This is necessary for normal pulmonary vascular development as murine studies show that HIFα deficiencies lead to early embryonic death (HIF-1α) or fatal neonatal RDS (HIF-2α).[32,33] In the neonatal pulmonary diseases studied, especially pVHL and HIF-1α seem to play a major role.

Hypoxemia is a major problem in patients suffering from neonatal respiratory disease. These infants are often treated with high levels of oxygen. These supranormal levels of oxygen may disrupt the fine-tuning of HIF/VEGF balance and thereby disturb normal angiogenesis and stimulate abnormal vascular remodeling. In control lungs HIF-1α is not degraded in veins and capillaries, suggesting relatively little oxygen leaves the lung ensuring high levels of HIF-1α. Infants treated with high concentrations of oxygen such as our patient groups (PHN and ACD) have low levels of HIF-1α in pulmonary capillaries and veins. The high levels of HIF-1α may be necessary for the normal postnatal disappearance of the double capillary bed and it is thereby important for postnatal alveolarization. This suggests

that for proper postnatal lung development a delicate controlled balance of the HIF/VEGF pathway is necessary. This poses a dilemma for treating these patients as it seems that lung development is benefited by lower levels of oxygen. Of course administering low levels of oxygen to these critically ill infants is not an option although in line with recommendations in neonatal resuscitation modulation of oxygen instead of 100% of oxygen remains an intriguing concept. Administration of oxygen is necessary to alleviate hypoxemia,[34] but it seems that it coincides with (abnormal) HIF-1α degradation in the developing capillary bed. This is potentially not beneficial for the process of the development of a normal low resistance pulmonary vascular bed and may therefore influence alveolarization. We showed that high levels of HIF-1α are beneficial for postnatal alveolarization.

Recent studies suggest that a normal vasculature is necessary for lung epithelial development. Blocking vascular development in fetal mouse lung explants inhibited branching morphogenesis.[14] Similarly, the anti-angiogenic protein EMAPII blocked neovascularization in a fetal lung allograph model and also disrupted epithelial morphogenesis, including differentiation of type II cells.[35] In postnatal rat lung, alveolarization was inhibited by several anti-angiogenic compounds.[36] Because the lung epithelium likely affects capillary morphogenesis through elaboration of angiogenic factors, a potential regulatory role for the vasculature on epithelial development suggests that a complex interplay of epithelial–endothelial cells is required for normal lung morphogenesis.

Disruption of either the epithelium or the vasculature, as might occur in MAS, (P)PH, ACD and BPD, can impair normal lung development. Therefore therapy should not only be directed at the immediate oxygenation but should also be directed at preventing remodeling into the wrong direction and it should include epithelial-mesenchymal interactions. Taking these molecular aspects into account, a number of therapies are under development as shown in *table 8.1*. Apart from inhaled NO and Prostacyclin all other therapies are still in the phase of evaluation as properly designed randomized control trials are not available.

Table 8.1 Vasoactive agents in treatment of PHN and BPD

Therapy	Use in neonates	Clinical significance
NO (37)	Yes	Vasodilation, angiogenesis
NOS (gene therapy) (38)	No	Vasodilation, angiogenesis
eNOS overexpressing stem cells (39)	No	Vasodilation, angiogenesis
VEGF (gene therapy or rhVEGF) (15, 40)	No	Angiogenesis
PHD inhibition (41)	No	Angiogenesis, preventing remodeling
HIF-1α (gene therapy) (41)	No	Angiogenesis, preventing remodeling
PDE-5 inhibitors (4, 42)	Experimental	Vasodilation, preventing remodeling
Prostacyclin (43)	Yes	Vasodilation
ET-1 receptor antagonist (44)	No	Vasodilation, preventing remodeling

Microvascular development consists of endothelial cell differentiation, proliferation, migration, tube formation, branching, vessel remodeling and maturation.[45] VEGF is important for the sprouting of new vascularization from existing ones and it is a potent mitogen for endothelial cells [for a review see 21]. Pharmacologic blockade of VEGFR2, or treatment with a neutralizing antibody to the receptor, resulted in decreased vascular density and impaired alveolar development suggesting that VEGF may also have a major role in alveolarization.[36,46] We also showed that *in vitro* treatment with a VEGF inhibitor resulted in a dramatic decrease in vascularization, however branching did not seem to be affected. VEGF profoundly affects several functional properties of endothelial cells, highly relevant to lung function and pulmonary vascular properties, such as nitric oxide (NO) and prostacyclin synthesis. VEGF activates endothelial cell NO synthase,[47,48] which in turn mediates the pro-angiogenic effects of VEGF.[49] VEGF overexpression through intra-tracheal VEGF gene therapy improved survival in a rat model of BPD and was able to preserve and restore normal alveolarization in this model, even when given late in the disease.[15] Also Kunig et al[40] show that treatment with recombinant human VEGF protein during recovery from hyperoxia improved lung architecture as demonstrated by increased septation and lung complexity with increased surface area and vascular density. Caution should be taken with the clinical application of VEGF therapy since pulmonary edema and hemosiderosis developed postnatally in mice overexpressing VEGF,[50] demonstrating that the balance is delicate. Too much VEGF production and action may lead to pleural effusion[51] and contribute to the increased vascular permeability[52] in acute lung injury.[53,54] This may be caused by the fact that VEGF stimulates NO synthesis and NO mediates the permeability effects of VEGF.[55] However, administering inhaled NO in premature infants who are at risk for developing BPD improves their pulmonary outcome.[37] Increasing NO production by administering mesenchymal cells overexpressing eNOS alleviates right ventricle hypertrophy in a pulmonary hypertension rat model.[39]

Another method for stimulating VEGF-dependent angiogenesis is activation of HIFs by inhibition of prolyl hydroxylase domain-containing proteins (PHDs). This is shown to improve oxygenation and lung compliance and this decreased pulmonary vascular resistance in premature baboons.[41]

Other new treatments like PDE-5 (phosphodiesterase) inhibitors become increasingly popular. Baquero et al show that in infants with severe PPHN Sildenafil (Viagra®) ameliorates oxygenation indices and SpO_2.[4] Although attempts have been undertaken to release intravenous Sildenafil, recently randomized control trials were stopped as not enough safety data are at present available for its intravenous use in critically ill newborns. In our observations based on over 40 patients treated with ECMO sildenafil in combination with inhaled nitric oxide reduced ECMO flow.[42] Intravenous prostacyclin (Epoprostenol, Flolan®) has been used for the treatment of pulmonary hypertension. This drug however had systemic side effects and children often needed volume correction and inotropic support.[43] As an alternative

endotracheal administration of prostacyclin protected from systemic side effects and was effective in neonates with PPHN[56] although again properly designed RCT's are not available in this particular age group.

The dual endothelial receptor antagonist Bosentan (Tracleer®) diminishes or abrogates endothelin induced smooth muscle cell contraction, hypertrophy and hyperplasia, and fibrosis and reduces both the hemodynamic and structural response to experimentally induced PH.[57,58] Bosentan improved clinical condition in children with idiopathic pulmonary hypertension although 60% needed additional intravenous Epoprostenol.[44] There is almost no experience with the use of Bosentan in newborns while appropriate pharmacodynamic parameters are hard to determine.

A major disadvantage of both Sildenafil and Bosentan is that absorption and distribution in their oral form is not investigated in newborns. Proper PK/PD (pharmacokinetic/pharmacodynamic) studies are not available in this particular age group.

In the future new treatment strategies such as PHD inhibitors and other HIF-1 inducers can be developed as angiogenesis inducers. Treatment with molecules downstream in this pathway, such as VEGF and NO should also be considered. These forms of therapy are all aimed at improving vascularization which is beneficial for alveolarization. The appropriate application form is another subject of ongoing debate and research.

CONCLUDING REMARKS

In this thesis we show that HIF-1 is a crucial molecule in the developing lung and that induction of the HIF/VEGF pathway may be beneficial for lung development. Overexpression of oxygen-insensitive HIF-1α in the embryonal murine lung leads to an increase in vascularization but not to an increase in branching morphogenesis. However, chemical stabilization of HIF-1α *in vitro* did not lead to a similar increase in vascularization, in contrast it decreased vascularization. However, over-expression of oxygen-insensitive HIF-1α in transgenic mice had an accelerating effect on postnatal alveolarization. Furthermore, we showed that in human lung HIF regulatory mechanisms are already in effect from week 8 of gestation and that these mechanisms do not diminish after the physiological rise in oxygen at week 10. In patients with PHN due to different causes we found no final common HIF/VEGF pathway. Differences between the various diseases associated with PHN where mainly found in HIF-1α and VHL expression. Most likely the cause lies in the different moments of onset of the various diseases. The main differences found in ACD patients compared to controls were also in expression of HIF-1α and VHL. Especially HIF-1 and VHL seem to play a major role in PHN and ACD. Potentially

these patients have mutations in these genes, therefore genetic analysis of these patient groups is a logical next step.

Interaction between basic and clinical research will continue to play an essential role in the field of lung development research. In addition the clinical implementation of basic research is an important aspect that should not be overlooked. Knowledge of basic pathways such as described in this thesis may aid in future improvement of treatment of congenital lung anomalies such as CDH as well as acquired diseases as BPD.

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chapter 9

SUMMARY

SAMENVATTING

SUMMARY

Chapter 1 is a general introduction to normal and abnormal lung development. It outlines the current state of knowledge of the molecular basis of normal fetal lung development and then discusses the importance of these molecules in aberrant human lung development. It highlights the most important growth and transcription factors in each phase of lung development and describes where neonatal disease may arise and deals with the possible culprits.

In **chapter 2** an outline of the thesis is given. The role of HIF during basic murine lung development and the impact of the HIF pathway in the etiology of clinical diseases such as PPHN and ACD are important for understanding the effect HIF has on angiogenesis and pulmonary development. With these new insights innovative treatment strategies may be contemplated.

Vessel formation in the lung is crucial for normal lung branching in the early embryonic phase as well as in the alveolar phase of lung development. In **chapter 3** we demonstrate that in early fetal lung development over-expression of oxygen insensitive HIF-1α (HIF-1α ΔODD) in mice leads to increased vascularization. This increase in vasculature seen in transgenic mice did not have an impact on branching. Low oxygen stimulated vessel growth in control and transgenic lungs as shown before. Pulmonary morphometric analyses of postnatal mice at various stages in alveolar development (postnatal days 2, 8, 14, 21) show an advancement of alveolarization in the HIF-1α ΔODD lungs. Moreover, small vessel density was increased in HIF-1α ΔODD lungs at E18.5 and postnatal days 2, 8 and 14. The HIF-1α ΔODD lungs had significantly higher VEGF mRNA expression than C57 control lungs on postnatal days 2, 8, and 14. The continuous activation of the HIF pathway and subsequent up-regulation of VEGF may account for this enhanced vascularization and subsequent alveolar formation.

As shown in **chapter 3** induction of HIF-1 and downstream molecules, such as vascular endothelial cell growth factor (VEGF), plays a major role in proper angiogenesis and branching morphogenesis in the developing prenatal and postnatal lung. Therefore, in **chapter 4** a study is presented describing *in vitro* chemical stabilization of HIF-1α. This is accomplished by adding either cobalt chloride ($CoCl_2$), dimethyloxalylglycine (DMOG), or desferrioxamine (DFO) to embryonic murine lungs in culture. In explants treated with $CoCl_2$ and DFO, the fine vascular network surrounding the developing lung buds disappeared, while the vessels along the trachea, main bronchi and larger airways remained intact. Although VEGF expression was up-regulated, VEGFR2 expression was downregulated by both treatments. Epithelial branching and vascularization were not affected by DMOG treatment. DMOG treatment also reduced VEGFR2 expression without affecting the peripheral vascularization, implying that VEGFR2 downregulation alone is not sufficient to explain the loss of peripheral

vascularization in $CoCl_2$ and DFO treated explants. The up-regulation of VEGF mRNA in the lung explants by both $CoCl_2$ and DFO treatments suggest stabilization of HIF-α, however, the increase in VEGF expression did not lead to improved vascularization. It is possible that the hypoxia was too extensive and led to cell death.

HIF-1α plays an important role in mediating oxygen-regulated events of pulmonary vascular development. However, the components regulating HIF-1α stability in the developing lung have remained uninvestigated so far. In **chapter 5**, we determined the spatial expression of various components of the HIF system in the human lung during the pseudoglandular stage of lung development (8 - 14 wks), when the primitive pulmonary vascular system develops. During the early phase of pregnancy oxygen supply to the fetus changes dramatically. The intervillous space in the placenta opens to maternal blood after 10 - 12 weeks of gestation and oxygen saturation of blood supplied to the fetus rises from 2 - 3% to 8 - 10%. We found that HIF regulatory mechanisms are already in effect from week 8 of gestation. Expression of HIF-1α did not decrease after week 10, and expression of molecules involved in degradation of HIF-1α did not increase. There was no obvious decrease in VEGF expression after the rise in fetal oxygen saturation. It could be that relatively oxygen rich blood is shunted away from the lung, through the open foramen ovale and the ductus arteriosus, due to the high pulmonary vascular resistance. This mechanism ensures a low oxygen environment in the developing lung, providing a good basis for angiogenesis.

Against the background of the developmental biological aspects of the HIF-1 pathway we evaluated, in both **chapter 6** and **chapter 7**, the expression of HIF-1α, VHL, VEGF, VEGFR2, eNOS, and iNOS in neonates dying from respiratory disease complicated or triggered by severe pulmonary hypertension.

In **chapter 6** we demonstrate a disturbed expression of the abovementioned proteins in patients with fatal persistent pulmonary hypertension of the neonate (PPHN). Especially pVHL and HIF-1α seem to play a major role. The differences we found between the various diseases suggest that these diseases are completely different entities, although vascular morphology in the end remains the same. Most likely the cause of the differences between the various diseases in HIF pathway related molecules lies in the different moments of onset. Our data support the notion that the HIF pathway is of importance for pulmonary angiogenesis and vascular remodelling in both normal pulmonary growth and in case of pulmonary hypertension.

Chapter 7 reveals the expression of the abovementioned proteins in a rare lethal pulmonary disease, Alveolar Capillary Dysplasia (ACD). Pulmonary veins share an adventitial sheath with the arteries in the bronchovascular bundle instead of their normal intra-acinar course away from the arterial branches and there is a decrease

in the number of alveoli and capillaries, which often are abnormally dilated. ACD is considered to be a disorder caused by an early disturbance of vascular assembly and possibly of mesenchymal-epithelial interaction. We show that HIF pathway related angiogenesis is disturbed in ACD. A striking aspect of the HIF pathway in ACD is the increase in pVHL expression with decreased HIF-1α levels. Other studies suggest that a defective endothelial nitric oxide synthase is the culprit for ACD, while our results suggest that the etiology of ACD lies upstream in the same pathway (pVHL and HIF-1α).

Taken together, the studies presented in this thesis, have delivered the following new insights into normal and abnormal lung development:

- Embryonal effects of over-expression of non-degradable HIF-1α in the lung are an increase in vascularization with a decrease in branching. Postnatal over-expression results into accelerated lung maturation shown by advancement in alveolarization.
- Chemical stabilization of HIF-1α *in vitro* in early embryonic lungs does not lead to an increase in vascularization.
- HIF regulatory mechanisms are already in effect from week 8 of gestation and are still operational after the increase in oxygen after opening of the intervillous space in the placenta at week 10.
- HIF pathway changes in patients with different causes of PPHN are not similar, although vascular morphology is the same.
- The cause for ACD may be sought upstream in the same pathway (HIF-1α, VHL) than researchers previously thought (eNOS).

SAMENVATTING

Een algemene inleiding tot normale en abnormale longontwikkeling wordt gegeven in **hoofdstuk 1**. Het vat de huidige kennis samen op het gebied van de moleculaire basis voor normale foetale longontwikkeling en vervolgens wordt de rol van deze moleculen besproken in afwijkende humane longontwikkeling. Het benadrukt de belangrijkste groei- en transcriptiefactoren in elke fase van de longontwikkeling en beschrijft waar neonatale ziektes kunnen opdoemen. Verder beschrijft het de mogelijke veroorzakers van neonatale ziektes.

Hoofdstuk 2 geeft kort de inhoud van het proefschrift weer. De rol van Hypoxia Inducible Factor (HIF) in basale longontwikkeling bij de muis en de invloed van de HIF-pathway in de etiologie van klinische ziektebeelden, zoals persisterende pulmonale hypertensie van de neonaat (PPHN) en Alveolaire Capillaire Dysplasie (ACD), zijn belangrijk voor het begrijpen van het effect dat HIF heeft op angiogenese en longontwikkeling. Met deze nieuwe inzichten kan over innovatieve behandelstrategieën worden nagedacht.

Vaatvorming in de long is essentieel voor een normale vertakking van de long in de vroege embryonale fase, maar ook in de alveolaire fase van longontwikkeling. In **hoofdstuk 3** tonen wij aan dat in vroege longontwikkeling overexpressie van zuurstofongevoelige HIF-1α (HIF-1α ΔODD) bij muizen leidt tot een vermeerdering van vaatvorming. Deze vermeerdering van het vaatbed in de transgene muizen heeft geen invloed op de vertakking van de long. Lage zuurstofconcentraties stimuleerden vaatgroei in controle- en transgene longen zoals eerder aangetoond. Morphometrische analyse van de long van postnatale muizen op verschillende momenten in alveolaire ontwikkeling (postnatale dagen 2, 8, 14, 21) laten een versnelling van alveolarisatie zien in de HIF-1α ΔODD longen. Verder was de dichtheid van kleine vaten verhoogd in HIF-1α ΔODD longen op E18.5 en postnatale dagen 2, 8 en 14. De HIF-1α ΔODD longen hadden een significant hogere VEGF mRNA-expressie dan C57 controle longen op postnatale dagen 2, 8 en 14. De continue activatie van de HIF-pathway en de opeenvolgende verhoging van VEGF zou de oorzaak kunnen zijn van de vermeerdering in vaatvorming en alveolus formatie.

Zoals getoond in **hoofdstuk 3** spelen inductie van HIF-1 en downstream moleculen, zoals VEGF, een grote rol in normale angiogenese en vertakking in de prenatale en postnatale long. Om die reden wordt in **hoofdstuk 4** een studie gepresenteerd waarin de *in vitro*-stabilisatie van HIF-1α wordt beschreven. Deze stabilisatie wordt bewerkstelligd door het toevoegen van cobaltchloride ($CoCl_2$), dimethyloxalylglycine (DMOG) of desferrioxamine (DFO) aan embryonale muizenlongen in kweek. In explants behandeld met $CoCl_2$ en DFO verdween het fijne vasculaire netwerk om de longknoppen, terwijl de bloedvaten rond de trachea, de bronchi en de grote luchtwegen intact bleven. Ondanks de stijging van de VEGF-expressie bleek dat de

VEGFR2-expressie daalde door beide behandelingen. Epitheliale vertakkingen en vascularisatie werden niet door de DMOG-behandeling beïnvloed. Behandeling met DMOG verminderde ook de VEGFR2-expressie zonder dat het de perifere vaatvorming beïnvloedde. Dit impliceert dat VEGFR2-vermindering alleen niet voldoende is om het verlies van perifere vaatvorming in met $CoCl_2$ en DFO behandelde explants te verklaren. De verhoging van VEGF mRNA in de met $CoCl_2$ en DFO behandelde explants suggereert stabilisatie van HIF-α, alhoewel de verhoging in VEGF-expressie niet tot verbeterde vascularisatie leidde. Het is mogelijk dat de hypoxie te uitgebreid was en heeft geleid tot celdood.

HIF-1α speelt een belangrijke rol in het mediëren van zuurstofgereguleerde gebeurtenissen in de pulmonale vasculaire ontwikkeling. Echter, de componenten die HIF-1α-stabiliteit in de zich ontwikkelende long reguleren zijn tot op heden niet onderzocht. In **hoofdstuk 5** bepaalden wij de ruimtelijke expressie van de verscheidene componenten van het HIF-systeem in de humane long tijdens de pseudoglandulaire fase van longontwikkeling (8 – 14 weken), wanneer het primitieve pulmonale vasculaire systeem zich ontwikkelt. Tijdens de vroege fase van de zwangerschap verandert de zuurstoftoevoer naar de foetus. De intervilleuze ruimte in de placenta opent zich voor maternaal bloed na 10 tot 12 weken en de zuurstofsaturatie van het bloed dat naar de foetus gaat stijgt van 2 - 3% naar 8 - 10%. Wij merkten dat de regulatoire mechanismen van HIF al vanaf week 8 van de zwangerschap aanwezig zijn. Expressie van HIF-1α verminderde niet na de 10de week en expressie van de moleculen die betrokken zijn bij de afbraak van HIF-1α steeg niet. Er was geen opvallende daling in VEGF-expressie na de stijging van de foetale zuurstofsaturatie. Het zou kunnen dat het relatieve zuurstofrijke bloed niet door de long geleid wordt, maar dat het, vanwege de hoge pulmonale vaatweerstand, door het open foramen ovale en de ductus arteriosus gaat. Dit mechanisme verzekert de zich ontwikkelende long van een omgeving met lage zuurstof welke een goede basis is voor angiogenese.

Tegen de achtergrond van de ontwikkelingsbiologische aspecten van de HIF-1-pathway, hebben wij in **hoofdstuk 6** en **hoofdstuk 7**, de expressie van HIF-1α, VHL, VEGF, VEGFR2, eNOS, en iNOS onderzocht bij neonaten die overleden zijn aan een longziekte die gecompliceerd of getriggered werd door ernstige pulmonale hypertensie.

In **hoofdstuk 6** laten we zien dat er een verstoorde expressie is van bovengenoemde eiwitten bij patiënten met fatale persisterende pulmonale hypertensie van de neonaat (PPHN). Vooral pVHL en HIF-1α lijken een grote rol te spelen. De verschillen die we vonden tussen de verschillende ziektebeelden suggereert dat deze ziektebeelden totaal verschillende entiteiten zijn, hoewel de vasculaire morfologie uiteindelijk toch hetzelfde blijft. De oorzaak van de verschillen tussen de verschillende ziektebeelden ligt het meest waarschijnlijk in het moment waarop deze ziektebeelden ontstaan. Onze data ondersteunen het concept dat de

HIF-pathway van belang is voor pulmonale angiogenese en vasculaire reorganisatie in zowel normale longgroei als in het geval van pulmonale hypertensie

Hoofdstuk 7 onthult de expressie van de bovengenoemde eiwitten bij een zeer zeldzame dodelijke longziekte, Alveolaire Capillaire Dysplasie (ACD). Pulmonale venen delen een adventitia-omhulling met de arteriën in de bronchovasculaire bundel in plaats van hun normale intra-acinaire locatie, weg van de arteriële takken en er is een vermindering in aantal van de alveoli en capillairen, die vaak abnormaal verwijd zijn. ACD wordt beschouwd als een ziekte veroorzaakt door een vroege verstoring van de vaatsamenstelling en mogelijk van de mesenchymale-epitheliale interactie. Wij laten zien dat de HIF-pathway gerelateerde angiogenese verstoord is bij ACD. Een opvallend aspect van de HIF-pathway bij ACD is de verhoging in pVHL-expressie met verminderde HIF-1α-niveaus. Andere studies suggereren dat een ondeugdelijke endothelial nitric oxide synthase de veroorzaker van ACD is, terwijl onze studie suggereert dat de etiologie van ACD hoger in dezelfde pathway ligt (pVHL en HIF-1α).

Samengevat heeft het onderzoek, gepresenteerd in dit proefschrift, tot de volgende nieuwe inzichten in normale en abnormale longontwikkeling geleid:

- Embryonale effecten van overexpressie van niet-afbreekbaar HIF-1α in de long zijn: een vermeerdering in vascularisatie met een vermindering in vertakking. Postnatale overexpressie resulteert in een versnelde longrijping, aangetoond door een versnelling van alveolarisatie.
- Chemische stabilisatie van HIF-1α *in vitro* in vroege embryonale longen leidt niet tot een vermeerdering in vascularisatie.
- HIF-regulatoire mechanismen zijn al vanaf de 8ste week van de zwangerschap aanwezig en werken zelfs na verhoging van zuurstof, door het openen van de intervilleuze ruimte in de placenta in week 10.
- HIF-pathway veranderingen bij patiënten met verschillende oorzaken van PPHN zijn niet gelijk, terwijl de vaatmorfologie uiteindelijk hetzelfde is.
- De oorzaak van ACD kan wellicht hoger in dezelfde pathway (HIF-1α, VHL) worden gezocht dan waar de onderzoekers eerst dachten (eNOS).

APPENDICES

list of publications

Groenman FA, Selvaratnam J, Wang J, Tseu I, Huang Z, Caniggia I, Luo D, van Tuyl M, Tibboel D, Post M. Epithelial expression of constitutive active HIF-1α stimulates postnatal lung development. Submitted.

Groenman FA, de Krijger RR, Hammond S, Michalsky MP, van Heijst A, Hulsbergen-van de Kaa CA, Rottier R, Tibboel D. Alveolar Capillary Dysplasia; angiogenesis gone awry. Submitted.

Groenman FA, Wang J, Caniggia I, Tibboel D, Post M. Chemical stabilization of hypoxia-inducible factor does not lead to a hypervascular lung in early development. Provisionally accepted, Am J Physiol Lung Cell Mol Physiol.

Groenman FA, van Heijst A, de Rooij JD, Hulsbergen-van de Kaa CA, Rottier R, de Krijger RR, Tibboel D. Pulmonary hypertension of the newborn: Different causes of onset, same final common pathway? Submitted.

Groenman FA, Rutter M, Caniggia I, Tibboel D, Post M. Hypoxia Inducible Factors in the first trimester human lung. J Histochem Cytochem 55: 355-363, 2007.

van Tuyl M, Groenman FA, Wang J, Kuliszewski M, Liu J, Tibboel D, Post M. Angiogenic factors stimulate tubular branching morphogenesis of sonic hedgehog deficient lungs. Dev Biol 303: 514-526. 2007.

van Tuyl M, Liu J, Groenman FA, Ridsdale R, Han RN, Venkatesh V, Tibboel D, Post M. Iroquois genes influence proximo-distal morphogenesis during rat lung development. Am J Physiol Lung Cell Mol Physiol 290: L777-L789, 2006.

Michalsky MP, Arca MJ, Groenman FA, Hammond S, Tibboel D, Caniano DA. Alveolar Capillary Dysplasia: a logical approach to a fatal disease. J Pediatr Surg 40: 1100-5, 2005.

Groenman FA, Unger S, Post M. The Molecular Basis for Abnormal Human Lung Development. Biol Neonate 87: 164-77. Epub 2004 Dec 9. Review, 2005.

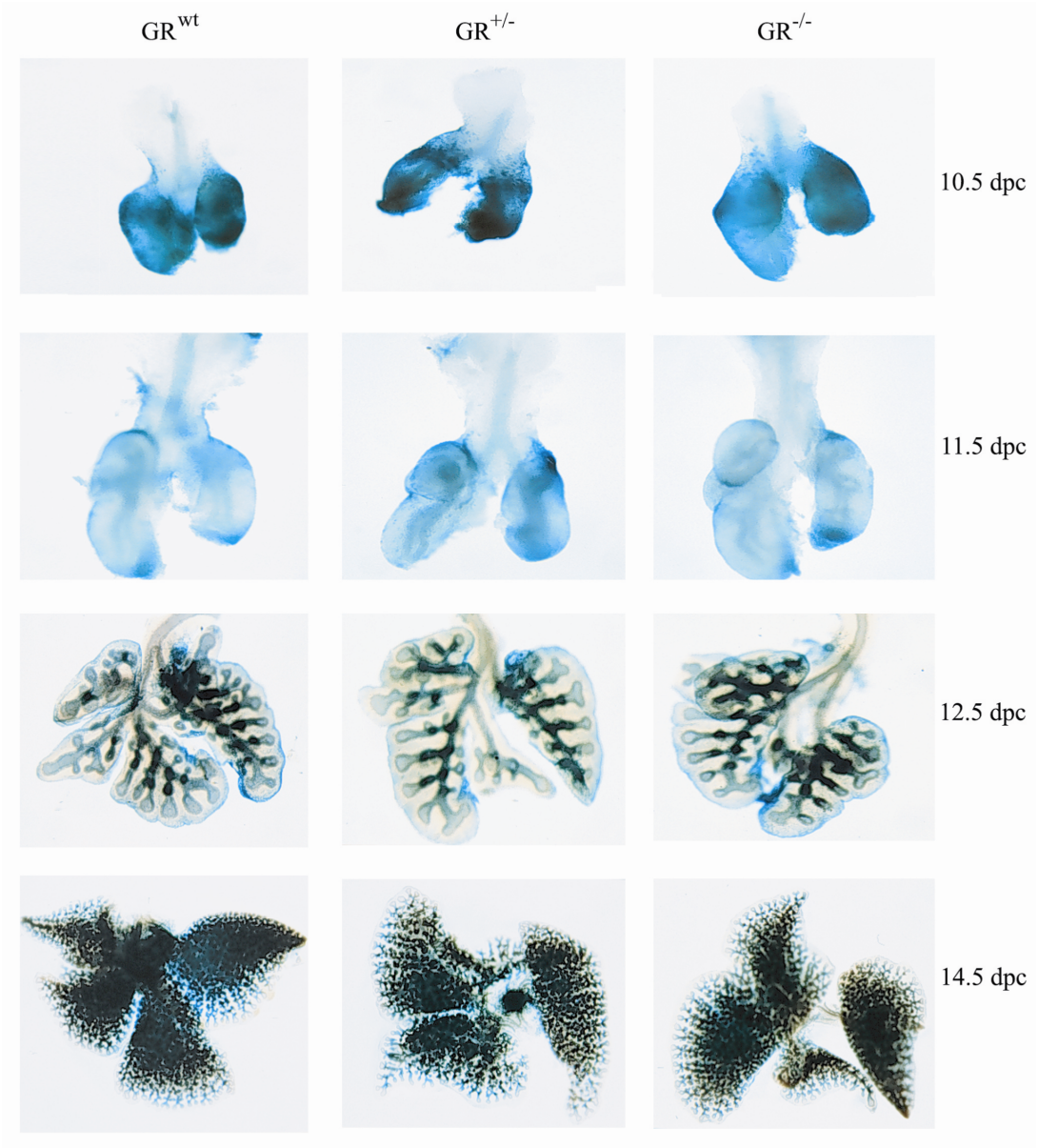
van Tuyl M, Groenman FA, Kuliszewski M, Ridsdale R, Wang J, Tibboel D, Post M. Overexpression of Lunatic Fringe Does Not Affect Epithelial Cell Differentiation in the Developing Mouse Lung. Am J Physiol Lung Cell Mol Physiol, 2004.

de Rooij JD, Hosgor M, Ijzendoorn Y, Rottier R, Groenman FA, Tibboel D, de Krijger RR. Expression of angiogenesis-related factors in lungs of patients with congenital diaphragmatic hernia and pulmonary hypoplasia of other causes. Pediatr Dev Pathol 7: 468-77, 2004.

van Heijst A, Haasdijk R, Groenman FA, van der Staak F, Hulsbergen-van de Kaa C, de Krijger R, Tibboel D. Morphometric analysis of the lung vasculature after extracorporeal membrane oxygenation treatment for pulmonary hypertension in newborns. Virchows Arch 445: 36-44, 2004.

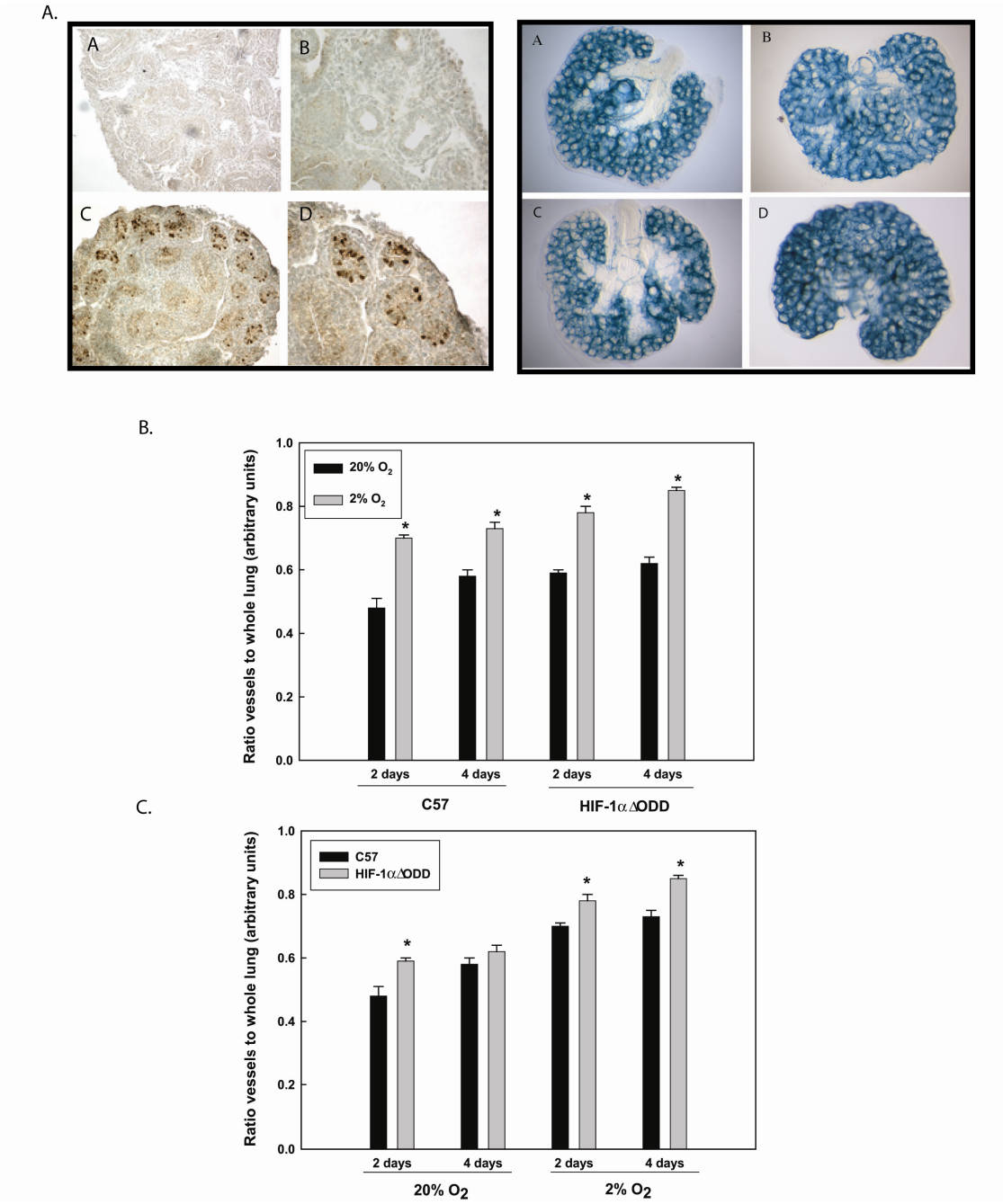
color figures

Figure 1.2 (*see page 10)* Normal early lung branching in GR knockout mice



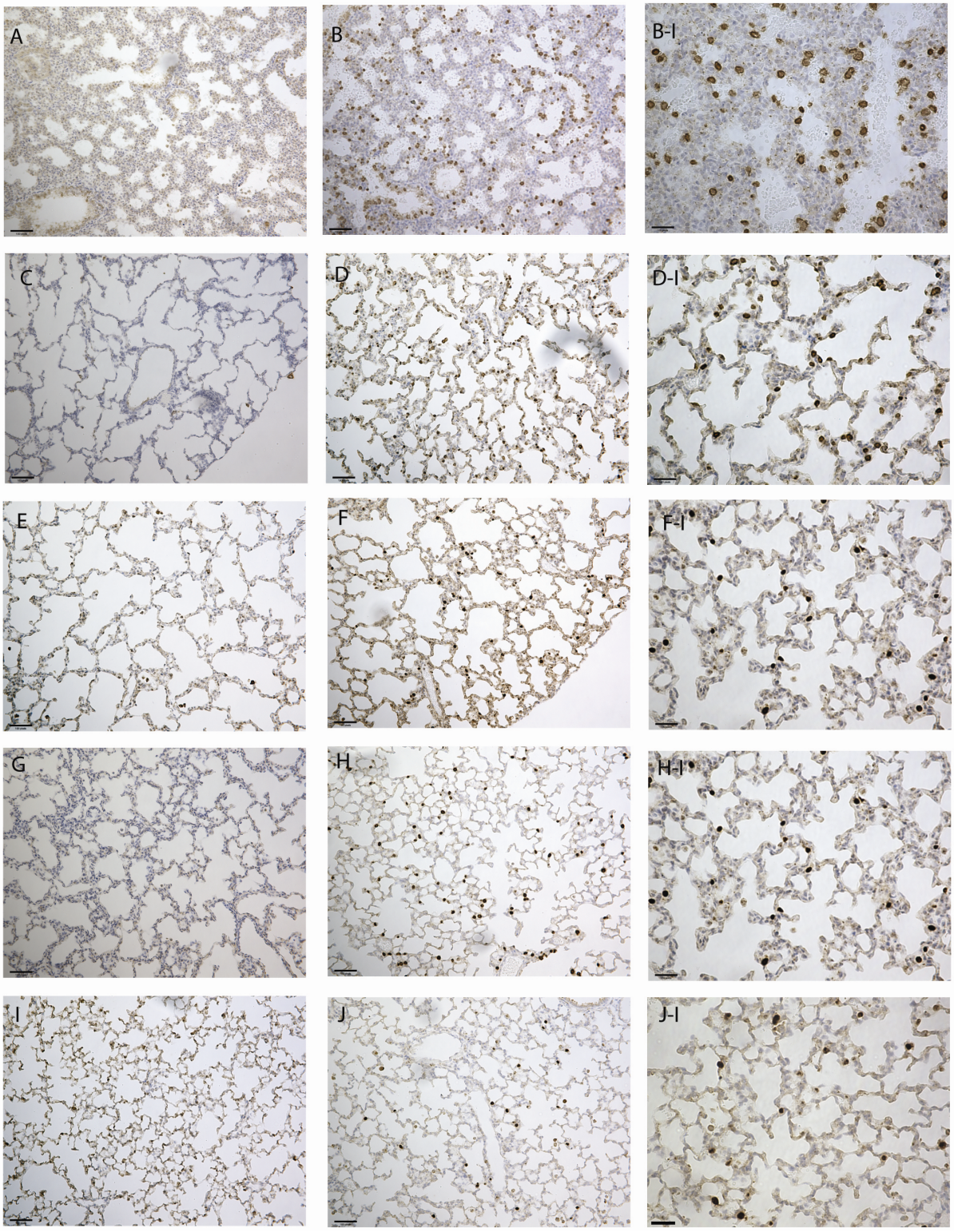
GR mice were crossed with cordon blue(C101)-LacZ transgenic mice, which displays LacZ gene expression specifically in epithelial cells. X-gal staining of lungs of GR/C101-LacZ mice at E10.5–14.5 visualized the complex branching pattern which was not affected by loss of GR signaling.
GR^{wt} *Wildtype mice*
GR+/– *heterozygous mice*
GR–/– *GR null mice*

Figure 3.1 *(see page 44)*



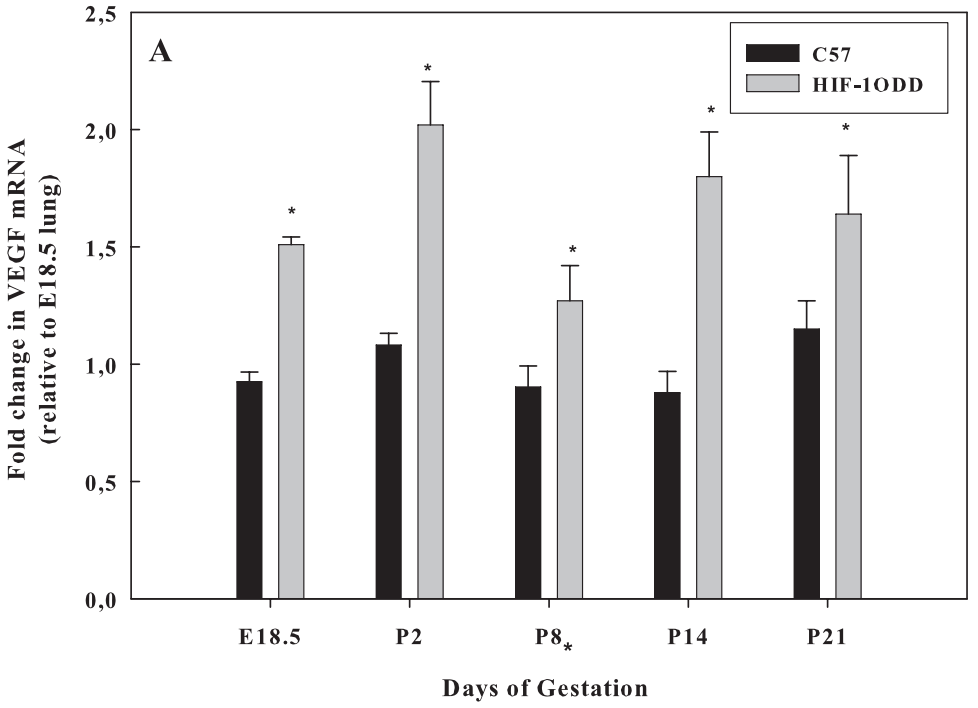
*Effect of oxygen on vessel growth in lung explants (**A-left panel:** HIF-1α ΔODD was constitutively expressed independent of oxygen concentration (2 vs. 20%) as demonstrated by immunostaining using HIF-1α antibody (A-B: immunoreactivity in C57 lung explants kept at 20% O_2; C-D: immunoreactivity in HIF-1α ΔODD lung explants kept at 20% O_2. Same staining pattern was observed at 2% O_2. .A-C: Bar = 100 μm; B-D: Bar = 25 μm). (**A-C**) Low oxygen enhanced vessel growth in C57 and HIF-1α ΔODD lungs, (**A-right panel**: (A) C57 20% O_2, (B) C57 2% O_2, (C) HIF-1α ΔODD 20% O_2, (D) HIF-1α ΔODD 2% O_2. All explants were cultured for 4 days). (**B**) Quantitative measurement of vascular density showed an increase in vascularization in both C57 and HIF-1α ΔODD lungs when kept at 2% O_2 compared to explants culture at 20% O_2. (**C**) Vessel density was greater in HIF-1α ΔODD lungs independent of O_2 concentration. Values are means ± s.e.m. for 10 lungs in each group. *P < 0.05.*

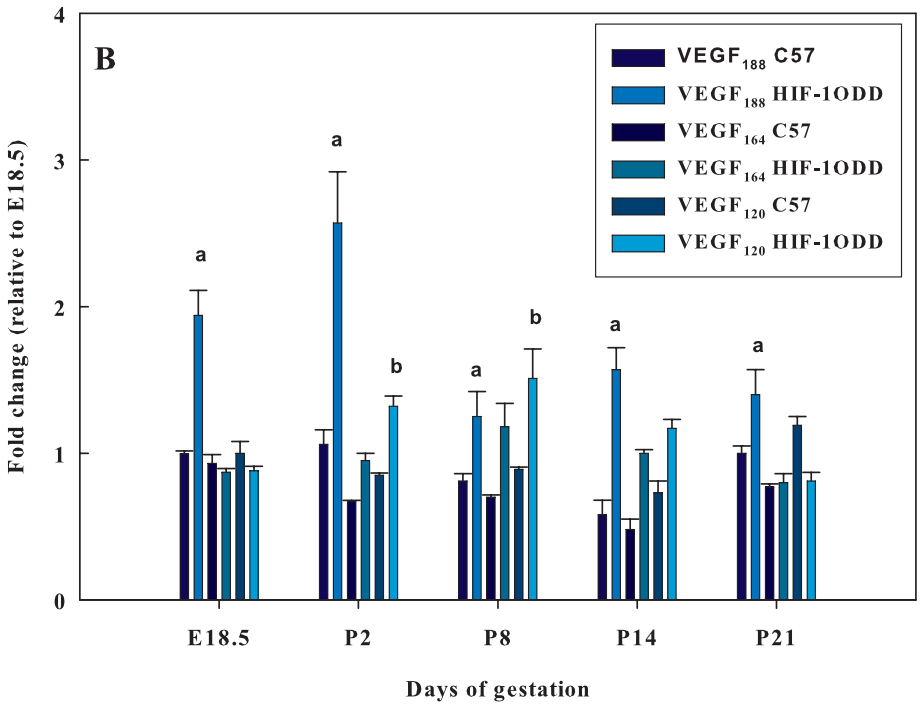
Figure 3.4 *(see page 48)* Immunohistochemical analysis of HIF-1α in C57 control and HIF-1α ΔODD lungs



Strong positive brownish staining for HIF-1α is noted in the nuclei of distal airway epithelial type II cells of HIF-1α ΔODD mice (B, B-I, D, D-I, F, F-I, H, H-I, J, J-I), but not C57 control mice (A,C,E,G,I) during all postnatal ages (E18.5: A,B,B-I; P2: C, D, D-I; P8: E, F, F-I; P14: G, H, H-I; P21: I, J, J-I. Bar: 200 μm (A-J); 50 μm (B-I, D-I, F-I, H-I, J-I).

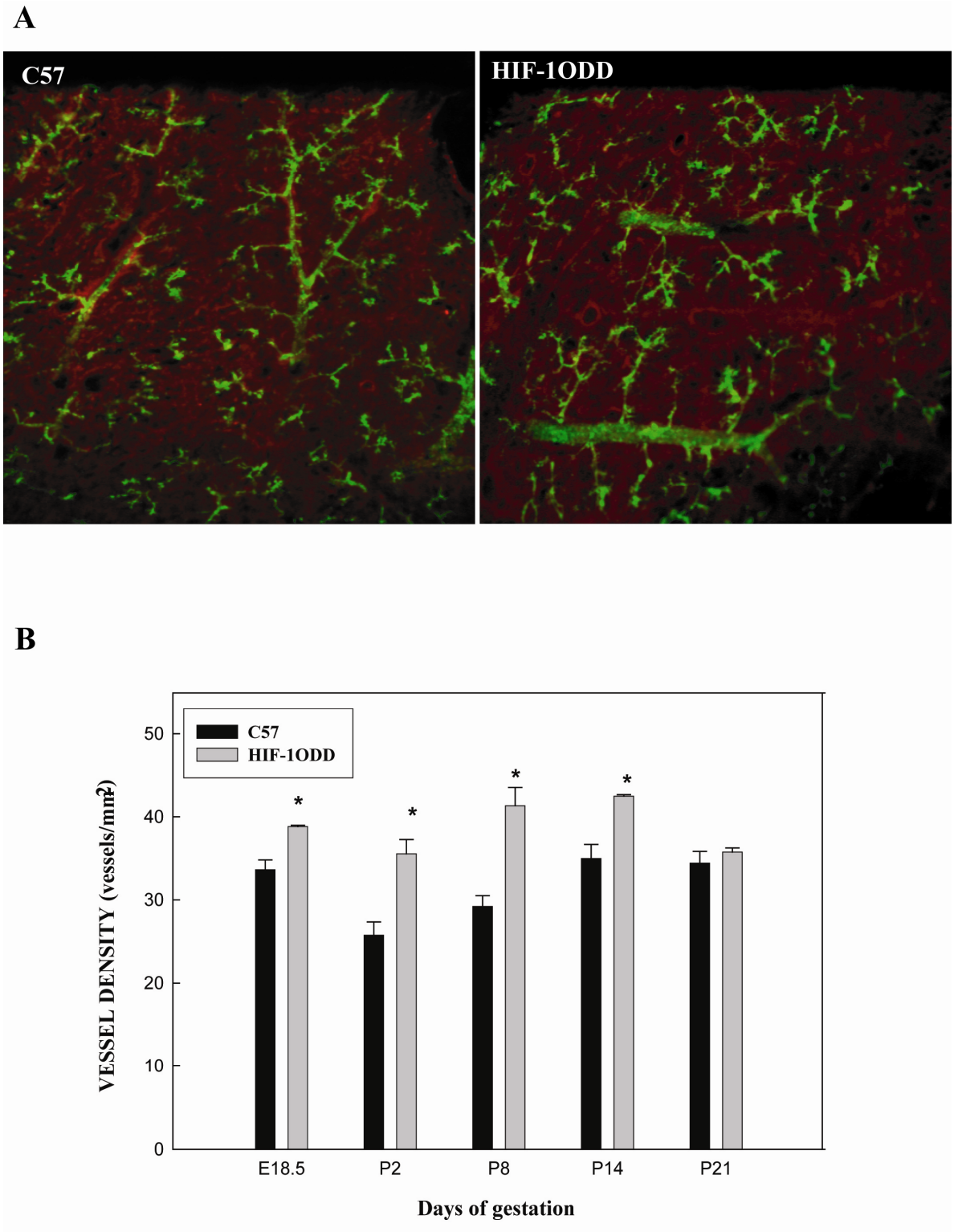
Figure 3.5 *(see page 49)* VEGF gene expression in C57 control and HIF-1α ΔODD lungs during postnatal development





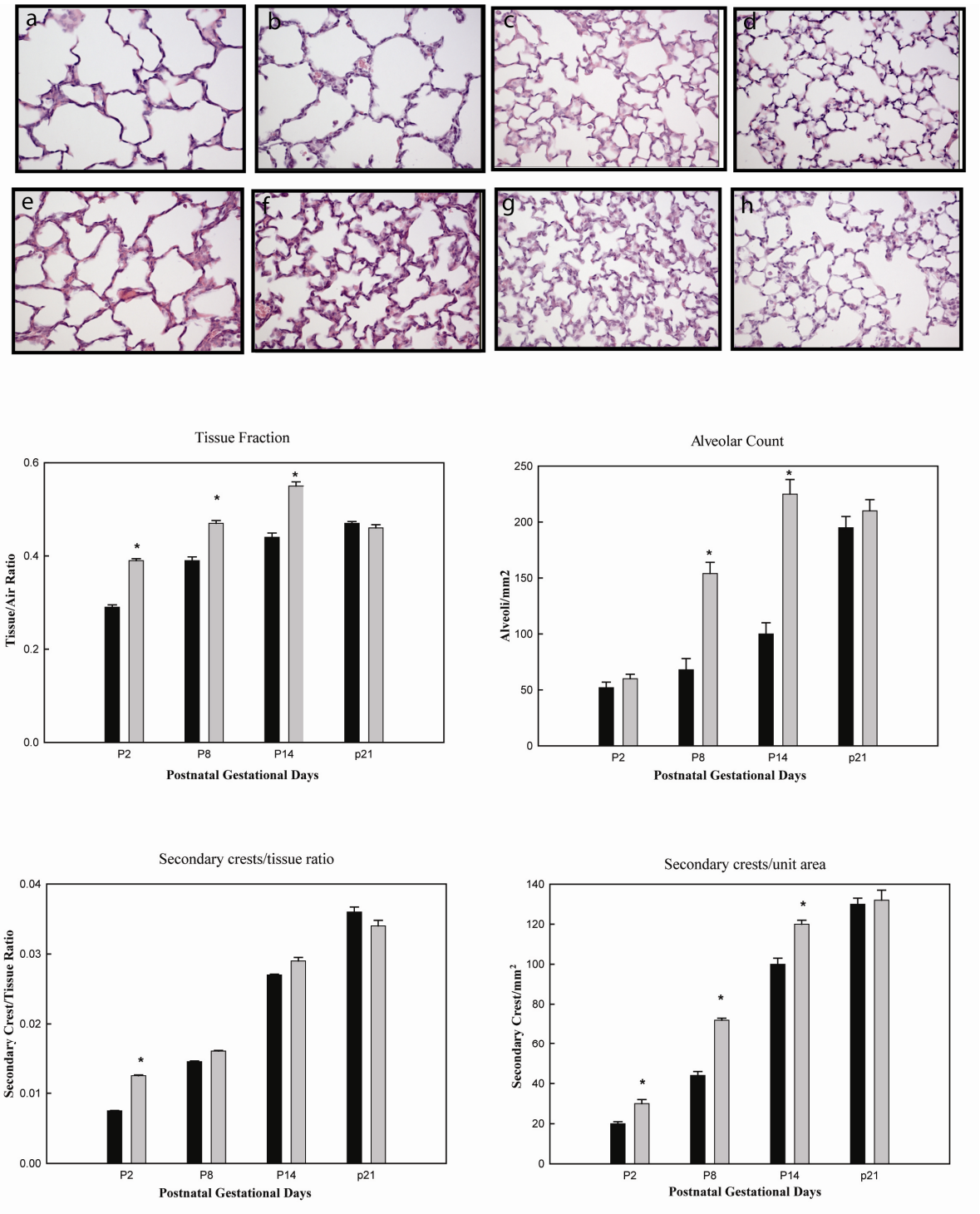
***A**) Expression of total VEGF mRNA and (**B**) expression of $VEGF_{188}$, $VEGF_{164}$ and $VEGF_{120}$ mRNA in murine lung, as assessed by real-time qPCR. Data are expressed as relative fold changes in expression when compared to E18.5 lungs. Values are mean ± s.e.m., for 4 lungs in each group. *P < 0.05.*

Figure 3.6 *(see page 50)* Effect of constitutive active HIF-1α expression on vascular formation during postnatal development



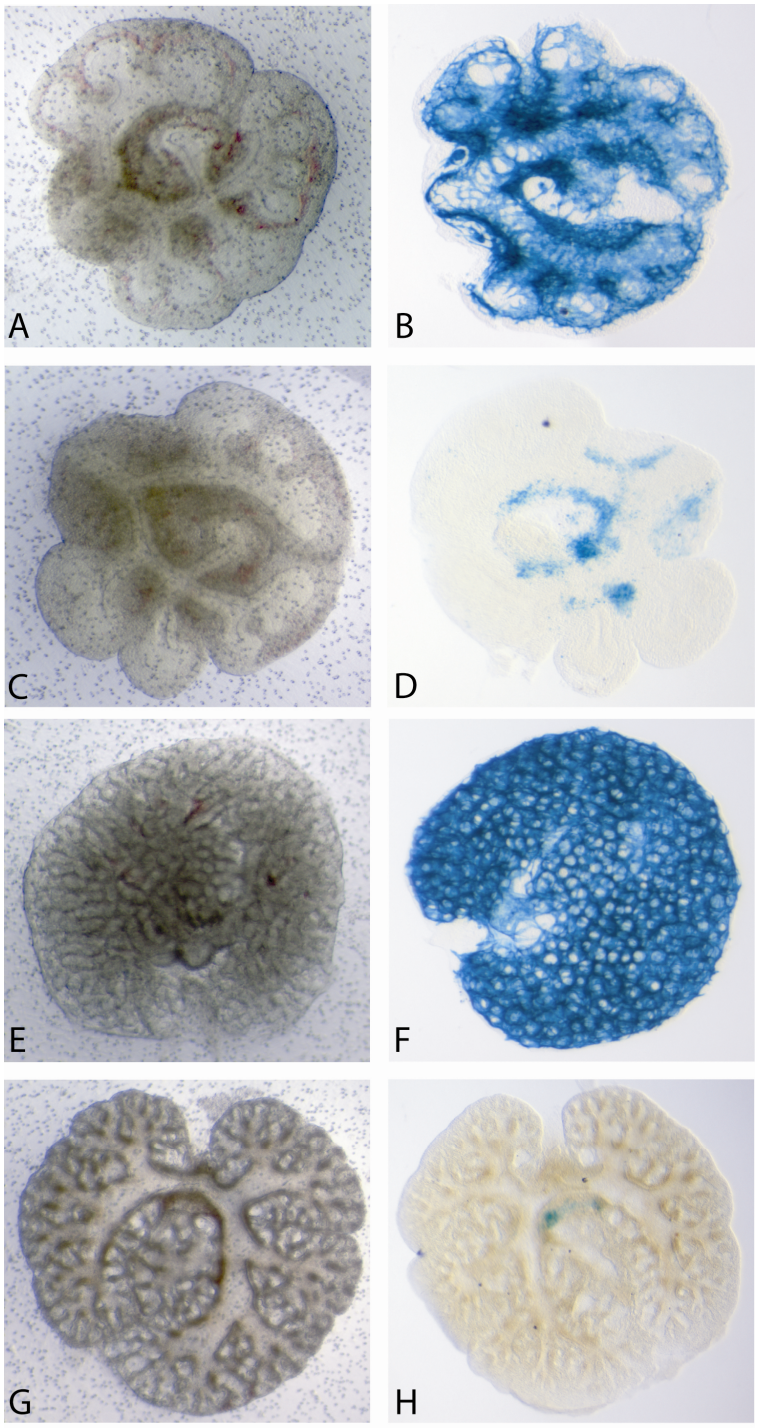
*(**A**) Fluorescent angiography of E18.5 lungs revealed no differences between C57 and HIF-1α ΔODD lungs. (**B**) Small vessel (diameter range 20 to 65 μm) number per unit area was increased in HIF-1α ΔODD lungs compared to C57 control lungs at all gestational ages.*

Figure 3.7 *(see page 51)* Lung histology and morphometry of neonatal C57 control and HIF-1α ΔODD mice



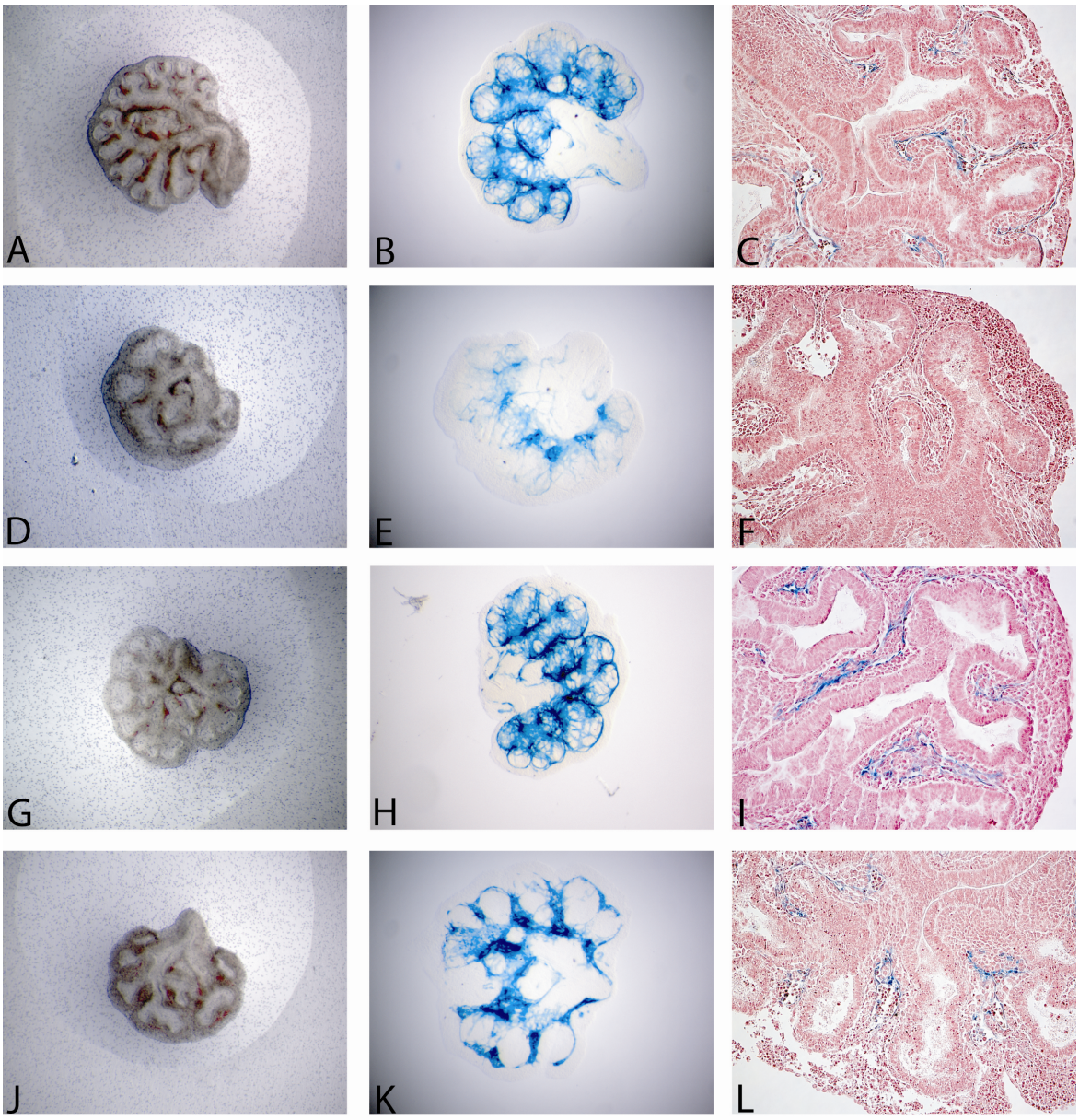
*(a-h) Haematoxylin and eosin staining of HIF-1α ΔODD lungs (e-h) showed increased parenchymal tissue per unit area of lung and smaller distal airspaces compared to C57 lungs (a-d) consistent with enhanced alveologenesis (Bar = 200 μm). Tissue fraction and number of alveoli per unit area were significantly increased in HIF-1α ΔODD lungs at postnatal day 2, 8 and 14. There was no significant difference in secondary crest/tissue ratio between HIF-1α ΔODD and C57 control pups except for postnatal day 2. Secondary crest number per unit area was significantly increased in HIF-1α ΔODD lungs at postnatal days 2, 8 and 14. Values are means ± s.e.m. for five pups in each group. *P < 0.05*

Figure 4.1 *(see page 64)* SU5416 inhibits vascularization in early lung explants



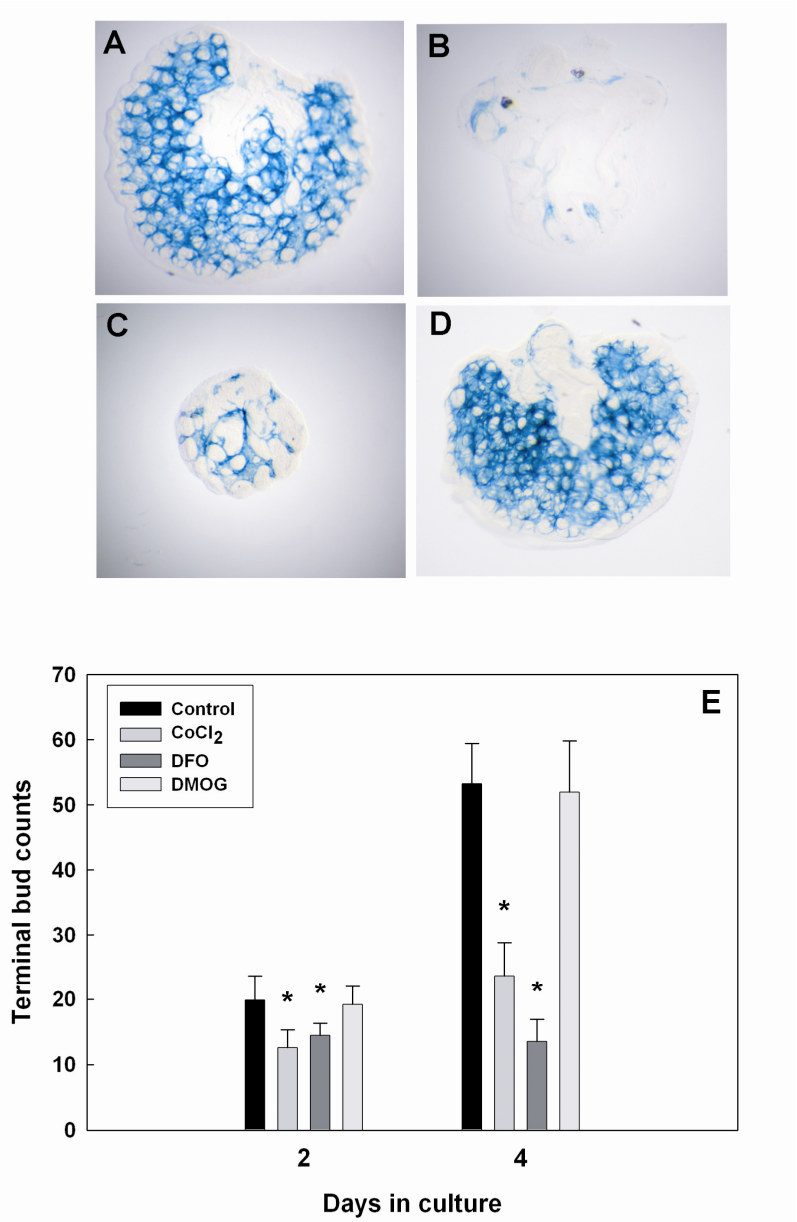
E11.5 Tie-LacZ lung explants were maintained in 2% O_2 with (C, D, G, H) and without (A, B, E, F) SU5416. Vascularization was assessed after 24 (A-D) and 144 hours (E-H) by X-gal staining (B,D,F,H). Unstained control explants (A, C, E, G). Blue color represents positive X-gal staining in the vessels.

Figure 4.4 *(see page 67)* Cobalt chloride and desferrioxamine, but not dimethyloxalylglycine, reduce vascularization in early lung explants



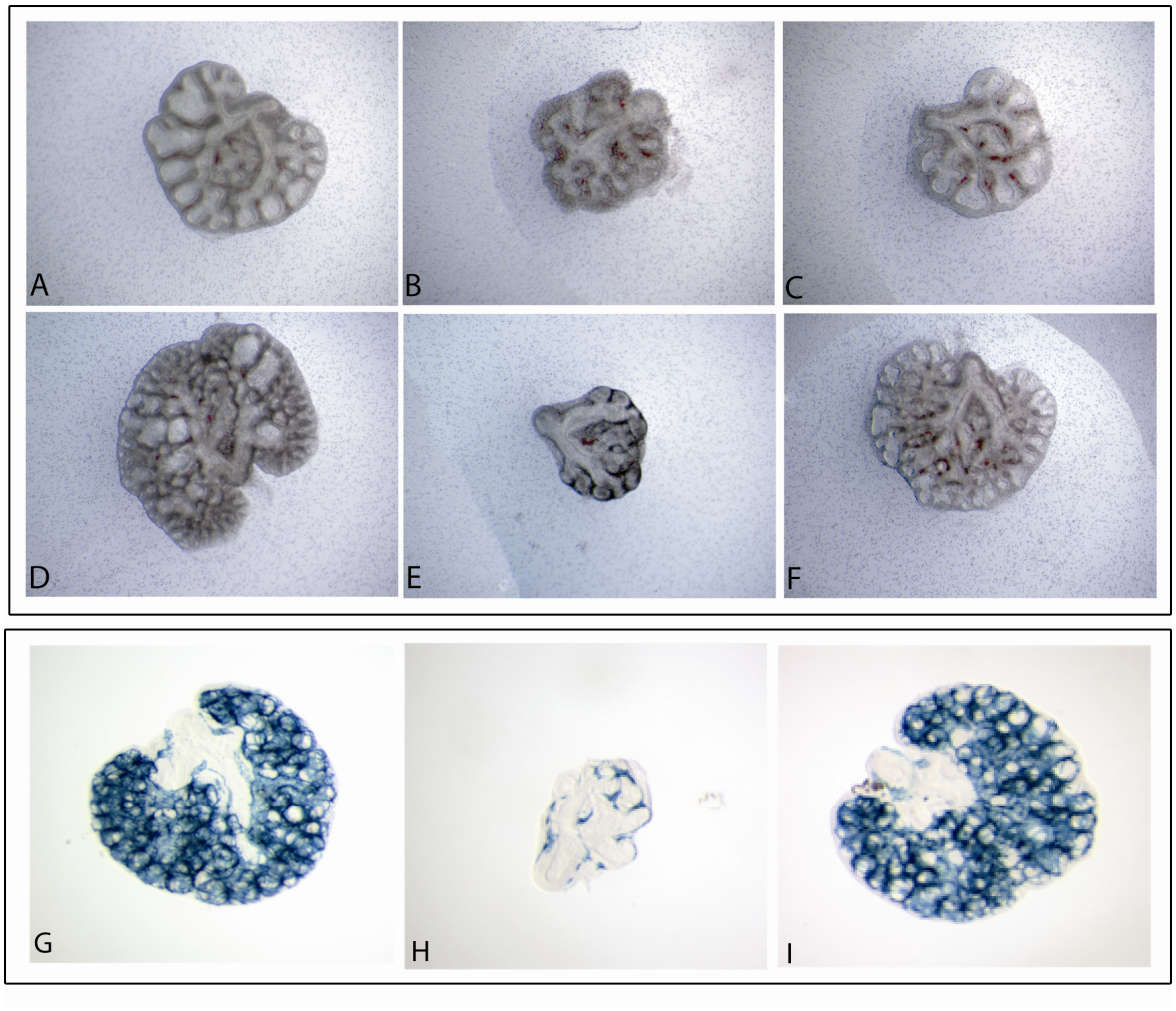
E11.5 Tie-lacZ lung explants were maintained in 20% O_2 with and without $CoCl_2$, DFO and DMOG. Vascularization was assessed after 48 hours by X-gal staining. A-C: control, D-F: $CoCl_2$, G-I: DMOG, and J-L: DFO. Blue color represents positive X-gal staining in the vessels (B, E, H, K) of the explants. Sections of X-gal stained explants (C, F, I, L) were counterstained with nuclear fast red.

Figure 4.5 *(see page 68)* Cobalt chloride and desferrioxamine, but not dimethyloxalylglycine, reduce branching morphogenesis



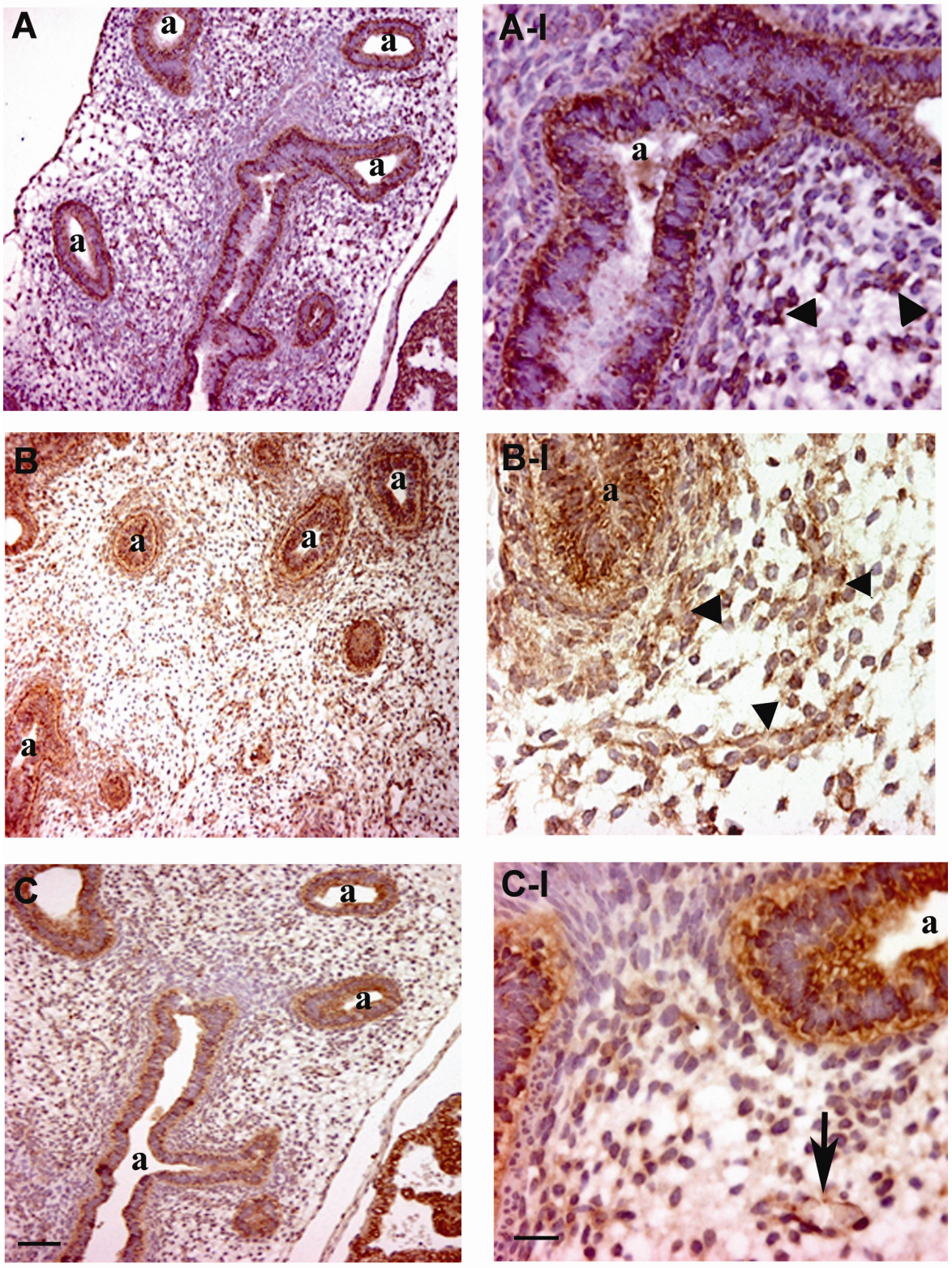
E11.5 Tie-lacZ lung explants maintained in 20% O_2 for 96 hrs with and without $CoCl_2$, DFO and DMOG. A-D: X-gal staining (A: control, B: $CoCl_2$, C: DFO, and D: DMOG). E: terminal buds counts, mean ± s.e.m., n ≥ 25 explants per treatment.

Figure 4.6 *(see page 69)* Addition of iron chloride rescues the desferrioxamine-induced decrease in vascularization and branching



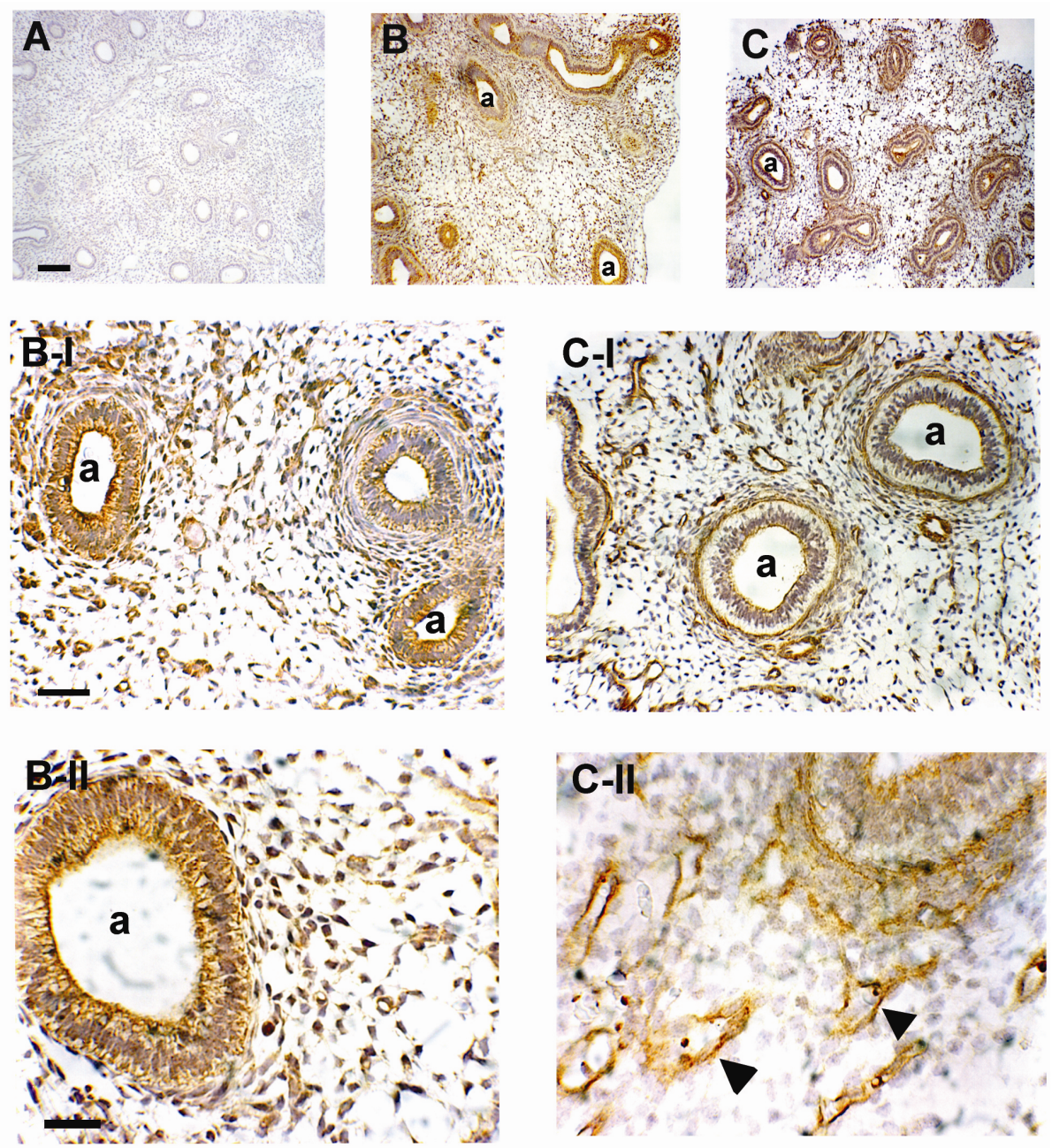
E11.5 Tie-lacZ lung explants were cultured for 48 (A - C) and 96 hours (D -I) without (A, D, G) and with 10 μM DFO (B, E, H) or 10 μM DFO + 50 μM $FeCl_2$ (C, F, I). X-gal stained explants (G, H, I).

Figure 5.1 *(see page 84)* Immunohistochemical analysis of HIF subunits in first-trimester (10-weeks) human lung



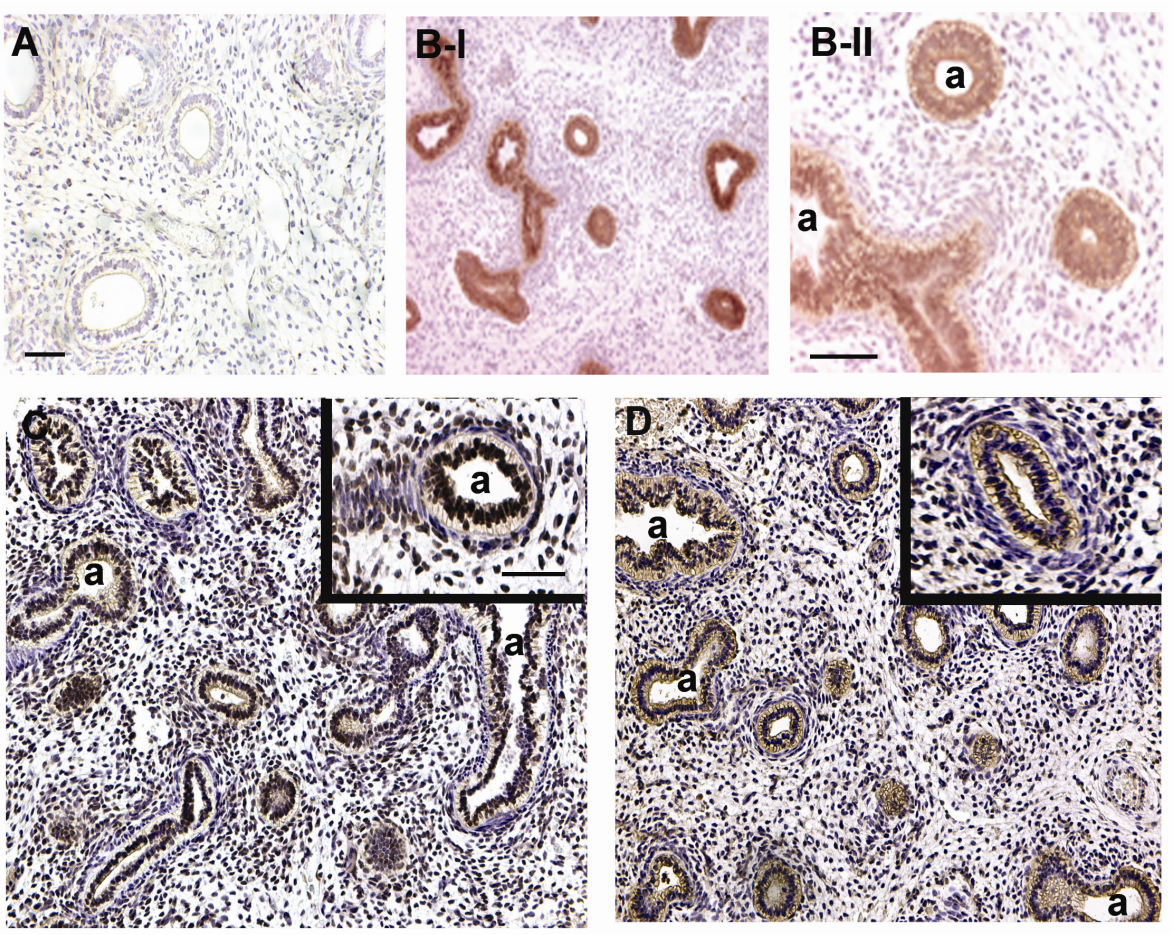
Airway (a) epithelium shows positive brownish staining for HIF-1α (A, A-I), HIF-2α (B, B-I) and ARNT (C, C-I). Positive signals are also detected in the lung parenchyma (arrow heads and arrow). Bar: 100 μm (A,B,C); 25 μm (A-I, B-I, C-I).

Figure 5.2 *(see page 85)* Immunohistochemical analysis of VEGF and VEGFR2 in first-trimester (10-weeks) human lung



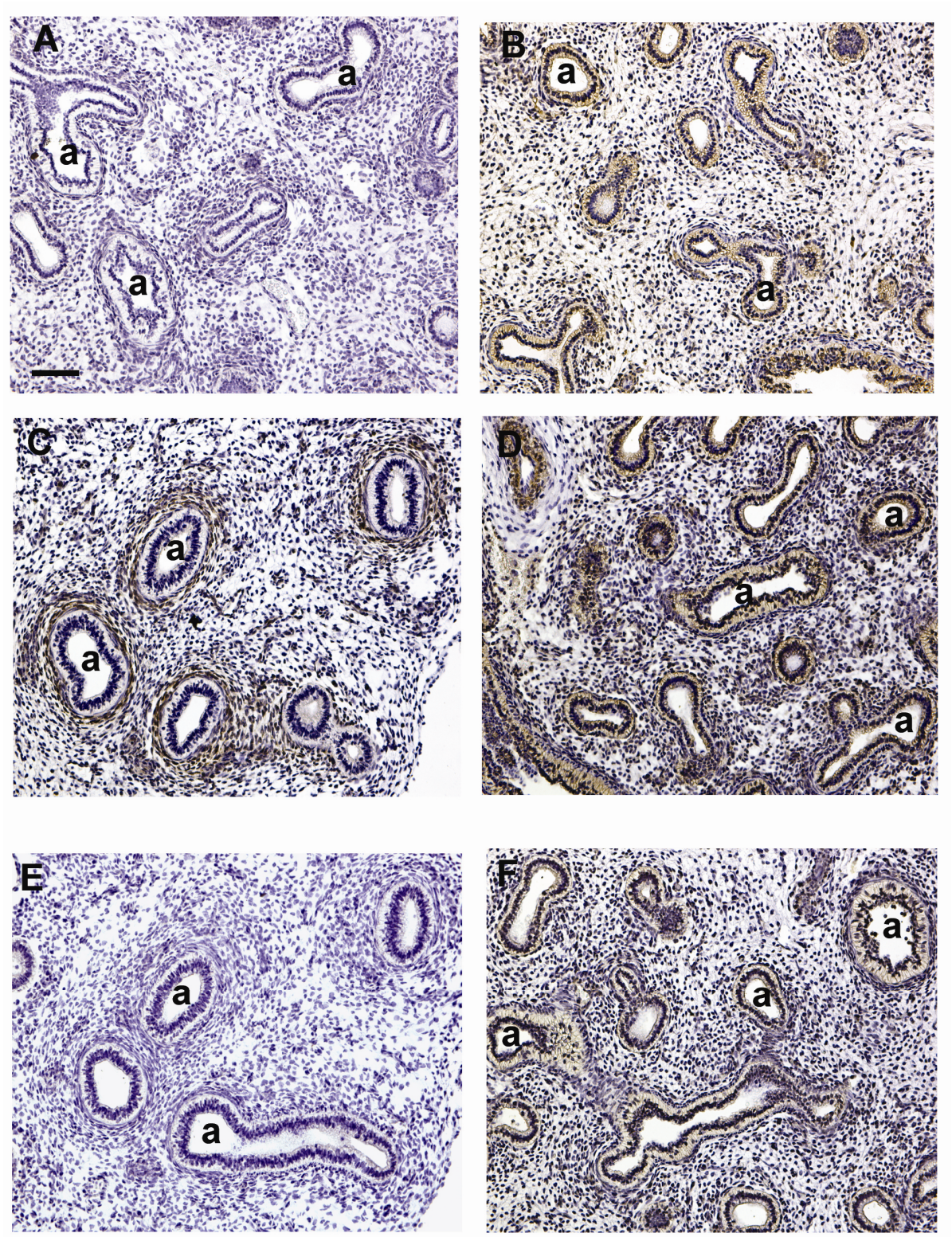
Strong positive brownish staining for VEGF (B, B-I, B-II) is noted in the airway (a) epithelium, but VEGF is also detectable in some mesenchymal structures. The VEGFR2 signal (C, C-I, C-II) is localized to the mesenchymal cells immediately underlying the epithelium and vascular structures (arrow heads) in the parenchyma. Weaker VEGFR2 staining is detectable in the luminal airway epithelium. Negative control staining is shown in panel A. Bar: 100 μm (A, B, C); 100 μm (B-I, C-I); 50 μm (B-II, C-II).

Figure 5.3 *(see page 86)* Immunolocalization of components of the VEC complex in first-trimester (10-weeks) human lung



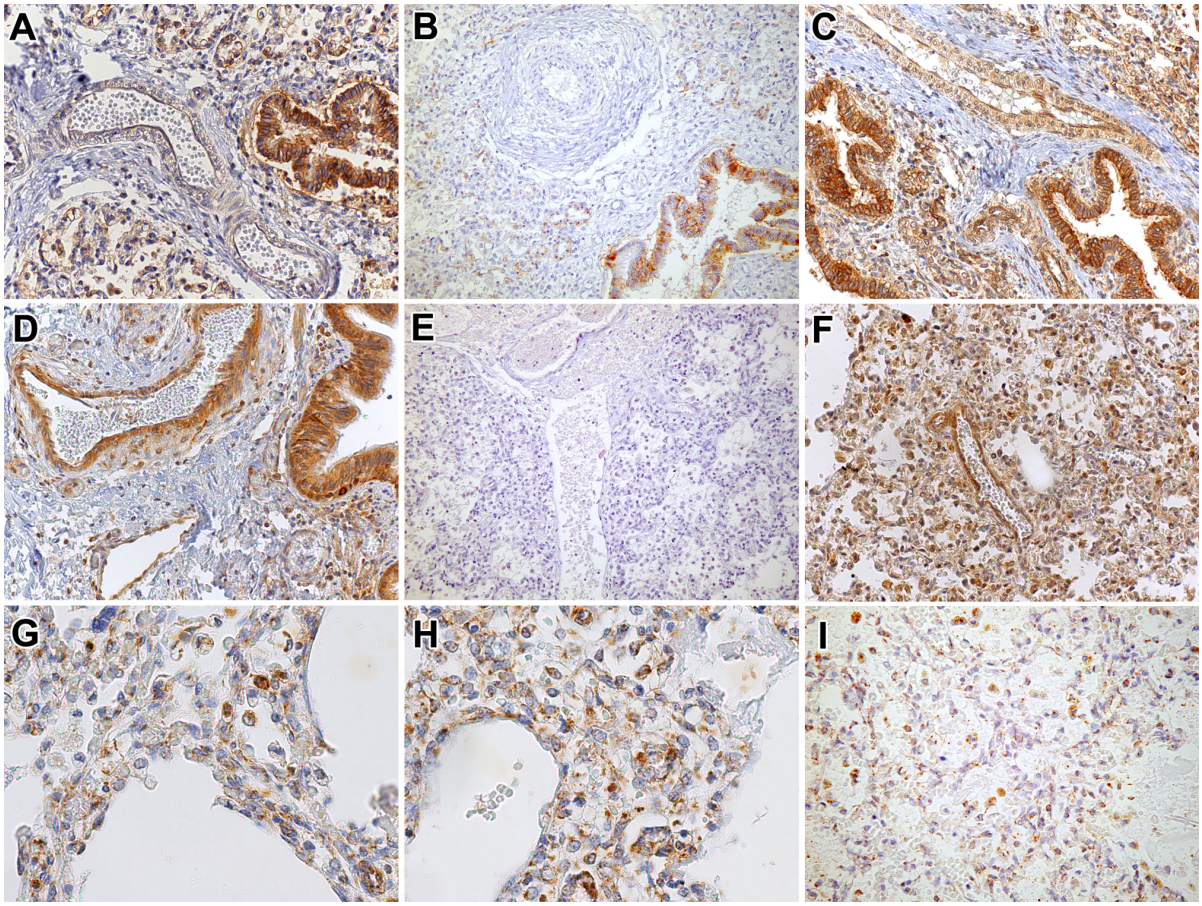
Positive brownish staining for VHL is evident in the airway (a) epithelium (B-I, B-II), while a monoclonal antibody control is negative (A). Airways (a) show positive staining for Cullin2 (C) and NEDD8 (D). Some positive brownish staining is also detected in the lung parenchyma, but the mesenchymal cells immediately subjacent to the epithelium are negative (insets in C and D). Bar: 100 μm (A, B-I, C, D); 50 μm (B-II, inset C and D).

Figure 5.4 *(see page 88)* Immunolocalization of PHDs and SIAH1 in first-trimester (10-weeks) human lung



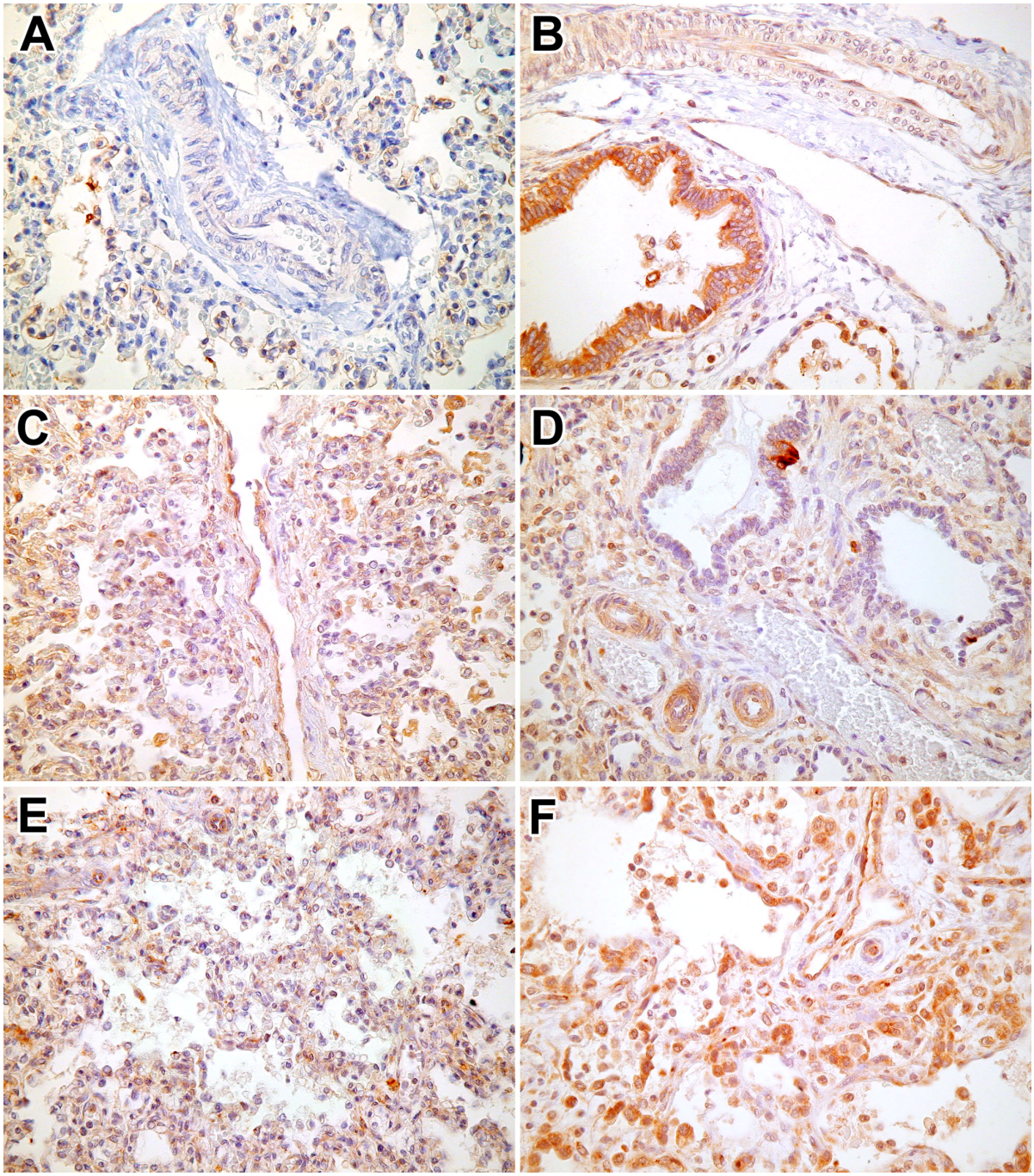
Airway (a) epithelium stains positive for PHD1 (B), PHD3 (D) and SIAH1 (F), while PHD2 protein localized to the lung parenchyma, especially to the mesenchymal cell layer subjacent to the epithelium (C). Negative control staining for primary rabbit and goat polyclonal antibodies are shown in panels A and E, respectively. Bar: 100 μm (A-F).

Figure 6.4 *(see page 102)*



A-C: VHL staining of normal control lungs (A), lungs from idiopathic PPHN (B) and from hypoplastic lungs (C), showing decreased expression of VHL protein in the arterial endothelium and medial smooth muscle cells in idiopathic PPHN as compared to controls, whereas hypoplastic lungs have increased expression in these cell types.
D-F: HIF-1α staining of normal control lungs (D), lungs from idiopathic PPHN (E) and from MAS (F), showing no staining at all in veins and capillaries of idiopathic PPHN whereas MAS showed a decrease in staining compared to controls.
G-I: iNOS staining of normal control lungs (G), lungs from idiopathic PPHN (H) and from MAS (I), showing capillary staining in MAS lungs compared to controls and idiopathic PPHN.

Figure 7.4 *(see page 117)*



A, B: pVHL expression is higher in endothelium, medial smooth muscle cells, venous endothelium and capillaries of ACD lungs (B) compared to controls (A).
C, D: HIF-1α expression is higher in veins and capillaries of controls (C) than ACD (D).
E, F: eNOS expression in capillaries is increased in ACD (F) compared to controls (E).

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curriculum vitae

Frederick Groenman was born on September 4th, 1978, in Haren (Groningen), the Netherlands. In 1997 he passed his VWO high school exam at the Maartenscollege in Haren. In the same year he started medical school at the Erasmus University in Rotterdam.
From 1998 to 2001 he worked as nurse assistant in the Thoracic Surgery Intensive Care Unit at the Thorax Center, Erasmus MC in Rotterdam.
In 2001 he interrupted his medical training to start working as a PhD student in Lung Development at the Department of Pediatric Surgery at the Erasmus MC-Sophia Children's Hospital in Rotterdam under guidance of Prof. dr. D. Tibboel. During the period from January 2001 to May 2003 he worked as a PhD student in the Pediatric Surgical Intensive Care Unit and the Neonatal Intensive Care Unit combining clinical studies and experimental pathology related work. In 2002 he obtained fellowships from the Sophia Foundation for Scientific Research and the Gerrit Jan Mulder Stichting enabling him to continue his study in Toronto. As a part of this research project he worked from May 2003 to September 2005 in the laboratory of the Department of Lung Biology at the Hospital for Sick Children in Toronto under guidance of Prof. dr. M. Post.
In September 2005 he resumed his medical studies, which he will finish in 2007.