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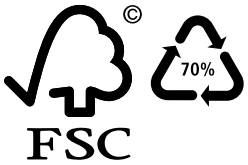

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Congenital Diaphragmatic Hernia

A pulmonary vascular point of view

Congenitale hernia diaphragmatica

Over de pulmonale circulatie

Proefschrift

ter verkrijging van de graad van doctor

aan de Erasmus Universiteit Rotterdam

op gezag van de rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties

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Voor Jules

Contents

Chapter 1

Therapeutic targets in neonatal pulmonary hypertension---Linking pathophysiology to clinical medicine

General introduction

Abstract

Treatment of pulmonary hypertension (PH) in neonates is a major challenge on the intensive care unit and involves high morbidity and mortality. However we have gained considerable insights into the pathophysiology of PH leading to an increasing number of possible treatment targets. Translation of these novel targets into clinical application requires multi-centre randomized controlled trials. Furthermore, considering the underlying pathology is important in therapy choice. New therapies will not only target vasodilatation, but also reduce vascular remodeling and enhance postnatal lung development. This review provides an overview of currently available drugs and promising new targets in the treatment of PH in newborns.

Introduction

Pulmonary hypertension (PH) in the neonate is a potential fatal condition. Due to the heterogeneous nature of this disease, standardization of treatment is difficult. Although efforts are being made to base treatment strategies on scientific evidence, many aspects of this condition still need to be further explored. PH is a consequence of failure to decrease the pulmonary vascular resistance (PVR) at birth, or even a progressive increase in pulmonary vascular resistance. This sustained high PVR leads to right-to-left shunting, which results in severe hypoxemia and eventually right heart failure. Both carry high morbidity and mortality, making the care of these newborns a major challenge for the neonatologist and pediatric intensivist.

Clinically, newborns with PH exhibit central cyanosis and respiratory distress. Commonly, the diagnosis of PH in neonates is made by echocardiogram or a difference between the preductal and the postductal arterial oxygen tension.[1] However cardiac catheterization remains to be the golden standard, as it allows accurate measurement of pulmonary arterial pressure and vascular resistance.[2]

Pulmonary hypertension is in fact a symptom complex that is associated with various neonatal diseases, including congenital heart disease, lung disease and liver disease.[3] Specific neonatal lung diseases associated with PH are for example respiratory distress syndrome (RDS), bronchopulmonary dyplasia (BPD), congenital diaphragmatic hernia (CDH), meconium aspiration syndrome (MAS), and interstitial lung diseases.[4] In premature infants perinatal asphyxia, oligohydramnion and pulmonary hypoplasia are associated with the development of PH.[5]

Treatment of PH in newborns is mainly supportive and partially based on specific pharmacological therapies. Current treatment modalities range from supplementary oxygen to modulation of systemic blood pressure, alkalization, and inhaled nitric oxide (iNO) to extracorporeal membrane oxygenation (ECMO). Despite these different treatment modalities, morbidity and mortality remain high. Furthermore, the rapidly emerging new insights in the regulation of pulmonary vascular tone around birth and in the pathogenesis of PH, provides us with an increasing numbers of new therapeutic targets. Nevertheless, translation of these targets to evidence-based

medicine in clinical practice lags behind. Even in adults, the treatment of PH cannot be considered an evidence-based area, as is shown in a recent meta-analysis of trials of PH.[6]

This review focuses mainly on the pharmacological treatment of neonatal PH associated with lung diseases and will briefly summarize the use of these therapies in other forms of PH. An overview of the pathogenesis and current treatment of PH is given in relation to intracellular trafficking and pathways. Also, potential therapeutic targets based on new insights in the pathogenesis will be discussed.

Development of the pulmonary vasculature and adaptation at birth

In general, the development of the pulmonary vasculature follows that of the airways. By the end of the pseudoglandular stage (16 weeks of gestation) the bronchial tree is fully developed and the pre-acinar vessels are formed by vasculogenesis.[7] The intra-acinar vessels start to develop later in fetal life (canalicular stage) and form simultaneously with the outgrowth of alveoli until 2 years of age. In this period, the pulmonary vascular bed expands by growth in size and lumen of existing arteries and outgrowth of new arteries, so called angiogenesis. Besides the intra-acinar arteries, the alveolar duct arteries and the alveolar capillaries are formed. They are the main determinant of pulmonary vascular resistance and the gas-exchanging surface of the lung.

In the fetus, the pulmonary vascular resistance is high and as a consequence, only 10% of the circulating blood passes the lung. When the gas exchanging function of the lungs emerges at birth, the pulmonary vascular resistance has to drop dramatically. With the first breath it already drops to 50%, enabling good arterial oxygenation of the blood.[8] Mechanical ventilation, increase in oxygen tension and, shear stress give rise to this initial fall in pulmonary vascular resistance. Subsequently, an increase in production of potent vasodilators, such as endogenous nitric oxide (NO) and prostacyclin (PGI_2) maintains a low pulmonary vascular resistance. In the first months after birth, structural remodeling of the pulmonary

vessels takes place to ensure the low pulmonary vascular resistance. The vascular wall becomes thinner and spread around a large lumen, by flattening of the endothelium, thinning of smooth muscle cells and reorganization of the extra cellular matrix. [9]

When these systems fail and a normal increase in compliance does not occur or excessive vasoconstriction is induced by hypoxia, acidose, or hypercabia, the pulmonary vascular resistance remains high. As the fetal circulation is still available, blood starts to shunt through the ductus arteriosus and foramen ovale. Consequently, it becomes only partly oxygenated and the right ventricle is exposed to pressure-overload, which will lead to significant hypoxemia and right ventricular failure.

Structural basis of neonatal pulmonary hypertension

Pulmonary hypertension in neonates encompasses a spectrum of pathologies best classified by their anatomical appearance; maladaptation, maldevelopment, and underdevelopment.[10]

The maladaptation group shows normal development of the lung, but functionally they don't adapt well to extra-uterine life. Either their pulmonary vascular resistance remains high or vasoconstriction is triggered by perinatal distress caused by for example a hypoxic event, acidosis, hypoglycemia and/or hypercarbia. Other underlying triggers for persistent PH are increased blood viscosity and large ventricle septum defects. The pathophysiological mechanisms that cause the pulmonary vasculature to maladapt are not fully understood. However as the mechanism underlying PH in maladaptation is more functional rather than structural, response to vasodilator therapy is most likely.

Maldevelopment of the lung is characterized by excessive muscularization of the pulmonary arteries, as seen by increased medial wall thickness and muscularization of normally non-muscular arteries. Besides the abnormalities of the peripheral arteries, especially the lung growth is normal. This is seen in idiopathic persistent pulmonary hypertension of the neonate (PPHN), meconium aspiration syndrome (MAS) and PH associated with congenital heart diseases. The maldevelopment is believed to happen late in gestation or as in case of the congenital heart diseases, mostly after birth. Due to increased flow or inflammation the pulmonary arteries remodel and start to develop

plexogenic lesions.[11] This makes PH based on maldevelopment potentially reversible. Treatment in these cases should be started early and not only be based on vasodilatation, but also incorporate anti-proliferative strategies to reduce or even reverse the maldevelopment of the pulmonary arteries. Obviously in cardiac anomalies early repair with reduction of L-R shunt is the treatment of choice.

The third group shows underdevelopment of the lung or lung hypoplasia. These lungs are characterized by a lower number of bronchial generations (early gestational effect) and/or less and smaller alveoli (later effect). Also the pulmonary arteries are reduced in number and size, even relative to the already underdeveloped lung. Similar to maldevelopment of the lung, there is increased vascular wall thickness and extensive muscularization of the pulmonary arteries. Examples of disorders associated with pulmonary hypoplasia are congenital diaphragmatic hernia (CDH), and oligohydramnion caused by renal dysplasia, renal agnesis or chronic leakage of amnionic fluid. Noticeable, the morphology in this group looks as if lung development stagnated during gestation. Premature babies, who develop "new" BPD could be considered part of this group to.[12] As premature babies of 24 weeks of gestation survive, their lungs will be exposed to high levels of oxygen and mechanical ventilation, while they are still in their canalicular stage. Consequently, normal development is impaired, and the lung shows simplification of alveoli and less pulmonary capillaries leading to PH.[13] Presumably because of these anatomical abnormalities, patients with PH based on underdevelopment of the lung respond poorly to vasodilatation therapy. In the future these patients may benefit from new therapeutic modalities that will restore/enhance lung growth, such as gene therapy and stem cell therapy.[14,15] Also, lung growth triggered by fetal tracheal occlusion is an example of new therapeutic modalities, which is already used in the treatment of severe lung hypoplasia.[16]

This classification of PH in neonates is not absolute. Most of the underlying disorders present a combination of these anatomical abnormalities. However in the future it will be important to target treatment to this underlying pathobiology. Therefore studies into pulmonary biology and well-defined clinical research are needed to ensure that new therapies are based on evidence in order to improve the outcome of neonates with PH.

Cellular pathobiology of neonatal pulmonary hypertension

Although there are different causes, the histopathology of PH in newborns shows many similarities. Key factors in the pathobiology of PH in the neonate are endothelial dysfunction and hyperreactivity, as well as increased proliferation of smooth muscle cells.

Endothelial cells (EC) are believed to have a central and critical role in the initiation of PH.[17] The endothelial cells display anomalies in their production of vasoactive mediators like, nitric oxide, prostaglandins and, endothelin. This results in an imbalance between vasoconstriction and vasodilatation, and between apoptosis and proliferation.[18]

Vascular smooth muscle cells (SMC) react to this imbalance with a state of increased vasoconstriction and proliferation. This is characterized by migration of the SMC into non-muscular arteries and medial wall hypertrophy of muscle arteries, caused by both hypertrophy and hyperplasia of the SMC.[19]

The extra cellular matrix (ECM) thickens and an increase in glycoproteins, such like tenascin and fibronectin enhances the proliferative state.[20] The formation of plexiform lesions (obliteration of the vascular lumen with overgrowth of EC) like in adults and in cardiac defects with fluid overload is rarely seen in newborns with PH.[21]

Finally, the hemodynamic stress can inhibit vascular growth and impair alveolarization in the postnatal developing lung.[22] Thus, this is a vicious circle; PH itself can accelerate pulmonary vascular injury, sustaining a high PVR. As a result the disease may progress rapidly and become more refractory to therapy. Moreover, a healthy vasculature of the lung contributes to normal alveolar development. Therefore it is important to start vasodilator therapy early in these neonates, as treating them during their period of rapid lung growth might potentate normal alveolarization.

Treatment of neonatal pulmonary hypertension

Supportive care of neonates with pulmonary hypertension consists of optimizing cardic output and systemic blood pressure, to minimize further right-to-left shunting. Mechanical ventilation and supplemental oxygen should be given to maximize recruitment of the gas-exchanging surface and a sufficient oxygen gradient for optimal diffusion. Furthermore, oxygen tension dilates pulmonary arteries by increased oxygen radicals through a complex oxygen sensing mechanism.[23] Further acidosis should be avoided as it increases pulmonary vascular resistance.

Magnesium sulfate (Mg_2SO_4) is used in the treatment of PH in newborns and case studies report that it might be effective.[24,25] Mg_2SO_4 modulates vascular contractility and reactivity, however it is not a potent or selective pulmonary vasodilator.[26,27] Its beneficial effects might be due to sedation, muscle relaxation and alleviation of oxidant-mediated tissue injury, or by it associated alkalosis.[28] Since uncontrolled clinical trials suggest a potential benefit from Mg_2SO_4 and it is inexpensive, randomized controlled trials are recommended.[29-31]

As a last resort, extracorporeal membrane oxygenation (ECMO) can be used to allow the lungs to recover. ECMO is used to attempt adequate tissue oxygenation and avoidance of further lung injury, while the pulmonary vasculature has a chance to decrease its resistance. Moreover ECMO is shown to partially reverse the adventitial thickening, which is seen in persistent stages of PH associated with for instance CDH.[32]

Linking pathophysiology to pharmacological therapy

Prostaglandins

Prostaglandins are formed through the metabolizing of arachidonic acid by cyclo-oxygenase and subsequent synthesis by prostacyclin synthetase to produce prostaglandin I_2 (prostacyclin or PGI_2). Prostacyclin activates adenylate cyclase in the SMC, which in turn increases the level of cAMP. (Figure 1.1)

Prostacyclin is an important vasodilator in the pulmonary circulation. Its synthesis increases during fetal life and decreases during normal transition to extra-uterine life. The prostacyclin pathway may not be of great importance for normal maintenance of the low PVR. However, it plays a role in hypoxic PH, as in hypoxia, the relaxation of the vasculature in response to PGI2 is increased.[33] Moreover, overexpression of prostacyclin synthetase in transgenic mice protects them from hypoxia-induced PH.[34] In addition to this, plasma ratios of prostaglandin over thromboxane are lower in newborns with hypoxemic respiratory failure, supporting the involvement of the prostacyclin pathway in the pathophysiology of PH in newborns.[35]

Epoprostenol, a prostacyclin analogue has been the mainstay in treatment of adults with severe PH over the past two decades and demonstrated sustained improvements in symptoms and mortality.[36] Alternative prostacyclin analogues, as the inhaled form Iloprost, oral Beraprost, and subcutaneous Treprostinil are proven not as effective as intravenous Epoprostenol.[4] However, inhaled Iloprost demonstrated a potent pulmonary vasodilatation; which was more pronounced than inhaled nitric oxide in adults with pulmonary arterial hypertension.[37]

Prostacyclin forms a promising pathway in the treatment of PH in neonates. Recent case reports show the successful use of prostacyclin in the treatment of newborns with PH.[38-40] Especially the newer, more stable form of Iloprost, which is administered endotracheally showed improved oxygenation without clinically relevant alterations in systemic arterial pressure.[41] Randomized controlled trials are needed to fully evaluate its place in treatment of PH in neonates. Also, combination therapy with phosphodiesterase inhibitors (see below) might be considered, as they potentiate the pulmonary vasodilator effects of prostacyclin.[42]

Nitric Oxide

The discovery of nitric oxide (NO) as possibly the most potent endothelium-derived relaxing factor lead in 1992 to the use of inhaled NO (iNO) in the treatment of PH in neonates.[43] NO is formed in the endothelial cell by the conversion of L-arginine to L-citrulline (figure 1.1). For this reaction NO synthase (NOS) needs to form a homodimer, which requires several cofactors, such as NADPH and tetrahydrobiopterin (BH4). After NO is formed, it directly diffuses to the nearby smooth muscle cell, where activation of soluble guanylate cyclase (sGC) leads to an increase in cGMP. The effects of cGMP are mediated through activation of several effector-proteins; cGMP-dependent protein kinase, cGMP-regulated phosphodiesterases, and cGMP-gated ion channels. The resulting membrane hyperpolarization, inhibition of calcium influx and decreased calcium sensitivity lead to the relaxation of the vascular SMC.[44] The action of cGMP is limited by hydrolyzation of cyclic nucleotides by phosphodiesterases (see below).

The importance of NO during lung development and in mediating normal vasoregulation is well established.[45] Disruption of the NO pathway is shown to play a pivotal role in the pathophysiology of PH. Studies in different animal models of PH in neonates show decreased NO synthase, lower sGC expression and increased phosphodiesterase activity.[8]

Inhaled NO (iNO) induces a significant pulmonary vasodilation, without systemic side effects and optimizes the ventilation-perfusion match. Randomized trials show a reduction in mortality and need for ECMO, when iNO is used in the treatment of PH in term and near-term infants. However in patients with PH due to congenital diaphragmatic hernia, a beneficial effect of iNO is doubtful.[46,47]

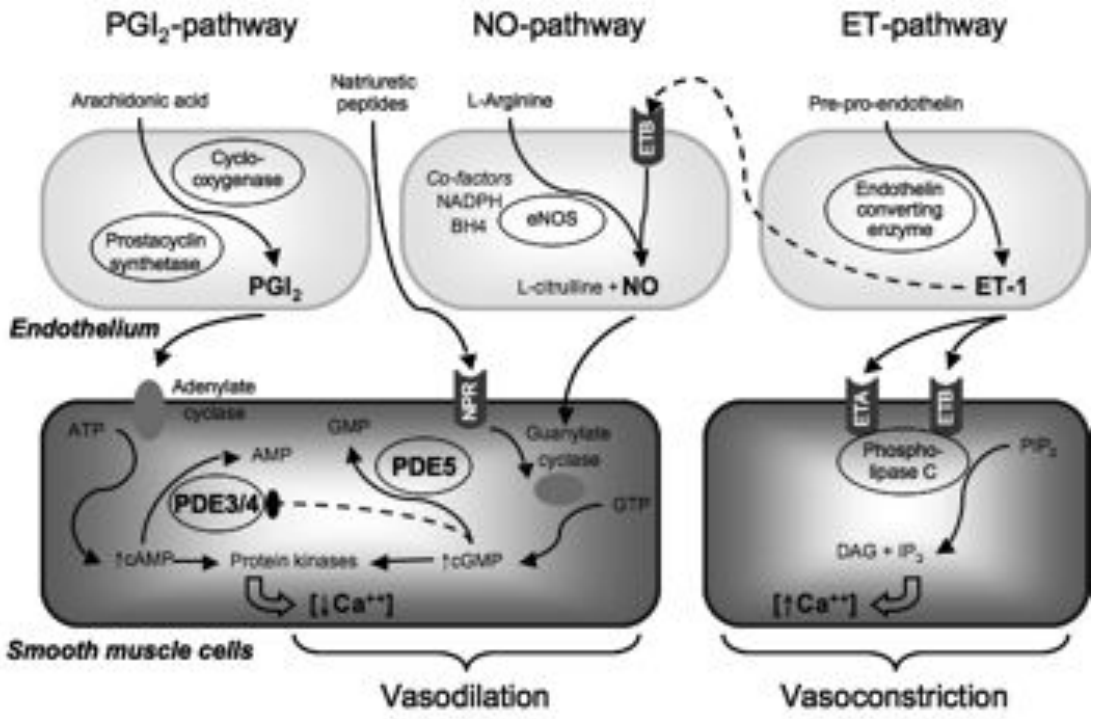

Figure 1.1 Main pathways in the balance of pulmonary vascular tone.
PGI_2: prostacyclin; PDE: phosphodiesterase; NO: nitric oxide; eNOS: endothelial nitric oxide synthase; NADPH: nicotinamide adenine dinucleotide phosphate; BH_4: tetrahydrobiopterin; NPR: natriuretic peptide receptor; ET(-1); endothelin; ETB: endothelin receptor B; ETA: endothelin receptor A; PIP_2: phosphatidylinositol 4,5,-biphosphate; DAG: diacylglycerol; IP_3: inositol 1,4,5-triphosphate

Besides exogenous NO, several other targets in the NO pathway are promising directions for future therapy.[45] Firstly, downstream inhibition of phosphodiesterase 5 will retain a high level of cGMP, as will be discussed below. Further, direct activation of sGC by BAY 41-2272 has been shown to reduce pulmonary vascular resistance in fetal and neonatal sheep with severe PH induced by ductus ligation.[48] Thirdly, availability of cofactor BH4 protects mice from hypoxia induced PH and controls pulmonary vascular tone in the same model.[49]

Although iNO is the first choice therapy for neonates with PH, approximately 30 % of patients are unresponsive to it.[50] Further appraisal of possible targets in the NO pathway and close monitoring of the efficacy of iNO as therapy in PH in newborns is therefore warranted. Furthermore, combination therapy of iNO and for example phosphodiesterase inhibitors are a promising option, that needs to be explored.

Phosphodiesterase inhibitors

Phosphodiesterases (PDEs) are a large family of enzymes that hydrolyze cyclic nucleotides (cGMP and cAMP). Inhibition of PDEs leads to vasodilator and anti-mitogenic effects. Currently, 13 different PDE families are known; some hydrolyze cGMP, some cAMP, and some both. Furthermore, the level of cyclic nucleotides regulates the activity of some PDE isoforms themselves, providing a 'cross-talk' between cGMP and cAMP (Figure 1.1).[51]

PDE5, a cGMP-specific phosphodiesterase is expressed most abundantly in the lung, but also PDE3 and 4, which preferentially hydrolyze cAMP, are also shown to be active in the pulmonary circulation. PDE5 is developmental regulated in the lung, showing a high concentration in the fetal lung that rapidly falls after birth.[52] Both PDE5 and PDE3 are upregulated in the lungs of rats with chronic hypoxia-induced PH, suggesting they play a role in the pathogenesis of PH.[53] Moreover a higher expression of PDE5 is found in adults with pulmonary arterial hypertension.[54]

Sildenafil, a selective PDE5 inhibitor, is used in the treatment of PH in adults and shows improvement of WHO functional class in these patients in a randomized controlled trial.[55] First reports also suggest that treatment with Sildenafil in newborns with PH is successful.[56] Sildenafil substantially lowers the PVR and it has a synergistic effect on iNO, supporting combination therapy.[57] As its effects last longer than those of iNO, Sildenafil is shown to be beneficial in weaning from iNO, preventing serious rebound PH.[58] Caution is needed however in the treatment of PH associated with diseases of lung parenchyma (i.e. MAS), as oral Sildenafil can worsen the ventilation-perfusion match in these conditions.[59]

Besides Sildenafil, several other specific PDE5 inhibitors are available, like Tadalafil and Vardenafil. Sildenafil, however, shows higher selectivity for the pulmonary circulation and improvement of arterial oxygenation.[60] Besides acute vasodilation, inhibition of PDE5 in pulmonary SMC cultures has shown to have an antiproliferative effect, which might be significant in chronic treatment of PH.[54] Moreover Ladha et al. showed that Sildenafil might preserve alveolar growth and lung angiogenesis in the hyperoxia-induced BPD rat model.[61] Additionally, prenatal administration of Sildenafil given to the mother in the nitrofen-induced CDH rat model increased the

survival of the affected pups and their lung morphology showed a less severe medial wall thickening.[62]

Sildenafil forms a promising target in the treatment of a subgroup of newborns with PH. Unfortunately Sildenafil can only be administered orally, which is a drawback in intensive care patients. Nonetheless safety and effectiveness of Sildenafil in the treatment of PH in newborns still needs to be established.[63] The use of Sildenafil should be restricted within the context of randomized controlled trials, in which evaluation points should not only be on short term reduction of pulmonary vascular resistance, but also include long term effects on lung development.

Besides inhibition of PDE5, inhibition PDE3 by Milrinone is introduced in the therapy of PH in newborns.[64] Milrinone, known for its inotropic and vasodilative effects, is already been routinely used in newborns with cardiac disease.[65] Several short case studies in neonates with PH show that intravenous Milrinone improves oxygenation without compromising the systemic blood pressure.[65,66] Earlier animal studies raise the concern that the vasodilative effects of Milrinone might not be selective for the pulmonary circulation.[67] It seems, however, that Milrinone may only have a selective effect on the pulmonary vasculature in the presence of PH.[68,69] Moreover, the use of the inhaled variant of milrinone might even induce more pulmonary selectiveness.

A second use of Milrinone is in combination therapy with prostacyclin or iNO. Milrinone is shown to potentate the effectiveness of prostacyclin by sustaining a high level of cAMP.[70] Further, cAMP is shown to be down-regulated during the use of iNO and this might cause part of the rebound PH of iNO withdrawal. Milrinone is shown to preserve cAMP concentration during iNO treatment and thereby prevents the rebound PH.[71]

As the understanding of the function of PDE3 in the pathophysiology of PH in neonates is not complete, further basic research is necessary. Although perspectives of Milrinone in the treatment of PH in neonates are effective, all evidences are uncontrolled and preliminary.

Endothelin inhibition

Endothelin (ET-1) is a very potent vasoconstrictor in the pulmonary circulation.[72,73] Vasoconstriction is mediated through its receptors ET-A and ET-B on the SMC, activating mechanisms involving phospholipase C and inositol triphosphate (IP_3), leading to release of Ca^+ from the intracellular calcium stores.[74] In contrast, activation of the ET-B receptor located on the EC leads to vasodilatation through a NO-cGMP dependent pathway and clearance of the circulating ET-1.[4]

During fetal life, the level of circulating ET-1 and ET-A expression is high, suggesting they maintain the normal high PVR. After birth, ET-1 levels decrease and more ET-B receptors on the EC appear, probably contributing to a lower vascular tone.[7,75] In piglets with PH however, the circulating levels of ET-1 remain high and an increase in ET-A receptor expression is seen.[76,77] Also, in the nitrofen-induced CDH rat model, expression of ET-1 and ET-A is upregulate.[78] The same is seen in human newborns with PH. Moreover, increased levels of ET-1 correspond with a poorer prognosis of PH in the newborn.[79]

Antagonists to the ET receptors are currently used in the treatment of adult and older pediatric patients with PH. Orally taken Bosentan, an ET-A/ET-B antagonist shows an increase in exercise capacity and improvement of haemodynamics in a randomized placebo-controlled study.[80,81] However, a Cochrane review on the use of ET receptor antagonists correctly points out that no data on mortality rates are available yet.[82] A major concern of Bosentan is its hepatotoxicit.[83] But first studies on Bosentan therapy in newborns with PH report effective and safe use.[84]

Beside Bosentan, Sitaxsentan and Ambrisentan might be promising new drugs. Both are ET-A specific antagonists, thereby keeping the dilative effect through the ET-B receptor. In recent randomized controlled studies, the efficacy of Sitaxsentan in adult PH patients is shown.[85] Even more promising is the use of Ambrisentan, which is shown to be clinically efficient with lower incidence of hepatotoxicity in a phase 3 study in adult PH patients.[86]

The therapeutic utility of ET receptor antagonists in newborns with PH has not been established. Because of the complex hemodynamic effects of ET-1 (vasoconstriction as well as vasodilation), therapeutic intervention within this pathway and prediction of its outcome is difficult. Nevertheless the use of ET receptor antagonists in adults with PH in conjunction with conventional therapy has shown to be beneficial.

Targets for future pharmacological therapies

Rho kinase

The regulation of pulmonary vascular tone is determined by the phosphorylation/dephosphorylation of myosin light chain (MLC) in the vascular SMC. An increase in cellular Ca^{2+} activates MLC kinase (MLCK), which phosphorylates MLC and thereby causes the SMC to contract. Besides the increase in cellular Ca^{2+} concentration, Ca^{2+} sensitization is known to play a role in SMC contraction.[87] Rho A, a small GTPase and its effector protein Rho-kinase (ROCK) are believed to be important modulators of Ca^{2+} sensitivity.[88]

Rho A and ROCK are highly expressed in the pulmonary arteries and are key regulators of vascular tone and structur.[89] In the normal fetal lung the Rho A-ROCK-pathway maintained a high PVR and might be of importance for fetal lung morphogenesis.[90] However, continued activation of the pathway after birth contributes to the development of PH.[91] Inhibition of ROCK is shown to prevent development of PH in monocrotalin and hypoxia induced PH in rats.[92]

Selective ROCK inhibition by Fasudil or Y-27632 showed acute vasodilatative effect in patients with PAH with little influence on the systemic pressure.[93,94] Inhibition of ROCK is a new promising class of drugs in the treatment of PH in neonates. Nevertheless, its use is still in an early experimental phase and further studies are needed.

VEGF

Vascular endothelial growth factor (VEGF) is a pluripotent growth factor that plays a central role in angiogenesis, endothelial barrier function and vascular tone.[95] VEGF is highly expressed in the lung and crucial for lung development and maintenance of the pulmonary vasculature in adult life.[96]

In the pathogenesis of PH VEGF is also involved. In several animal models, VEGF receptor inhibition impairs vascular growth and alveolarization, mimicking PH in newborns.[97,98] Furthermore, expression of VEGF receptor 2 in models of PH is decreased.[99,100] In clinical studies, tracheal aspirates of PPHN patients show decreased VEGF levels.[101] While, expression of VEGF is higher in pulmonary resistance arteries of patients with CDH.[102]

As a therapy, VEGF improves vasodilatation through the NO pathway and reduces vascular remodeling in PH caused by ductus ligation in fetal lam.[103] Also, gene therapy with VEGF of newborn rats with hyperoxia-induced BPD, improves survival and preserves postnatal alveolarization.[104]

The regulation of VEGF expression in the lung is a very fine balance. Depending on location and underlying pathology, VEGF caries out different functions. And although several animal studies show the potential benefit of therapy with VEGF, actual use in human patients is probably hazardous. Because VEGF also increases endothelium permeability leading to leaky vessels and overexpression is speculated to be a risk factor for tumor growth.[105] However, the idea of stimulating angiogenesis in the pulmonary hypertensive lung of neonates and thereby enhancing their lung growth is appealing.

PHD

Hypoxia inducible factor (HIF) is an oxygen-sensitive transcription factor. An increase in HIF activates a range of target genes, which are involved in angiogenesis and vascular remodeling.[106] One of its target genes is VEGF. The availability of HIF is regulated by post-transcriptional hydroxylation by prolyl hydroxylase domain-containing proteins (PHDs).[107]

The lung develops in a relative low-oxygen environment and HIF expression is developmentally regulated, suggesting that HIF-regulated pathways are involved in proper vascular development of the pulmonary circulation.[108] Moreover, a decrease in HIF expression is found in preterm lambs with RDS.[109] Asikainen et al. showed that inhibition of PHDs activates HIF and stimulates VEGF-dependent angiogenesis.[110] Furthermore, the pathophysiological features of BPD in premature baboons are improved by HIF stimulation[111]

Modulation of HIF activity may represent a novel and effective therapeutic approach in the treatment of PH in newborns. However, caution is warranted, as stimulation of HIF might beneficially enhance angiogenesis and thereby lung growth, but it might also induce unwanted vascular remodeling of the existing vessels.

Concluding remarks

In this review we summarized the main pathways in the regulation of pulmonary vascular tone related to the pathogenesis of pulmonary hypertension in neonates and we evaluated therapeutic targets within these pathways (table 1.1). When discussing pulmonary hypertension in neonates, it is important to realize that the lung is still developing. It is likely that treating a developing system has consequences on the effectiveness of the agents and the other way around these agents may unknowingly affect the normal development. Likewise, the response to therapy is dependent on the specific etiology of neonatal PH. A patient with a functionally maladaptation at birth might response well to short-term vasodilative therapy. Whereas a patient with structural underdevelopment of the lung probably needs chronic therapy and might benefit more from drugs not only providing vasodilation, but also prevent vascular remodeling and enhances normal lung growth.

Translational research is the key ingredient in the field of PH in neonates. We feel that with the rising number of drugs, it is of the utmost importance that these patients are treated in trial connection. Multicenter randomized controlled trials are mandatory to reach an area of evidenced based pharmacological therapy for newborns with PH. Moreover, well-conducted basic research will lead to a better understanding of the

complexity and interaction of the pathways involved in lung development and regulation of pulmonary vascular tone. And this will provide novel targets within known and unknown pathways. Eventually increasing understanding of the underlying pathophysiology will make it possible to target treatment specific to the different forms of PH in neonates.

Lastly, when drugs in clinics do not provide the expected outcome in a specific group of PH patients, like iNO in CDH, a closer look into the pathway in more specific animal models might reveal a restricting factor of the pathway or enclose interactions with other pathways that could be accounted for by using for example combination therapies.

In conclusion, apart from iNO clinical trials are largely underpowered and drugs studies based on small series don't provide level 1 evidences nor insight into optimal combination therapies (table 1.1). Therefore randomized controlled trials are warranted.

The field of treatment of pulmonary hypertension in newborns is moving rapidly. Some new promising drugs are recently introduced in clinical practice. They will be evaluated in multicenter randomized controlled trials, so their place in the treatment of PH in neonates will become clearer in time. As understanding of lung development, transition at birth and the regulation of pulmonary vascular tone evolves; the armamentarium of the neonatologist and pediatric intensivist will be extended with new possible therapeutic targets. Also novel treatment modalities as stem cell and gene therapy are future options in the field of PH in neonates. And in the specific group of antenatal pulmonary hypoplasia with risk of PH prenatal treatment with lung growth enhancing mediators belongs to the prospect. All will lead to a better outcome for newborns with pulmonary hypertension.

Pathway	Target	Available drugs	Status
Prostaglandin	PGI_2	Epoprostanol	Pediatric case studies
		Iloprost	Pediatric case studies
		Beraprost	Adult case studies
		Treprostinil	Adult case studies
Nitric oxide	NO	Inhaled NO	Pediatric randomized controlled trial
	sGC	Bay 41-2272	Experimental
	BH4		Experimental
Phosphodiesterase	PDE5	Sildenafil	Pediatric case studies
		Tadalafil	Adult clinical trial
		Vardenafil	Adult case reports
	PDE3	Milrinone	Pediatric case reports
Endothelin	ET-A/ET-B	Bosentan	Pediatric case reports
	ET-A	Sitaxsentan	Adult clinical trials
		Ambrisentan	Adult clinical trials
Natriuretic peptide	BNP	Nestritide	Pediatric case reports

Table 1.1 Pathways involved in treatment of neonatal pulmonary hypertension

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Chapter 2

Why study the pulmonary vasculature in congenital diaphragmatic hernia?

Outline of the thesis

Congenital diaphragmatic hernia

Congenital diaphragmatic hernia (CDH) is an anomaly that occurs in about 1 per 2000 to 3000 life births worldwide.[1,2] In the Netherlands, each year approximately 60 newborns are born with CDH. CDH represents a major cause of mortality and severe morbidity in the newborn period and remains a significant challenge facing the neonatologist, pediatric intensivist and pediatric surgeon.

In the past, CDH was considered solely as a result of a defect in the diaphragm that theoretically could be easily corrected after birth by removal of the herniated viscera and subsequent closure of the diaphragm. Since the 1980s, treatment has moved from an emergency surgical closure of the diaphragm to delayed surgery following initial resuscitation and stabilization of the newborn with CDH.[3] In many clinics around the world supportive therapy during the first hours of life consist of immediate intubation, ventilation with peak pressures as low as possible, decompression of the abdominal contents by a nasogastric tube, blood pressure support to prevent severe right-to-left shunting and comforting the patient by sedation and anesthesia.[4]

Despite advances in treatment modalities, mortality rates in life-born patients range from 10 to 60 %.[5-7] Today, CDH is considered more as a complex lung disease than as an anomaly resulting only from the diaphragm failing to form. Evidence suggests that failure of both lung parenchyma and pulmonary vascular development may occur independently of the defect in the diaphragm and as a result of defects in signaling pathways.[8,9] The degree of pulmonary hypoplasia and the severity of the pulmonary vascular abnormalities are key determinants of survival in CDH.[10,11]

Pulmonary vascular disease in CDH

Clinical an important part of the disease spectrum in CDH involves aberrant pulmonary vascular development, maladaptation of the pulmonary circulation at birth and subsequent persistence of the high pulmonary resistance. Therefore we propose to name CDH associated vascular anomalies: "pulmonary vascular disease".

The lungs of a newborn with CDH have fewer and smaller airways, an abnormal architecture of the respiratory acinus, a decreased number of vascular generations and increased pulmonary vascular wall thickness with muscularization extending too far into the periphery.[12] Although the morphology is well described and much progress is made in the determinants of normal lung development, our understanding is still deficient to identify the signaling pathways that instigate the abnormal lung development in CDH.

Increased pulmonary vascular resistance is an almost universal finding in CDH even when not clinically manifested as pulmonary hypertension (PH).[13] PH is essentially a reversible process, but may become chronic if treatment should fail. Present pharmacological treatment of PH consists of inhaled nitric oxide, prostaglandin, and more recently phosphodiesterase inhibitors.[14] As therapies proven to be effective in randomized control trials are not available, the treatment of the individual CDH patient should still be considered a matter of "trail and error". The (prenatal) identification of patients at risk is still a matter of debate.[15]

Apart from the initial morphological differences, the normal vascular changes that should occur in the neonatal period are hampered in CDH patients by persistent high pulmonary vascular resistance, exposure to hypoxic and hyperoxic episodes in addition to ventilator induced lung injury and inflammatory mediators. Little is known about the changes in the physiological remodeling process of the neonatal pulmonary vasculature that contribute to the development of refractory PH and consequently overall morbidity and mortality of CDH.

Research therefore needs to focus on understanding pulmonary vascular development, modulating pulmonary vascular reactivity, and prevention of a disordered process of postnatal pulmonary vascular remodeling in order to improve the outcome of CDH patients.

Aim of the studies described in this thesis

The main objective of our study is to identify new potential therapeutic targets aimed at the different aspects of pulmonary vascular disease in CDH.

In the first part of this thesis we determined the expression of various angiogenic factors of the HIF-VEGF pathway during normal human lung development and identification of potential differences in CDH patients (Chapter 3 and 4). In part two of this thesis we investigated downstream signaling of the NO-pathway. We used the nitrofen-induced CDH rat model to gain insight in the expression and function of phosphodiesterases inhibitors and various potassium channels (Chapter 5 and 6). Third part of this thesis is focused postnatal pulmonary vascular remodeling. We described the heterogeneity of vascular smooth muscle cell (SMC) in human CDH (Chapter 7) and studied the disordered extracellular matrix in postnatal injured lungs in animals and humans (Chapter 8).

In chapter 9 the results of our studies are incorporated into the discussion of the remaining questions about pulmonary vascular disease CDH. Furthermore suggestions are given for future research and new treatment strategies are contemplated.

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Chapter 3

Expression of hypoxia inducible factors in normal human lung development

Abstract

Pulmonary vascular development is essential for proper lung development and its disturbance can lead to neonatal morbidity and mortality, as exemplified in congenital diaphragmatic hernia. Hypoxia-inducible factors (HIFs) appear to be key molecules in physiologic angiogenesis and in certain forms of lung pathology, such as bronchopulmonary dysplasia. Little is known about the qualitative and quantitative expression of HIFs in normal human fetal lung development. Therefore, we investigated the expression of HIF-1α, HIF-2α and HIF-3α along with their upstream regulators and downstream targets, von Hippel-Lindau protein (pVHL), vascular endothelial growth factor A (VEGF-A) and its receptor, VEGFR-2 in 20 normal human fetal lungs (13.5 wk gestation till term) and five adult lungs. Quantitative PCR demonstrated a positive correlation between HIF-2α and VEGF-A expression and gestational age. Although there appeared to be a decreasing trend in HIF-3α expression during pregnancy, it did not reach statistical significance. Immunohistochemistry for HIF-1α and HIF-2α revealed that HIF-1α is expressed in the epithelium while HIF-2α is expressed in both interstitium and epithelium. Our data suggest that hypoxia-inducible factors, most notably HIF-2α, appear to exert an important role in angiogenesis during human fetal lung development especially in the last phases of pregnancy, preparing the fetus for extra-uterine life. As such our results form the baseline data for the evaluation and interpretation of abnormal pulmonary vascular development.

Introduction

Fetal lung development is an intricate process which is orchestrated by numerous growth and transcription factors, and morphogens.[1] Structurally, the airway branches and pulmonary vessels are closely aligned, suggesting an intimate interaction during lung development. A vascular network has been shown to be present from the earliest stage of lung development and pulmonary vascular development seems to be rate-limiting for airway branching.[2-6] The process of vascular development, in particular angiogenesis, is mainly driven by hypoxia. Moreover, the low-oxygen environment during fetal life is essential for normal development of the lung.[5,7]

Vascular endothelial growth factor A (VEGF-A) is a potent hypoxia-inducible growth factor for vascular patterning during lung development.[8] For instance, VEGF blockage impaired alveolar development and decreased pulmonary angiogenesis in newborn rats, mimicking bronchopulmonary dysplasia.[9] Conversely, postnatal VEGF gene therapy improved survival and increased lung capillaries and alveolarization in this rat model. VEGF-A binds and activates two tyrosine kinase receptors namely VEGFR-1, also known as fms-like-tyrosine kinase (Flt)-1, and VEGFR-2, also known as KDR in humans, or fetal liver kinase Flk-1 in mice.[10,11] VEGFR-2 null mutant mice die *in utero* due to lack of vasculogenesis and a defect in development of endothelial cells, indicative of the key role of VEGFR-2 in differentiation and/or proliferation of endothelial cells and vascular biology.[12]

One of the most potent activators of VEGF transcription is hypoxia-inducible factor (HIF), which is a heterodimer composed by one of three oxygen-sensitive alpha subunits (HIF-1α, HIF-2α, HIF-3α) and a constitutive nuclear protein, the beta subunit (HIF-1β/ARNT). The alpha subunit is rapidly hydroxylated by prolyl hydroxylases (PHDs) under normoxic conditions, leading to its association with the von Hippel-Lindau protein (pVHL). Subsequently, the hydroxylated alpha subunit is targeted for proteasomal degradation. Since the PHDs require oxygen for their activity, the crucial hydroxylation step does not occur under hypoxic conditions and the alpha subunit remains stable and dimerizes with the beta subunit to form an active HIF transcription factor.[13,14] Modifications to the HIF pathway have underlined its importance in development. Embryos with inactivating mutations of HIF-1α or HIF-2α all die in

mid or late gestation.[15-18]

Despite these studies in mice, very little is known about the expression of the VHL-HIF-VEGF pathway in human lung development. Although differential expression of the proteins HIF-1αand VHL has been observed in the lungs of CDH patients,[19] quantitative analysis of HIFs during normal and abnormal lung development is limited. We therefore investigated the mRNA expression of VHL, HIF-1α, HIF-2α, HIF-3α, VEGF-A and its receptor, VEGFR-2, by quantitative RT-PCR. In addition, we studied the expression of HIF-1α and HIF-2α by immunohistochemistry in normal human fetal lungs, to determine their spatial expression in normal lung development.

Material and methods

Tissue specimens

Normal human lung tissue (n=25) was retrieved from the archives of the Department of Pathology, Erasmus MC, Rotterdam, following approval of the experimental design and protocols by the Erasmus MC Medical Ethical Committee. Fetal and neonatal lungs (n=20) were obtained from terminations of pregnancy and newborns that had died within 1 hour of birth. None of these fetuses or newborns had abnormalities related to the lungs and none of the newborns received supplemental oxygen or mechanical ventilation. All tissues were harvested within 24 hours after death. The lung samples were divided into four groups (n=5) according to the lung developmental stages i.e. pseudoglandular (7-17 wk gestation), canalicular (18-26 wk gestation), saccular (27-35 wk gestation), and alveolar (36 wk gestation – term). As a fifth group, unaffected lung adjacent to surgical resections for lung carcinoma was obtained from 5 adults (28-49 yr). Tissue specimens were snap frozen, stored in liquid nitrogen until used for RNA isolation. For immunohistochemical studies, tissues were fixed by immersion in 4% buffered formalin and embedded in paraffin. Five μm thick paraffin sections were mounted on 3-amino-propyl-trioxysilane coated slides (Sigma, St. Louis, MO) and processed for immunohistochemistry.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from frozen lung tissues using Trizol reagent (Life Technologies, Rockville, MD) following the manufacturer's instructions. RNA was quantified by measuring the absorbance at 260 nm and the purity was checked by the 260/280 nm absorbance ratio. cDNA synthesis was carried out in a final volume of 20 µl, containing 1 µg of total RNA, 50mM Tris-HCL (pH 8.3), 75 mM KCl, 3 mM MgCl2, 108 mM DTT, 100 ng random hexamer primers, 0.5 mM dNTPs (Roche Diagnostics, Basel, Switzerland), 10 U RNase inhibitor, and 200 U Moloney Murine Leukaemia Virus reverse transcriptase (all reagents from Invitrogen, San Diego, CA). These samples were incubated for 1 hr at 37ºC followed by incubation for 15 min at 99ºC. Negative controls were prepared by omission of the Moloney Murine Leukaemia Virus enzyme. ºC

Real-time PCR was performed using an iCycler IQ Real time PCR detection system (Bio- Rad, Veenendaal, The Netherlands) and qPCR Core kit for SYBR Green I (Eurogentech, Seraing, Belgium). Gene-specific primers used in this study are shown in Table 1. Gene amplification was carried out by activation of Hot Goldstar enzyme at 95ºC for 10 min, followed by 40 cycles of 95ºC for 30 sec, 58ºC for 30 sec, 60ºC for 45 sec, and 75ºC for 15 sec. Each sample was run in triplicate and negative control samples and samples without cDNA templates were run in parallel. After each assay, products were run on a 1.5% agarose gel to check the size and specificity.

PCR results are shown as the relative expression level of normalized samples (ΔCt) in relation to the expression of the "calibrator" sample of 13.5 wk gestation ($2^{-\Delta\Delta Ct}$), which was arbitrarily set at 100% (arbitrary value = 1). The Ct value refers to the cycle number at which the PCR plot crosses the threshold line; ΔCt is calculated by subtracting the Ct value of the corresponding endogenous reference gene; RNA Polymerase II, Subunit A (POLR2A) from the specific Ct value of the target gene, and ΔΔCt is obtained by subtracting the ΔCt of each experimental sample from the ΔCt of the "calibrator" sample.

Gene	Forward Primer (5'□3')	Reverse Primer (5'□3')
HIF-1α	GCT CAT CAG TTG CCA CTT CC	CCT CAT GGT CAC ATG GAT GAG
HIF-2α	CCA ATC CAG CAC CCA TCC CAC	GTT GTA GAT GAC CGT CCC CTG
HIF-3α	ACC TGG AAG GTG CTG AAC TG	AAT CCT GTC GTC ACA GTA GG
VHL	CTC TCA ATG TTG ACG GAC AG	CCA GAT CTT CGT AGA GC
VEGF-A	AGA ATC ATC ACG AAG TGG TG	TGT TGT GCT GTA GGA AGC TC
VEGFR-2	CAG AGT GGC AGT GAG CAA AG	TAC ACG ACT CCA TGT TGG TC
POLR2A	CGG ATG AAC TGA AGC GAA TG	AGC AGA AGA AGC AGA CAC AG

Table 3.1 Primer sequences for quantitative PCR

Statistical analysis

Data from real-time RT-PCR are shown as mean $2^{-\Delta\Delta Ct}$ + SEM. Differences in mRNA expression between groups were analyzed with one-way ANOVA with the *post hoc* least significant difference (LSD) test. The correlations between mRNA expression and gestational age were determined by nonparametric Spearman's correlation. $P < 0.05$ was considered statistically significant. All statistics were calculated using SPSS (version 11.0; SPSS Inc., Chicago, IL).

Immunohistochemistry

Antibodies used were mouse monoclonal anti-human HIF-1α H1α67 (Neomarkers, Fremont, CA) 1:1000, and rabbit polyclonal anti-mouse HIF-2α PM8 antiserum 1:10,000.[20] Formalin fixed paraffin embedded sections were dewaxed in two sequential xylene baths, and rehydrated using graded ethanol washes. For antigen retrieval, sections were immersed in preheated DAKO target retrieval solution (DAKO, Carpinteria, CA) and treated for 90 seconds in a pressure cooker. Incubation time with primary antibodies was 1 hour at room temperature. Antigen/antibody complexes were revealed by means of the Catalyzed Signal Amplification system (DAKO) according to the manufacturer's instructions. Sections were counterstained with haematoxylin for 15 seconds, dehydrated in graded ethanol washes, and mounted in DPX (Lamb, Eastbourne, United Kingdom). Negative control staining was performed by omitting the primary antiserum and did not show any background staining (data not shown).

Results

Temporal expression (quantitative RT-PCR)

All investigated factors were expressed from 13.5 weeks gestation onwards to term, and in adults. In figure 3.1, the relative mRNA expression of each of the 6 factors investigated is depicted for each of the developmental stages and in the adult lung. Although there are reciprocal trends in the expression of HIF-2α and HIF-3α, with the former showing an increase throughout pregnancy and the latter showing a decrease, this does not reach statistical significance.

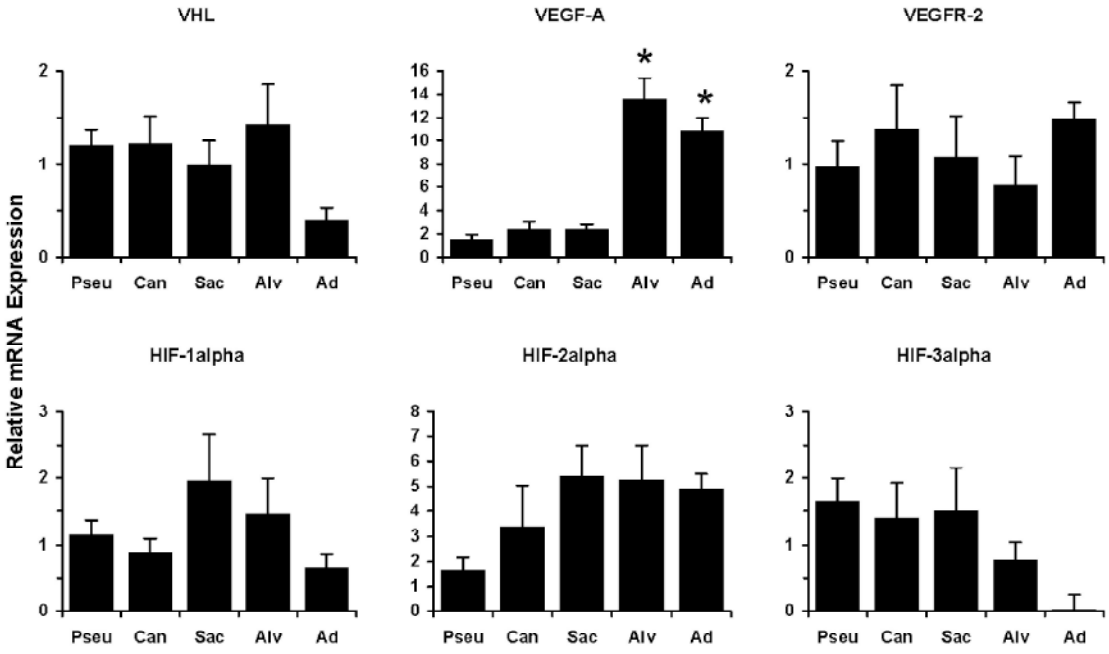

Figure 3.1 mRNA expression of VHL, VEGF-A, VEGFR-2, HIF-1α, HIF-2α, and HIF3-α in human lungs.
Samples were grouped according to their developmental stages i.e. Pseudoglandular (Pseu), Canalicular (Can), Saccular (Sac), Alveolar (Alv) and Adult (Ad); n = 5 per group. Data are shown as mean 2-ΔΔCt ± SEM (as outlined in *Materials and Methods*). VEGF-A expression is significantly higher in the alveolar stage and in adult (*, $P < 0.01$). There was no significant difference in mRNA expression in other genes ($P > 0.05$).

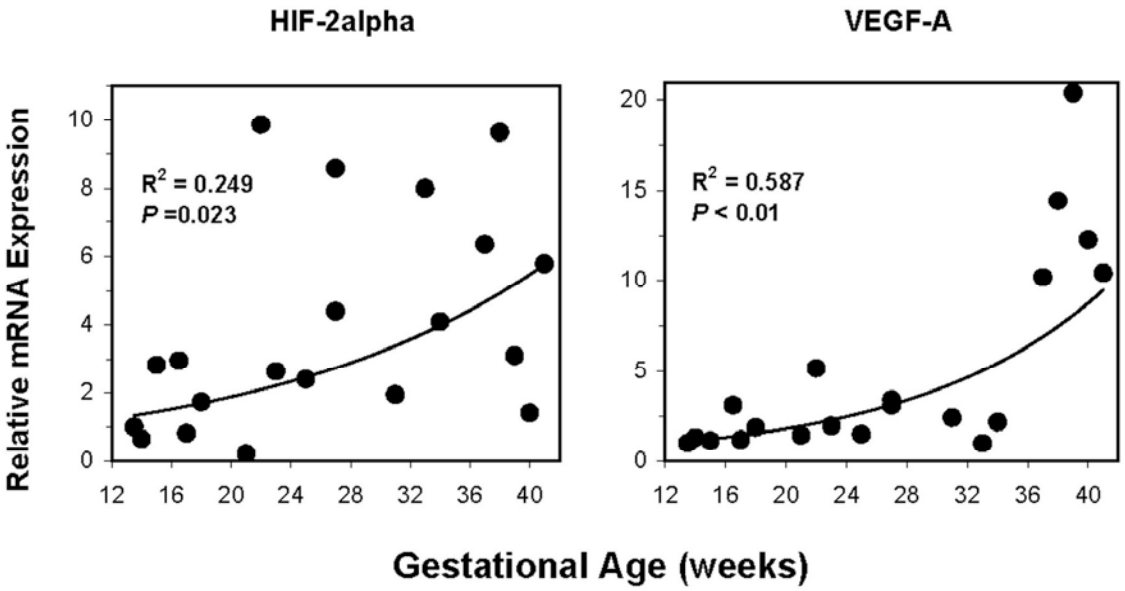

Figure 3.2
Positive correlation between HIF-2α and VEGF-A mRNA expression and gestational age of 20 samples from 13.5 to 41 weeks of gestation. The r-values show Spearman's correlation coefficient with gestational age.

However, there is a clear increase in the expression of VEGF-A near the end of pregnancy, i.e. in the alveolar phase, which is also apparent in adult lungs ($p<0.01$ for alveolar or adult phase vs. pseudoglandular, canalicular, or saccular phase). No obvious increase or decrease in the expression of VHL, HIF-1α, or VEGFR-2 can be seen. When the expression levels of all individual fetal samples are plotted against gestational age, HIF-2α and VEGF-A show a positive correlation in the course of fetal lung development (HIF-2α: r=0.249, P=0.023; VEGF-A: r=0.587, P<0.01) (Figure 3.2).

Immunohistochemistry for HIF-1α and HIF-2α

The immunostaining for HIF-1α and HIF-2α revealed that both subunits were expressed in all tissues examined. HIF-1α immunoreactivity was primarily detected in the airway epithelium and showed the consistent pattern throughout gestation (Figure 3.3 a-d). HIF-2α reactivity was detected mainly in the interstitium at early stages of lung development (Figure 3.4a, b), whereas its expression in the airway epithelium, predominantly in type II pneumocytes, was detected later on the gestation (Figure 3.4c, d)

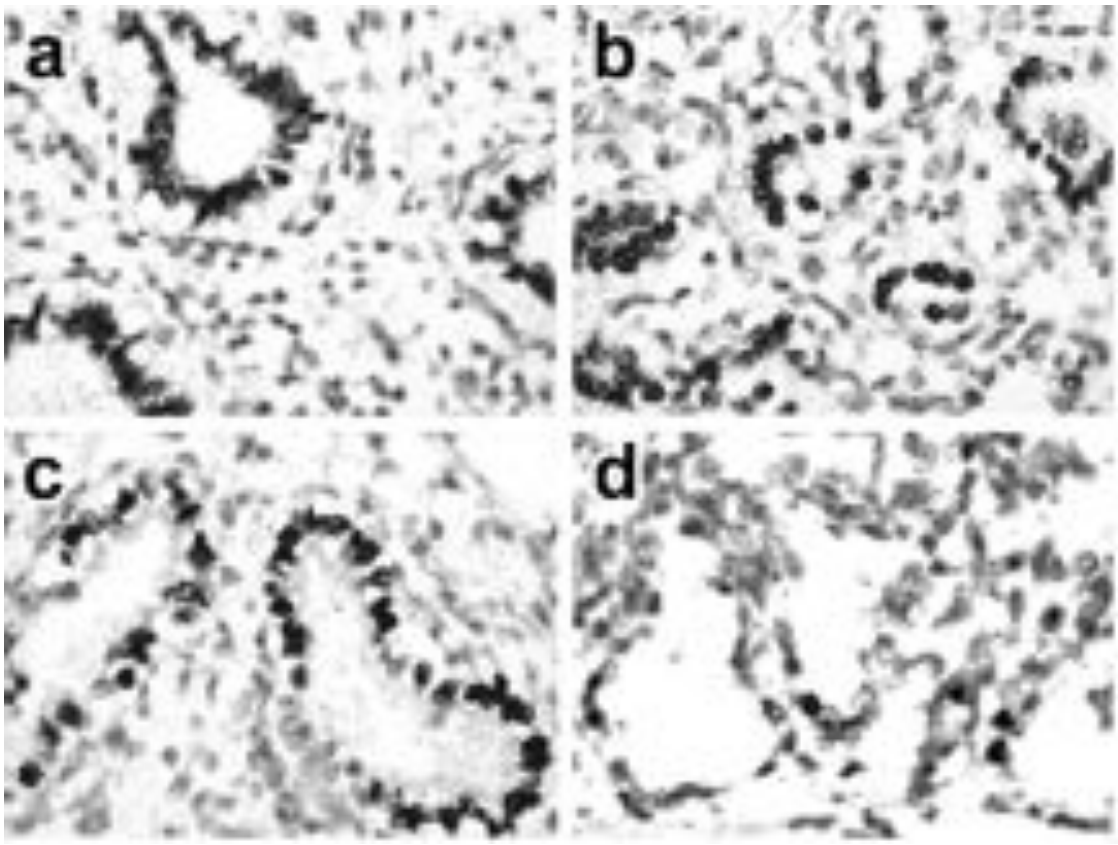

Figure 3.3 HIF-1α immunostaining (Color figure page 162)
Strong nuclear expression of HIF-1α in airway epithelium of human fetal lung at different gestational ages; (a) 16 wk, (b) 21 wk, (c) 27 wk, and (d) 31 wk. In Figure 3d staining is somewhat weaker than in the others. (Magnification, X 400)

Discussion

In this study, we analyzed the expression of factors involved in the VHL-HIF-VEGF pathway during human lung development. Our quantitative PCR data demonstrated that VHL, HIF-1α, HIF-2α, HIF-3α, VEGF-A and VEGFR-2 are expressed in human lung tissue from 13.5 weeks of gestation onwards. Moreover, HIF-2α and VEGF-A mRNA expression increases with advancing gestation. Immunohistochemistry was performed to show the site of HIF-1α and HIF-2α expression. HIF-1α expression was consistently detected in the airway epithelium throughout gestation. HIF-2α expression was first detected in the interstitium, while later on the gestation its expression was detected in type II pneumocytes. The site of expression of VHL and VEGF was shown in our previous work.[19]

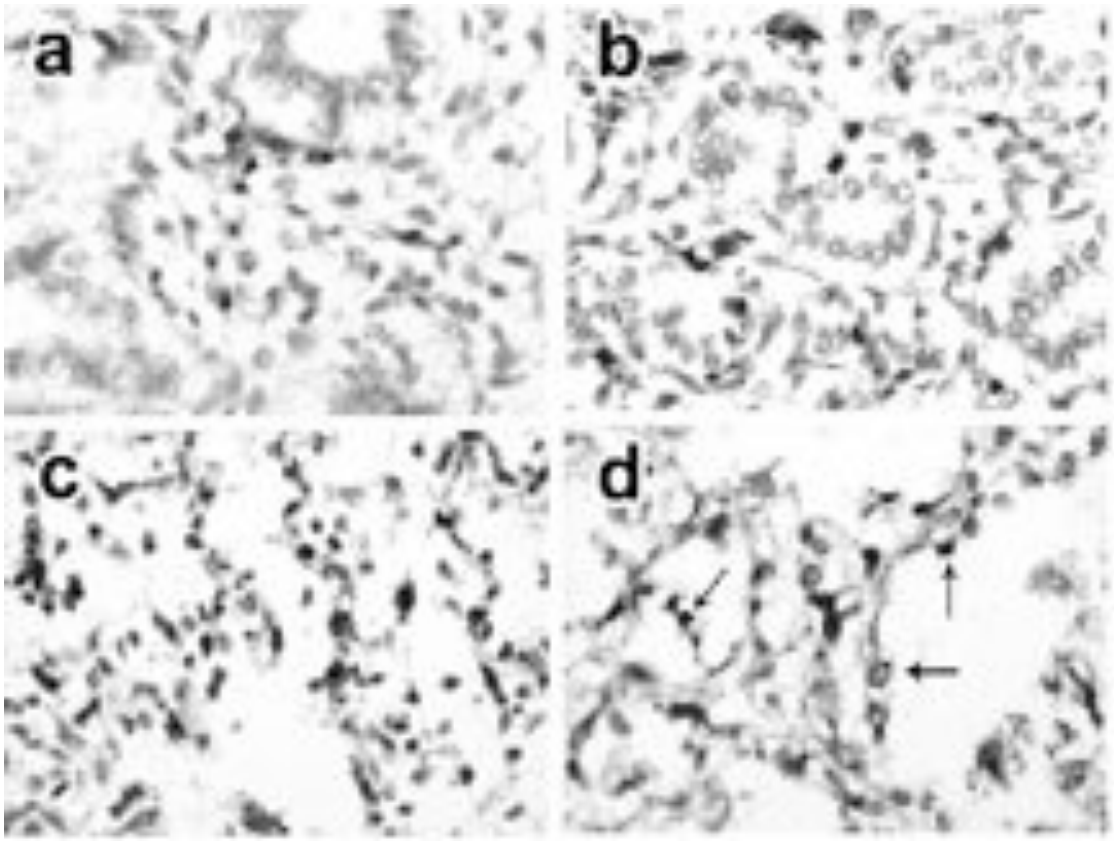

Figure 3.4 HIF-2α immunostaining (Color figure page 163)
Predominant nuclear reactivity of HIF-2α in the interstitium at early stages of human fetal lung development; (a) 16 wk, (b) 21 wk. Later on in gestation, HIF-2α expression was also detected in alveolar-lining cells; (c) 27 wk, and (d) 33 wk. Arrows indicate HIF-2α reactivity in alveolar-lining cells. (Magnification, X 400)

The intimate relationship between the pulmonary vasculature and the airways suggests that these two systems interact during development. Recent reports have indeed indicated an essential role for vascularization in airway development.[3-5,21] *In utero* the lung develops in a relatively hypoxic environment and one of the most potent angiogenic factors, VEGF-A, is actively transcribed under hypoxic conditions. VEGF-A transcription is upregulated in a hypoxic environment by one of oxygen sensitive HIF subunits.[22-24] Our PCR data showed a positive correlation between gestational age and mRNA expression of HIF-2α and VEGF-A. There is no significant change in the mRNA expression of HIF-1α and HIF-3α during fetal lung development. These results are compatible with previous studies in mouse lungs that

showed an increased expression of HIF-2α and VEGF during development, whereas HIF-1α remained constant.[15,22] Animal studies showed that HIF-2α deficiency and the lack of VEGF in later stages of lung development resulted in impaired lung maturation and insufficient surfactant production, the latter directly caused by VEGF regulation.[15] Using immunohistochemistry, we demonstrated that HIF-1α is mainly expressed in the bronchial epithelium throughout gestation, whereas HIF-2α is predominantly expressed in the interstitium in earlier stages and appears in the epithelium later in development. The expression of HIF-2α in the airway epithelial cells is in agreement with previous report in mouse lungs which demonstrated HIF-2α expression in type II pneumocytes.[15,22] Our findings are also in agreement with the recently published work of Groenman et al, who described HIF-1α and HIF-2α protein expression in human first trimester lungs in the same cell types.[25] The difference in the expression patterns between HIF-1α and HIF-2α may reflect their individual roles in lung development.

HIF-1α and HIF-2α share a high degree of structural and functional similarity as emphasized by their ability to interact with hypoxia response elements of the target genes and the subsequent induction of transcriptional activity.[13] However, ample evidences suggest that these two alpha subunits also have distinct physiologic roles. Whereas HIF-1α is ubiquitously expressed, HIF-2α transcripts are more restricted to particular cell types, for instance vascular endothelial cells, type II pneumocytes and liver parenchyma.[15,18,22,26] Analysis in embryonic stem cells suggested that HIF-1α, but not HIF-2α, plays an important role in hypoxia responses.[16,27,28] In contrast to the stem cells studies, HIF-2α has been shown to be crucial in stimulating several hypoxia-inducible genes (including tyrosine hydroxylase and VEGF) during embryonic development.[15,17,18] Moreover, there are a growing number of differences between HIF-1α and HIF-2α with respect to cell specific regulation, hypoxic and non-hypoxic stimuli, interaction partners, and downstream targets.[28-32] In animal studies, HIF-1α-/- mice exhibit midgestational lethality with severe blood vessel defects.[16] Although homozygous HIF-2α knockout embryos also showed a defect in vascular remodeling, with aberrant vascular formations,[17] a subset of the offspring survived to term but suffer from respiratory distress due to surfactant insufficiency.[15,17,18] The differences between HIF-1α and HIF-2α are further exemplified in a primate model of

bronchopulmonary dysplasia (BPD), in which it appears that HIF-1α, but not HIF-2α, expression decreases considerably at term, suggesting that both factors be related to the pathophysiology of BPD.[33]

A third HIF protein, HIF-3α, is also able to dimerize with HIF-1β and binds to hypoxia response elements. Among several splice variants of HIF-3α, inhibitory PAS domain protein (IPAS) is the best characterized. In adult mice under normoxic conditions, IPAS is predominantly expressed in Purkinje cells and corneal epithelium. However, IPAS can also be induced by hypoxia in the heart and lung. In contrast to the role of HIF-1α and HIF-2α, IPAS has no endogenous transactivation function and has been reported to act as a dominant negative regulator of HIF-1α.[23,34]

The VEGF signaling pathway has been shown to play an important role in embryonic vasculogenesis, particularly during fetal lung development.[8,10-12] The level of VEGF is critical for normal lung development. Overexpression of VEGF in distal lung altered vascularization and arrested airway branching,[8] whereas a decrease in lung VEGF resulted in poor septum formation and an emphysematous pattern.[35] In addition, reduced VEGF levels in tracheal aspirates were also observed in infants with bronchopulmonary dysplasia.[36,37]

It has been shown that the VEGF-pathway is important for both vascular development and branching morphogenesis.[2,6,38] The role of VEGF-A and its receptors in lung development was anticipated by their expression patterns. VEGF–A is expressed mainly in the epithelial cells,[39] while VEGFRs are expressed in the mesenchymal cells. VEGF-A is also detected in alveolar type 2 cells and has an effect on the synthesis of surfactant protein (SP). VEGF-treated human lung explants demonstrated increased SP-A and SP-C mRNA compared with control lungs.[40] Furthermore, VEGF can rescue HIF-2α+/- mice, which showed defective surfactant production, alveolar septum, vascular defects and respiratory distress at birth.[15] Our finding of increasing VEGF-A mRNA expression in fetal human lung during development suggests that the role of VEGF-A is not restricted to the initial phase of pulmonary development. Previous studies in VEGF-A knock-out mouse embryos have shown that VEGF-A is also necessary for events later in gestation such as vessel sprouting and maintenance of vessel integrity.[41,42]

VEGFR-2 appears to be the main receptor mediating the effect of VEGF-A on lung maturation. The study in mice with antibodies against VEGFR-1 and VEGFR-2 indicates that only VEGFR-2 mediates the effects of endogenous VEGF on lung maturation *in vivo*.[15] Thus far, there are limited data on the ontogeny of VEGFR-2 in human lung development. In this study, we demonstrated no significant change of VEGFR-2 mRNA expression during human fetal lung development.

Taken together, we have shown that hypoxia-inducible factors, particularly HIF-2α, appear to exert an important role in vascular growth and airway branching morphogenesis in human fetal lung, especially in the last phases of pregnancy. The suggestive anti correlation between HIF-2α and HIF-3α indicates a putative important regulatory role. This action occurs by regulation from interstitial and epithelial cells through an effect on downstream target genes, such as VEGF-A. These data contribute to our basic knowledge about normal pulmonary (vascular) development and may serve as a reference for the interpretation of pathological states such as primary pulmonary hypertension and congenital diaphragmatic hernia.

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Chapter 4

Expression of hypoxia inducible factors, its regulatory and target genes in congenital diaphragmatic hernia patients.

Abstract

Congenital diaphragmatic hernia (CDH) is associated with lung hypoplasia and pulmonary hypertension and has a high morbidity and mortality. The cause and pathophysiology of CDH are not fully understood. However, impaired angiogenesis appears to play an important role in the pathophysiology of CDH. Therefore, we examined different components of an important pathway in angiogenesis: hypoxia inducible factors (HIFs); their regulators VHL and PHD3 and their target genes VEGF-A and VEGFR-2.

Quantitative PCR of lung tissue showed a significantly decreased expression of VEGF-A mRNA in the alveolar stage of lung development in CDH patients compared with matched controls. In the canalicular stage no differences for VEGF-A was seen between lungs of CDH patients and those of control patients. Other components of angiogenesis (VHL, HIF-1α, HIF-2α, HIF-3α, VEGFR-2 mRNA and PHD3 protein) that were analyzed showed no differences in expression between CDH patients and controls, independent of the developmental stage.

A lower expression of VEGF mRNA in CDH patients in the alveolar stage, possibly as a result of down regulation of HIF-2α might indicate a role for those factors in the pathophysiology of CDH.

Introduction

The probability of survival in congenital diaphragmatic hernia (CDH) is mainly determined by the severity of lung hypoplasia and its associated pulmonary hypertension.[1] The lungs in CDH patients are characterized by maldevelopment of airways, impaired vascular development and a disordered process of postnatal vascular remodeling.[2] Pulmonary vessel morphometry in CDH shows a decreased volume of the pulmonary vascular bed, an increased adventitial and medial thickness and muscularized distal pulmonary arterioles.[3,4] Newborns with CDH have a delayed transition to postnatal circulation due to respiratory failure. In a number of cases they develop pulmonary hypertension resulting in major treatment challenges and eventually death.

The most potent inducer of angiogenesis is vascular endothelial growth factor A (VEGF-A).[5] Furthermore, VEGF-A is abundantly expressed in parenchyma of the developing lung.[6] Also during postnatal alveolarization of the lung, angiogenesis in general, and VEGF-A in particular, has an important role.[7] The VEGF-A receptor, VEGFR-2, mediates the major part of VEGF-A-driven vascular growth.[5] The transcription of both VEGF-A and VEGFR-2 is mainly regulated by hypoxia inducible factor (HIF).[8]

The HIF transcriptional complex is a heterodimer composed by one of the three alpha subunits (HIF-1α, HIF-2α, HIF-3α) and a constitutive nuclear protein, the beta subunit (HIF-1β). Aside from common target genes, HIF-1α and HIF-2α also regulate unique sets of genes that play important roles during development.[8] Furthermore, HIF-3α appears to be reciprocally regulated in the response to hypoxia resulting in negative feedback.[9] Especially HIF-2α has an important role in the remodeling of the primary vascular network.[10]

Post-transcriptional regulation of the HIFα isoforms occurs through hydroxylation of specific proline residues by prolyl 4-hydroxylases (PHD), which are active at hypoxic conditions (Figure 4.1). At low oxygen concentration, the hydroxylated HIFα proteins associate with the Von Hippel-Lindau protein (pVHL) and are subsequently targeted for proteosomal degradation. PHD is expressed in early human lung development and

inhibition of PHDs prevents the degradation of HIFα at normoxia, which leads to amelioration of the pathophysiology in an animal model with brochopulmonary dysplasia.[11,12] This indicates that PHDs are potential therapeutic targets.

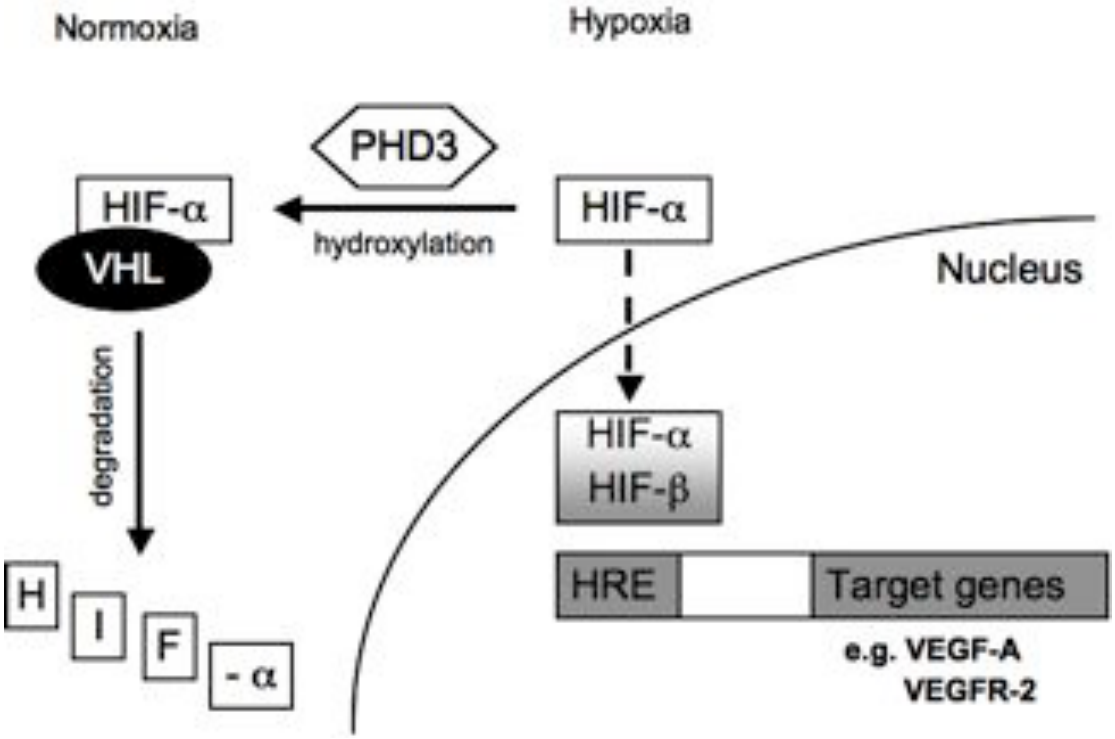

Figure 4.1 Overview of the regulation of HIF
Oxygen dependent degradation of HIF and the activation of its target genes involved in angiogenesis.

In an earlier study, we showed that HIF-2α and VEGF expression in the human lung correlate with increasing gestational age, underscoring their importance in pulmonary development.[13] However, little is know about the expression of the various HIFs and their target genes in CDH patients. We hypothesize that these factors might be involved in the early (canalicular) and late transitional (alveolar) stages of the pathophysiology of CDH. Therefore we analyzed with real time PCR the expression of *HIF-1α, HIF-2α, HIF-3α* and *VHL* and two targets of *HIF, VEFG-A* and *VEGFR-2*, in human control and CDH lung samples in the canalicular and alveolar stage. Furthermore, location and expression of PHD3, the predominant post-transcriptional regulator of HIF-2α, is evaluated by immunohistochemistry.

Material and methods

Human lung tissue was retrieved from the archives of the Department of Pathology of the Erasmus MC, Rotterdam, following approval by the Erasmus MC Medical Ethical Committee. From formalin-fixed and paraffin-embedded lung tissue of a series of CDH patients, we selected 16 CDH patients, who died within 24 hr after birth or from termination of pregnancy. Patients were grouped (n=10 alveolar stage and n=6 canalicular stage) based on their gestational age and histological appearance of their lung tissue. Neonates, deceased from non-pulmonary causes served as controls and were matched for gestational age (n=5 canalicular stage and n=6 alveolar stage), duration of postnatal survival and spontaneous breathing and/or ventilation (Table 4.1). Snap frozen lung tissue of 7 CDH patients (n=3 canalicular stage and n=4 alveolar stage) was used for RNA isolation.

	Developmental stage	n	Gestational age (wk)	Postnatal age (hr)	Birth weight (gr)	Lung/body ratio
Control	canalicular	5	21 (15-29)	0.2 (0-1)	522 (57-1480)	unknown
	alveolar	6	38 (35-40)	6.5 (1-24)	2863 (2390-3190)	unknown
CDH	canalicular	6	22 (15-29)	0.2 (0-1)	318 (30-813)	0.0095 (0.0017-0.0155)
	alveolar	10	37 (34-41)	7 (1-24)	2425 (1250-3800)	0.0040 (0.0013-0.0137)

Table 4.1
Characteristics of congenital diaphragmatic hernia (CDH) and control group used for immunohistochemistry.

Real time PCR

Total RNA was extracted from frozen lung tissue (30-60 mg) using Trizol reagent (Life Technologies, Rockville, MD) following the manufacturer's instructions. cDNA was synthesized using 1 μg of total RNA by reverse transcription and used in real-time PCR as previously described.[13] A qPCR Core kit for SYBR Green I-assay (Eurogentech, Seraing, Belgium) and gene-specific primers for target genes *VHL, HIF-1α, HIF-2α, HIF-3α, VEGF-A* and *VEGFR-2* were used for real-time PCR. All reactions were performed in triplicate and controlled using standard non-template as

negative control and an identical positive control. PCR results are shown as $2^{-\Delta\Delta Ct}$ using *POLR2A* as the endogenous reference control and a sample of 13.5 wk gestation as "calibrator", which was arbitrarily set at 100% (arbitrary value=1). Because we used the same calibrator sample and performed the experiments under the same conditions, comparisons can be made between CDH patients and previously published controls.[13]

Immunohistochemistry

Immunohistochemistry was performed on 4 μm sections of paraffin-embedded lung tissue, mounted on coated slides (Starfrost®, Berlin, Germany) according to standard protocol, using the EnVision™ detection system (DakoCytomation, Glostrup, Denmark).[13] Primary antibody was a polyclonal rabbit anti-PHD3 (1:2000, Novus Biologicals, Littleton, CO). Antigen retrieval consisted of boiling for 15 minutes in Tris-EDTA buffer (pH 9). Two independent observers (IvdH and PvdV) examined the slides for PHD staining and scored each sample as strong (3), clear (2), weak-but-visible (1) or negative (0).

Statistical analysis

For relative mRNA expression of the HIF pathway differences between CDH patients and controls were analyzed with unpaired *t* test. The chi-square test was used for proportions of PHD3 immunostaining scores of CDH patients vs. controls. A *p*-value of 0.05 was considered as statistically significant.

Results

Real-time PCR

Previously, we have shown that *VHL, HIF-1α, HIF-2α* and, *HIF-3α* and their target genes *VEGF-A* and*, VEGFR-2* are expressed during the canalicular and the alveolar stages of lung development.[13] In lung samples from CDH patients all abovementioned factors were expressed at both stages of development (Figure 4.2).

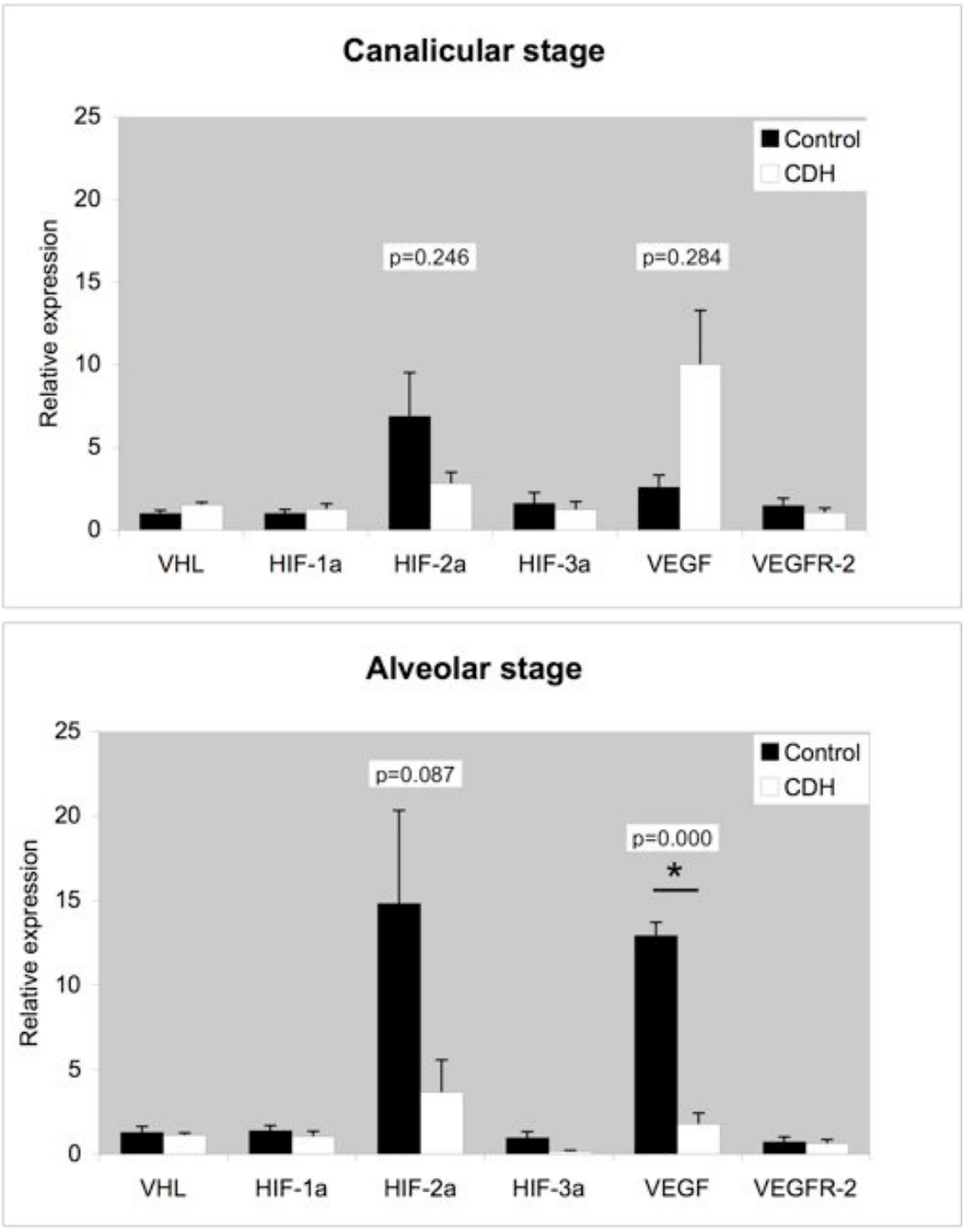

Figure 4.2
Relative mRNA expression of Von Hippel Lindau (VHL), hypoxia inducible factors (HIF-1α, HIF-2α and, HIF-3α) and vascular endothelial growth factor (VEGF) and its receptor (VEGFR-2) measured by quantitative real-time PCR in lung tissue of congenital diaphragmatic hernia (CDH) patients and matched controls in A) the canalicular stage (CDH n=3 and control n=5) and B) the alveolar stage (CDH n=4 and control n=5).

From all expressed factors *HIF-2α* and *VEGF-A* showed the highest relative expression in the canalicular and the alveolar stage. *VEGF-A* expression was significantly (p=0.000) lower expressed in CDH patients than in controls in the alveolar stage. *VEGF-A* expression in the canalicular stage was similar in CDH patients and controls. Expression of *VHL, HIF1-α, HIF-2α, HIF-3α,* and*, VEGFR2* showed no difference between CDH patients and controls, or in the alveolar or canalicular stage of lung development.

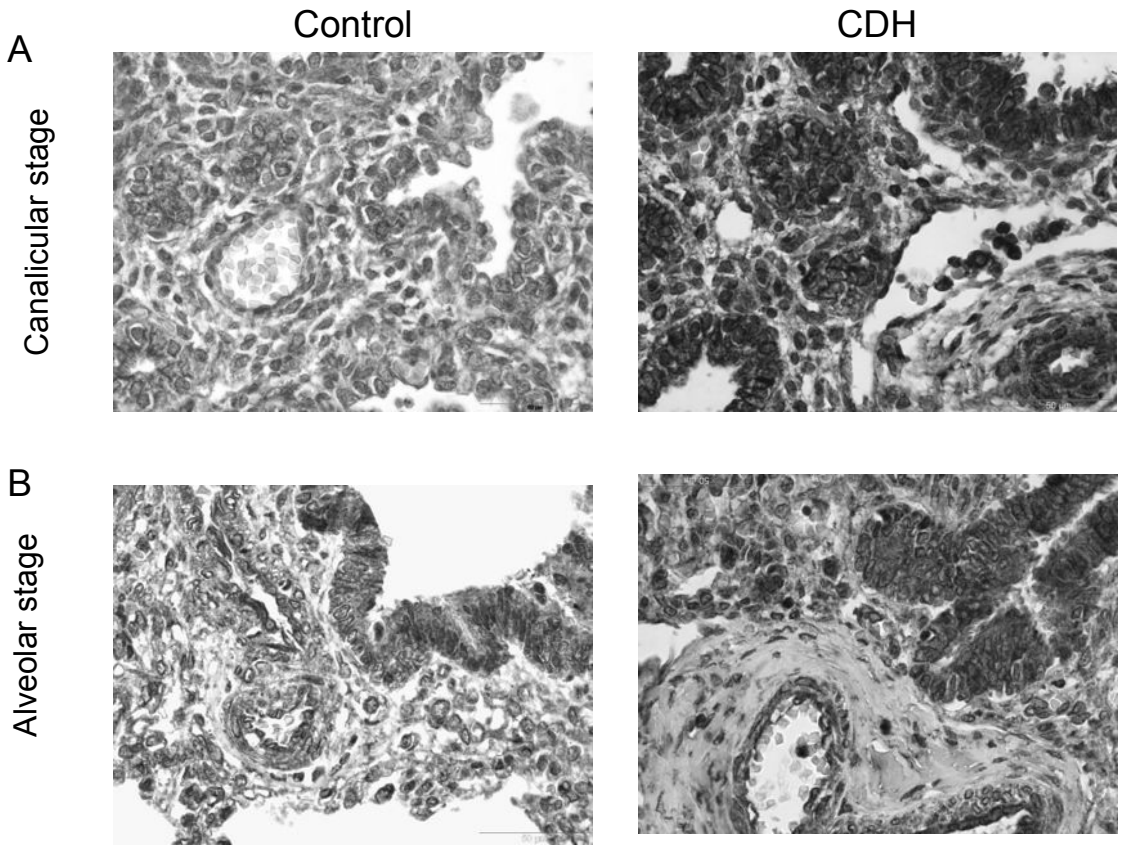

Figure 4.3 (Color figure p 164)
Immunostaining with anti-prolyl hydroxylase 3 (PHD3) of congenital diaphragmatic hernia (CDH) and control lung tissue in A) the canalicular and B) alveolar stage of lung development showed positive expression of PHD3 in all cell types of the lung, except for the arterial adventitia. All cases scored a clear to strong staining and no differences were found between CDH patients and controls for neither developmental stage. (Original magnification 400x)

Immunohistochemistry

For localization and expression of PHD3 we used immunohistochemistry. PHD3 showed a cytoplasmatic expression, which was strongly present in the bronchial epithelium and endothelium of arterioles. Moreover PHD3 was expressed in most cell types of the lung, except for the arterial adventitia (Figure 4.3). All cases scored a clear to strong staining in vessels of all sizes as well as bronchial structures of the lung. We did not observe differences between samples from CDH patients and controls in the canalicular or the alveolar stages.

Discussion

In this study we evaluated the mRNA expression of the various HIFs, their target genes VEGF-A and VEGFR2 and the regulatory protein PHD in human CDH lungs and found that the abovementioned factors were expressed in the canalicular stage as well as in the alveolar stage. For the first time, a significantly lower mRNA expression for VEGF-A was seen in CDH patients in the alveolar stage. Due to the limitation of human patient material available, we have only been able to perform measurements at specific time points in development. Although our material is unique, our conclusions relate only to these time points and not to other stages of lung development.

Interestingly, VEGF mRNA expression was significantly decreased in CDH patients compared with controls, who were treated with similar ventilation strategies. In this study we used lung tissue of patients that died within 24 hours after birth to look specifically at the transitional stage. In the transition to extra uterine life, pulmonary arteries have to adapt to the gas exchanging function and increased blood flow.[14] Therefore the capillary network has to be recruited, resistance arteries need to dilate, and the pulmonary vasculature has to remodel. VEGF is necessary for directing capillary sprouting and governing remodeling of the existing capillary network.[15] Inhibition of VEGF in this early postnatal period leads to impaired vascular maturation and subsequent arrest of alveolarization.[16] These features are seen in lung morphometry of deceased CDH patients.[17] Moreover, several animal studies show

that decreased VEGF mRNA expression is associated with pulmonary hypertension, which is very common in CDH patients.[18] Conversely, similar and increased VEGF protein expression has been reported by our group in deceased CDH patients.[19,20] A reason for this difference remains speculative, but the hypoxic circumstances in which these patients have died may add to the differences reported in VEGF mRNA and protein expression.[21]

Our results demonstrated similar mRNA expression of VEGF in CDH patients compared with controls in the canalicular stage. From several animal studies there are contradicting results: some report decreased expression of VEGF in canalicular stage lungs of nitrofen-induced CDH in mice and rat[22,23], while others report increased or no difference in VEGF expression in CDH rats during gestation.[24,25]

Expression of VEGF receptors has been suggested to play a role in the variation in VEGF function. However, no change in the amount of VEGFR-2 mRNA was seen during lung development, or between control and CDH lung tissue. Moreover, in the nitrofen-induced CDH rat model no change in VEGFR-2 was observed.[26] Although VEGF and VEGFR-2 are both transcription targets of HIF, there is no evidence for a reciprocal regulation of both factors, which could explain an increased expression of VEGF without an increase in VEGFR-2 expression.

In the alveolar stage, lungs of CDH patients seemed to express less HIF-2α. However this result did not reach statistical significance, probably due to small numbers. HIF-2α increases during gestation and stays stable after birth, whereas HIF-1α is postnatal downregulated.[27] This suggests a potential role for HIF-2α during the transitional period of the fetus to adapt to life in an oxygen-rich environment. Furthermore, HIF-2α is required for the remodeling of the primary vascular network into a mature pattern[10] and is closely related to VEGF expression.[27]

In the canalicular stage the expression of HIF-2α mRNA in CDH patients' lungs was comparable to controls. Moreover, loss of HIF-2α in HIF-2α -/- mice did not affect lung development during the canalicular stage.[28] The function of HIF-2α during early lung development and the consequences of altered expression remain elusive.

The expression of two HIF-2α-related genes, HIF-1α and HIF-3α did neither differ between control and CDH patients, nor in the canalicular stage or in the alveolar

stage. Previously, we showed that protein expression of pVHL and HIF-1α was decreased in CDH patients, comparable to HIF-2α mRNA expression.[20] HIF-1α is mainly post-translational regulated; its protein expression is increased by hypoxia.[27] As all of our investigated cases died under hypoxic conditions, this could account for the increased stability of the protein without increased mRNA expression.

Upstream in the regulation of HIF we showed similar protein expression of PHD3 in control versus CDH lung tissue as well as in canalicular stage and alveolar stage. Of the three known human PHDs (PHD1, PHD2, and PHD3) we were specifically interested in PHD3, as this isoform has the highest affinity for HIF-2α and is functionally important during prolonged periods of hypoxia.[29] Immunohistochemistry is not quantitative and therefore it may mask any possible differences in PHD expression. From the literature we know that HIFs and PHDs are reciprocally regulated and play key roles in the pathogenesis of hypoxia-induced pulmonary hypertension.[30] The possible therapeutic inhibition of PHDs and the consequently stabilization of HIF could enhance postnatal lung growth, which might be beneficial to CDH patients.[31]

The results of our study suggest that a lower VEGF-A expression, possibly as a result of selective downregulation of HIF-2α may contribute to the pathophysiology of the transitional/alveolar lung development of CDH patients.

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Chapter 5

Expression and function of various phosphodiesterases in experimental nitrofen-induced congenital diaphragmatic hernia in rats

Abstract

Background: Congenital diaphragmatic hernia (CDH) is an anomaly associated with pulmonary hypoplasia and pulmonary hypertension (PH). The limited efficacy of current approaches to treat PH in CDH, including inhaled nitric oxide (NO), drives the search for other therapies. Phosphodiesterases (PDEs) degrade cyclic nucleotide second messenger cAMP and cGMP downstream of NO thereby limiting the vasodilatory response to NO.

Objective: To identify therapeutic targets by cataloguing the expression and function of PDE isoforms in the pulmonary vasculature in nitrofen-induced CDH in fetal rats.

Methods/Results: Quantitative RT-PCR revealed PDE 1 to 5 and PDE 9 mRNA expression in pulmonary arteries (PAs) of control and nitrofen-induced CDH term fetal rats. In this order of potency, the PDE inhibitors Sildenafil (PDE5) >EHNA (PDE2) >Rolipram (PDE4) >Cilostamide (PDE3) all dilated isolated 3rd generation PA after preconstriction with the thromboxan analogue U46619. Hyperoxic pre-incubation of PAs significantly attenuated vasodilatation induced by the PDE5 inhibitor Sildenafil (65% vs. 33%, $P< 0.004$). CDH PAs dilated significantly less to PDE2 inhibitor EHNA compared to control (51% vs. 72%, $p<0.05$). Subsequently PDE2 protein expression was higher in PAs of CDH animals.

Conclusion: Most PDE isoforms exist in the pulmonary arteries of fetal rats and their inhibition causes pulmonary vasodilatation. PDE5 inhibition was the most potent vasodilator, however there were no differences between groups. PDE5-induced vasodilatation was attenuated by hyperoxic pre-incubation. PDE inhibitors might be considered therapeutic targets in combination with iNO in neonates with CDH.

Introduction

Congenital diaphragmatic hernia (CDH) is characterized by a defect in the diaphragm and lung hypoplasia. Lung hypoplasia and refractory pulmonary hypertension (PH) largely determine the high morbidity and mortality in CDH. With an incidence of 1 in 2,500 newborns, CDH remains one of the major congenital pathologies in pediatric surgery.[1] Randomized controlled trials showed that inhaled nitric oxide (iNO) significantly improves oxygenation and decreases the combined outcome death/need for extracorporeal membrane oxygenation in term and near term infants with persistent pulmonary hypertension (PPHN).[2-4] Yet, only 30% of CDH patients respond to iNO, underscoring our current limitation in managing PH in infants with CDH.[5]

NO causes pulmonary vasodilatation through activation of soluble guanylate cyclase (sGC). sGC generates cyclic guanosine monophosphate (cGMP) in the underlying vascular smooth muscle cell. cGMP in turn activates the cGMP-dependent protein kinase (PKG) that serves as a primary regulator of vascular tone. The level of cGMP in the cell is controlled by a family of catabolic enzymes, cyclic nucleotide phosphodiesterases (PDE). There are 11 different, but homologous PDE gene-families, based on substrate (cGMP or cAMP) affinity, selectivity and regulation mechanisms.[6] Auto-feedback mechanisms and cross-regulation mechanisms between cyclic nucleotides and phosphodiesterases make this a complex mechanism in the regulation of vascular tone. However the differential tissue distribution of PDEs and the importance of NO in dilating the pulmonary circulation at birth make them a desirable target for pharmacological intervention.[5]

It is suggested in an experimental rat model that impaired responsiveness to iNO in CDH patients is due to rapid degradation of cGMP by PDE5.[7] Inhibition of PDE5, a PDE that is cGMP-specific and largely expressed in the lung, is effective in lowering the pulmonary vascular resistances and a promising therapeutic option in PH.[8] Besides PDE5, other PDEs may also hold therapeutic promise in neonatal PH. However the expression and function of these PDEs in the neonatal pulmonary circulation is relatively unexplored. We hypothesized that PDE isoforms expression is

higher in nitrofen-induced CDH pulmonary arteries (PAs) and therefore PDE inhibitors cause increased vasodilatation in CDH PAs compared to control PAs.

Material and methods

Animal model

The Animal Health Care Committee of the University of Alberta approved all procedures and protocols. Time-pregnant Sprague-Dawley rats (Charles River) randomly received either 100 mg nitrofen dissolved in 1 ml olive oil or 1 ml vehicle by gavage on gestational day E9.5. Day of vaginal plugging was defined as gestational day E0.5. At E21.5 (term), fetus were delivered by caesarian section and killed by decapitation. From control fetus and nitrofen-exposed fetus with diaphragmatic defect, the heart and lungs were removed en-block and the third generation PA of the left lobe was dissected for RNA extraction and organ bath studies. Whole lungs were either flash frozen for protein extraction or formalin fixed and paraffin embedded for immunohistochemistry.

Real-time Polymerase Chain Reaction

Total RNA (n=4 for CDH and control) was extracted from at least 3 pooled isolated term fetal rat PAs using RNeasy Mini kit (Quiagen, Mississauga, Canada) and quantified with UV spectrophotometry. The TaqMan One-Step RT-PCR Master Mix reagent kit in combination with predesigned primers TaqMan® Gene Expression Assay (both Applied Biosystems, Foster City, CA) was used to quantify mRNA expression of PDE1A, PDE1B, PDE1C, PDE2A, PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D, PDE5A, PDE9A and, PDE11A. Levels of mRNA were normalized to a housekeeping gene (18S rRNA) and expressed as $2^{\Delta\Delta CT,}$ as described previously.[9]

Organ bath studies of fetal rat PAs

A third-generation branch of the main pulmonary artery (with a calculated internal diameter <60 μm and length of ≈2 mm segments) were mounted in microvascular wired-myograph (Danish Myo Technology, Denmark) 6-ml organ baths containing Krebs bicarbonate solution (NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and, glucose 8.3 (mM); pH7.4) aerated with normoxia (21% O_2/5% CO_2/74% N_2) or hyperoxia (95% O_2/ 5% CO_2) and maintained at 37ºC. Changes in contractile force were recorded with a PowerLab Data Acquisition System. Following a 30-min stabilization period, the internal diameter of the vessel segments was set to the experimentally derived optimal resting tension of 20 mmHg. After preconstriction with U46619 (0.1 μM) concentration response curves (CRCs) were created for inhibitors of PDE1 (8-IBMX 10^{-7} -10^{-5} M, CDH n=2 and control n=3), PDE2 (EHNA, 10^{-6}-10^{-4} M, n=8 for both groups), PDE3 (Cilostamide, 10^{-6}-10^{-4} M, n=8 for both groups), PDE4 (Rolipram, 10^{-9}-10^{-5} M, CDH n=7 and control n=10) and, PDE5 (Sildenafil, 10^{-6}-10^{-4} M, CDH n=11 and control n=12). To mimic the clinical situation in which most newborns are ventilated with an inspiratory fraction O_2 (FiO_2) of 1.0, we exposed PAs to hyperoxia. In a separate set of experiments PDE5 inhibition by Sildenafil (CDH n=6 and control n=17) was recorded under hyperoxic conditions, (pH=7.35-7.45, PO_2=±344 mmHg) starting 60 min prior to U46619 constriction.

Immunoblotting and Immunohistochemistry

As PDE2 inhibition by EHNA showed a decreased relaxation in CDH PAs we performed protein expression assays for this particular PDE. Flash frozen whole lungs (n=3 for both groups) were homogenized in RIPA buffer containing antiprotease cocktail (Sigma). Protein concentrations were calculated and equalized before being run on 6% SDS-PAGE gels. Protein expression was visualized with rabbit anti-PDE2A antibody (1:5000, Fabgennix, USA). Clip-170 (1:1000, ref) was used as protein loading control.[10] The same antibody (1:200, microwave antigen retrieval) was used in standard immunohistochemistry assay on paraffin embedded lung slides to locate PDE2 expression.[11]

Statistics and drugs

Values are expressed as mean ± SEM. Relaxation responses are expressed as a percentage of the contraction to U46619. Intergroup comparisons were performed with a *t* test or a factorial repeated-measures ANOVA, as appropriate. Fisher's protected least significant difference test was used for post hoc comparisons. A *p*-value of < 0.05 was considered to be statistically significant. All drugs, except Sildenafil, a generous gift from Pfizer Pharmaceuticals (Sandwich, England), were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Sildenafil, Cilostamide, Rolipram and 8-IBMX were dissolved in DMSO. EHNA was dissolved in water.

Results

Expression of PDEs in pulmonary arteries of term fetal rats

Quantitative RT-PCR showed expression of PDE-1A, 1B, 1C, 2A, 3A, 3B, 4B, 4D, 5A and, 9A mRNA in PAs from control and CDH term fetal rats (Figure 5.1). No amplification was found for PDE-4A, 4C and, 11A. The relative highest expression was found for PDE5A in both groups. Although not significant, PAs from CDH rats showed higher mRNA levels for all expressed PDEs except for PDE4D.

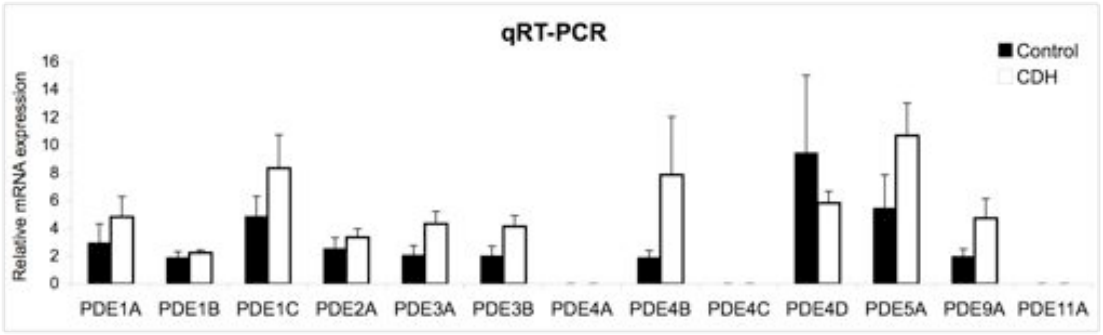

Figure 5.1 Relative mRNA expression of various phosphodiesterases (PDEs)
mRNA extracted of pulmonary arteries from term fetal rat, control or nitrofen-induced congenital diaphragmatic hernia showed expression of PDE-1A, 1B, 1C, 2A, 3A, 3B, 4B, 4D, 5A and, 9A. Each bar represents mean ± SEM of n=4 qRT-PCR with in each reaction at least 3 pulmonary arteries pooled.

PDE inhibition dilates pulmonary arteries of term fetal rat

Amongst the expressed PDEs, pharmacological inhibitors were available for PDE1, PDE2, PDE3, PDE4 and PDE5. Both, PAs of control and CDH term fetal rats dilated significantly in response to these PDE inhibitors except for PDE1 (Figure 5.2). Relaxation of PAs was approximately 65%, 60% and 35% for Sildenafil (PDE5 inhibitor), Rolipram (PDE4 inhibitor) and Cilostamide (PDE3 inhibitor) respectively. No vasodilatation was seen after PDE1 inhibition by 8-IBMX.

In response to the PDE2 inhibitor EHNA, CDH PAs relaxed significantly less compared to control PAs (51% vs. 72%, $p<0.05$) (Figure 5.2).

Hyperoxia decreases PDE5 induced pulmonary artery relaxation

Pre-incubation of PAs in hyperoxia significantly reduced PA relaxation induced by 0.1mM Sildenafil by 50% (65% vs. 33%, $P< 0.004$) (Figure 5.3) in both, control and CDH groups.

PDE2 protein expression is increased in nitrofen-induced CDH

Western blot analysis showed higher PDE2 expression in whole CDH lungs as compared to controls (Figure 5.4A). Immunohistochemistry showed that PDE2 expression was located in the media of the pulmonary artery wall, in the bronchus epithelium and also in type 2 alveolar cells (Figure 5.4B).

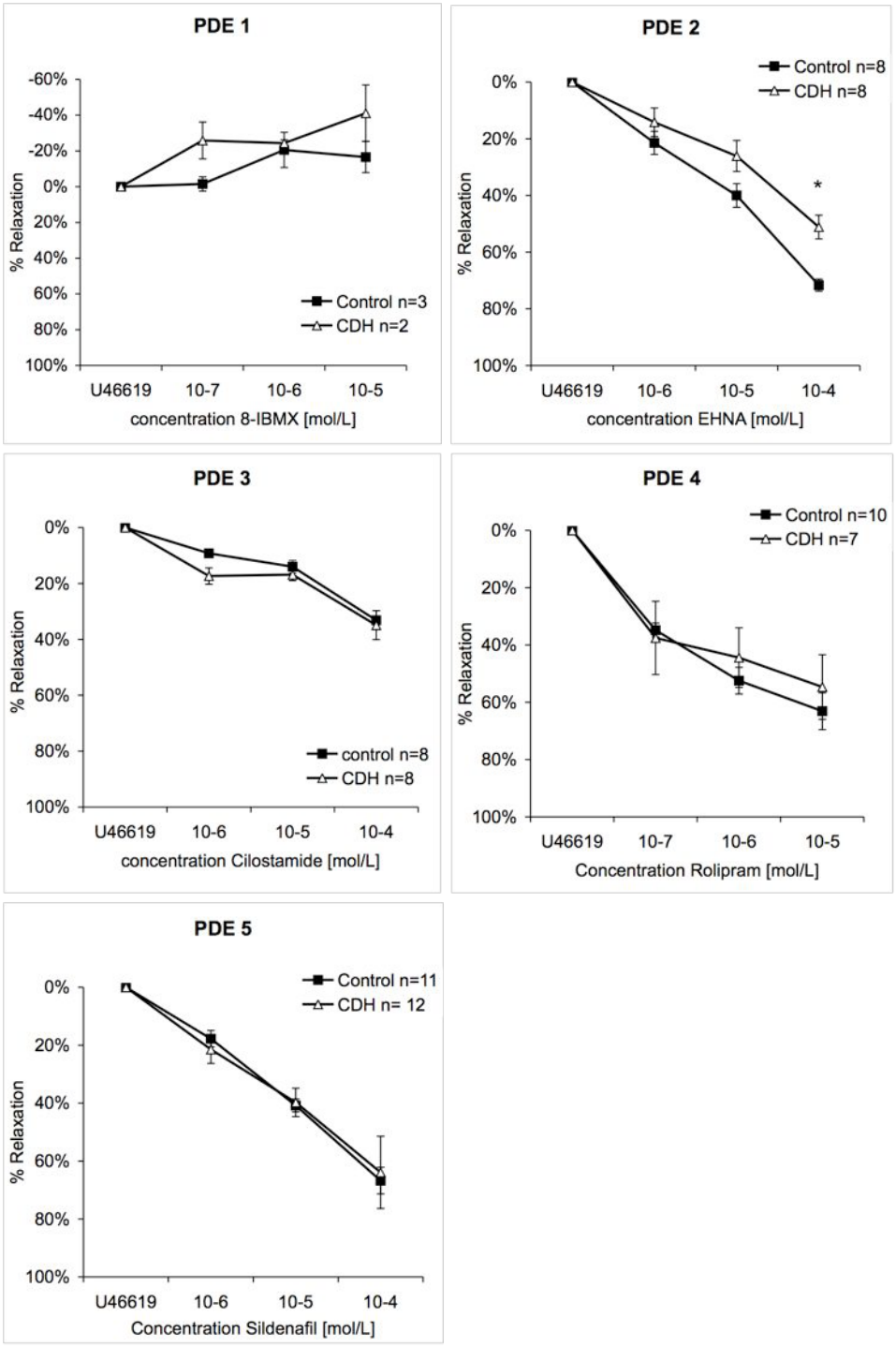

Figure 5.2 Concentration response curves of various phosphodiesterase inhibitors showed significant relaxation of third generation pulmonary arteries of control and CDH fetal rats in response to a) IBMX (CDH n=2, control n=3), b) EHNA (n=8 both groups), c) Cilostamide (n=8 both groups), d) Rolipram (CDH n=7, control n=10), and e) Sildenafil (CDH n=11, control n=12). Relaxation is shown as mean percentage ± SEM of preconstriction to U46619. A significant differences was found between CDH and control PAs for relaxation by 10^{-4} mol/L EHNA (51% vs. 72% $p<0.05$)

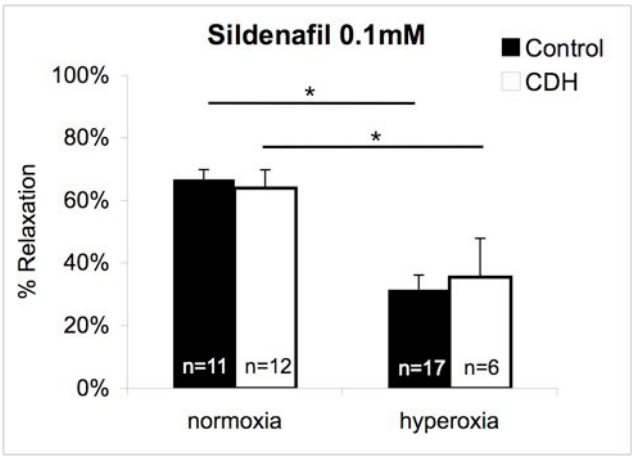

Figure 5.3
This bar graph shows mean percentage ±SEM relaxation of control (normoxia n=12, hyperoxia n=17) and CDH (normoxia n=11, hyperoxia n=6) pulmonary arteries by PDE5 inhibitor Sildenafil. Under hyperoxic condition PAs relax significantly less by 0.1mM Sildenafil in both groups (CDH 64% vs 35%, $p<0.006$, control 67% vs 32%, $p<0.001$).

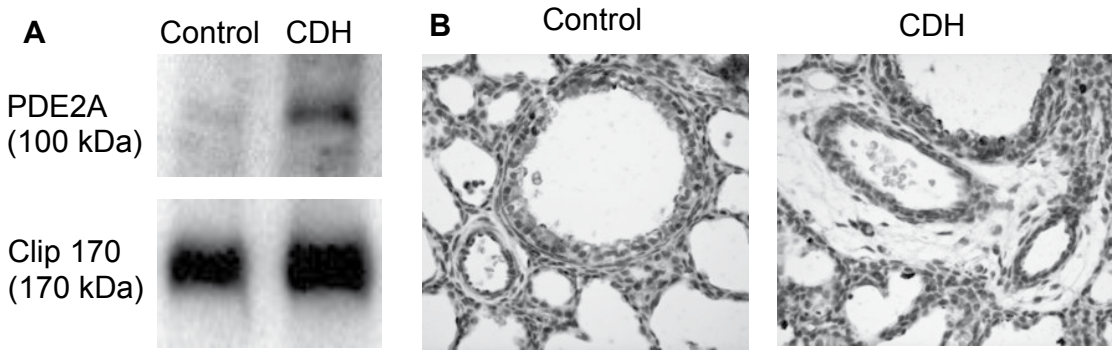

Figure 5.4 (Color figure page 165)
A) Western blot of protein extracted form whole lung showed lower PDE2 expression in CDH (n=3) fetal rat compared to control (n=3). Clip-170 was used as protein loading control.
B) Immunohistochemistry showed expression of PDE2 mainly in bronchial epithelium, arterial media and type 2 pneumocytes.

Discussion

This is a descriptive study showing the expression of PDE 1 to 5 and, 9 in the PAs of normal term fetal rat and in fetal rats with nitrofen-induced CDH. Except for PDE1 and PDE9 (not assessed), all available PDE inhibitors caused PA relaxation *in vitro* in both groups. This study is the first to assess the role of various PDEs in the normal transitional pulmonary vasculature and in experimental CDH in rats.

Our study demonstrates the expression of PDE1, PDE2, PDE3, PDE4, PDE5, and PDE9 in the PAs of term fetal rats. A similar expression profile has been described in the main PA in adult rats.[12] Different from our hypothesis, the results showed no increased PDE mRNA expression in PAs of nitrofen-induced CDH term fetal rat compared with control. Accordingly, no increased PA relaxation in response to the various PDE inhibitors was found in CDH fetuses, see discussion below. Only few studies in various animal models of pulmonary hypertension have shown up-regulation of some of the PDEs, such as PDE5 and PDE3.[13,14]

Growing evidence supports the involvement of several PDEs in the regulation of vascular tone. PDEs might have little impact on resting tension, but may modulate acute smooth muscle cell contraction.[6] Our data support a role for PDE2, PDE3, PDE4 and, PDE5 in the regulation of pulmonary vascular tone. Our experiments suggested that these PDE inhibitors induce the relaxation of fetal rat PAs in the following order of magnitude: Sildenafil > EHNA > Rolipram > Cilostamide. The potential of the various PDE isoform inhibitors to relax fetal PAs differed from studies described in adult rat PAs. Pauvert et al. showed that Cilostamide > Zaprinast (PDE5 inhibitor) > Rolipram >> EHNA relax adult rat PAs. We speculate this differences to be due to maturational regulation of PDE isoenzymes expression/activity from the neonatal period to adulthood. Moreover cyclic nucleotides are shown to hydrolyze faster in fetal arteries and to accumulate after birth contributing to the decrease in pulmonary vascular resistance at birth.[15]

In line with other studies in various animal models and humans we found Sildenafil inhibition of PDE5 most potent in relaxing the PAs.[16,17] PDE5 expression and activity is significantly decreased within 1 hour after birth suggesting its importance in the

pulmonary transition at birth.[18] Consequently, case studies in newborns with PPHN and PH associated CDH have already shown beneficial effects of Sildenafil on lowering the pulmonary resistance and improving cardiac output.[19,20] Interestingly, hyperoxic pre-incubation of PAs significantly attenuated the relaxation induced by Sildenafil. Farrow et al. previously showed that hyperoxia increases PDE5 expression and activity, leading to impaired vasodilatation in response to Sildenafil.[21] As ventilation with high FiO_2 is frequently used in CDH patients, the potential benefits of PDE5 inhibition may be reduced.

This study showed a significant dilatation to the PDE2 inhibitor EHNA, which is attenuated in PAs from fetal rats with CDH. Hypoxic pulmonary vasoconstriction is reversed by PDE2 inhibition by EHNA in isolated perfused rat lung.[22] Beside inhibition of PDE2, EHNA can also directly stimulate the adenosine induced endogenous NO production.[23] A malfunction in this second pathway could explain an attenuated relaxation to EHNA, while we found an increased PDE2 protein expression. These results suggest a possible role for PDE2 in the pathogenesis of PH in CDH.

Interestingly we found a substantial PA relaxation in response to Rolipram (PDE4 inhibitor) and Cilostamide (PDE3 inhibitor). Both PDE4 and PDE 3 are responsible for cAMP hydrolyses, indicating a potential role for these two inhibitors in combination therapy with prostaglandins,[24] an emerging therapy in CDH patients to relieve the right ventricle from excessive afterload by reopening the ductus arteriosus.[25]

We conclude that the inhibition of different PDEs might present therapeutic targets in the treatment of PH in newborns. Of the PDE inhibitors used in this study, Sildenafil is the most potent, justifying current investigations as a therapeutic option in CDH patients.[26] Our data further support the observation that PA vasodilatation is impaired by even short exposure to hyperoxia, therefore lower FiO_2 strategies might be considered in combination with the pharmacological treatment of PH.

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Chapter 6

Function and expression of potassium channels in pulmonary arteries of term fetal rats with nitrofen-induced congenital diaphragmatic hernia.

Abstract

Pulmonary hypertension (PH) contributes significantly to the mortality and morbidity in newborns with congenital diaphragmatic hernia (CDH). CDH patients respond poorly to the current vasodilator therapy, inhaled NO. NO acts partly through the large conductance Ca^{2+} sensitive potassium (K^+) channel (BK_{Ca}). K^+ channels are important for maintaining low pulmonary vascular resistance. K^+ channels are down regulated in adult forms of PH. Therefore we evaluated the expression and function of K^+ channels in the pulmonary circulation of a nitrofen-induced CDH model in rats.

Expression of BKca, Kv1.5, and Kv2.1 was assessed by qRT-PCR and Western blot. The pharmacological function of K^+channels was evaluated in isolated 3rd generation pulmonary artery (PA) rings of term fetal rats in a wired myograph. mRNA amplification showed significant higher expression of Kv1.5 in PAs from CDH animals and similar expression of BKca and Kv2.1 compared to controls. Western blot showed similar protein expression for BKca, Kv1.5 and Kv2.1 in control and CDH PAs. PA vasodilatation to the BKca channel opener NS1619 was less in term rat fetus with CDH compared to controls (37% vs. 57%, p=0.046). The Kv inhibitor 4-AP showed no differences in constriction between control and CDH PAs. The non-specific K^+ channel blocker TEA and BKca inhibitor IBX had no effect on baseline tension of the pulmonary arteries.

In conclusion, these data imply that the function of the BKca channel is impaired in term rat fetus with nitrofen-induced CDH, although this is not reflected by change in protein or mRNA expression. Activation of BKca channels might be considered to enhance pulmonary vasodilatation in CDH.

Introduction

Congenital diaphragmatic hernia (CDH) is one of the most common congenital anomalies in pediatric surgery with an incidence of approximately 1 in 3000 live births.[1] The relative high mortality in neonates with CDH compared to other major congenital anomalies is due to the pulmonary hypoplasia and in particular pulmonary hypertension (PH).[2] Although iNO is nowadays first choice in the treatment of neonatal PH, in CDH patients there is no evidence of improved outcome.[3] Moreover about 30% of the patients do not respond to iNO and triggers an ongoing search for effective vasodilators in CDH.[4,5] This raises the question if the NO pathway in CDH is impaired downstream of NO.

NO causes vasodilatation in part by opening the large conductance Ca^{2+} sensitive K^+ channel (BK_{Ca}).[6] Furthermore, the BK_{Ca} channels appear to mediate pulmonary vascular dilation at birth.[7] Failure of the pulmonary vasculature to dilate at birth induces PH. PH in newborns with CDH and the poor response to iNO may result from altered BK_{Ca} channels function, i.e. decreased function/expression of the BK_{Ca} channels.

Besides BK_{Ca} the pulmonary vasculature expresses more families of K^+ channels.[8] In the fetus and around birth, the BK_{Ca} channels control the resting membrane potential in pulmonary arterial smooth muscle cells.[9] After birth, there is a maturational shift to O_2-sensitive voltage-gated activated (Kv) channels. Kv channels mediate hypoxic pulmonary vasoconstriction (HPV).[10] HPV is intrinsic to the lung and essential in the regulation of an optimal ventilation/perfusion match, without increasing pulmonary vascular resistance.[11] In animal models of CDH HPV is blunted and might contributed to the persistent PH.[12] Moreover, in adult forms of PH the expression and function of O_2 sensitive Kv channels is decreased.[13]

The relative importance of the different type of K^+ channels in the transition at birth and in refractory PH in newborns with CDH, however, remains controversial. Therefore, we quantified the expression of K^+ channels and studied pharmacological modulation of the K^+ channels in the pulmonary vasculature *in vitro* in the nitrofen-induced CDH rat model, a well-established model that reliably mimics the pulmonary abnormalities seen in human CDH.[14-16]

Material and Methods

Animal model

The Animal Health Care Committee of the University of Alberta approved all procedures and protocols. Time-pregnant Sprague-Dawley rats (Charles River) randomly received either 100 mg nitrofen dissolved in 1 ml olive oil or 1 ml vehicle by gavage on gestational day E9.5 (day of vaginal plugging was defined as gestational day E0.5). At E21.5 (term), fetus were delivered by caesarian section and killed immediately by decapitation. From control fetus and nitrofen-exposed fetus with diaphragmatic defect, the heart and lungs were removed en-block and the third generation PA of the left lobe was dissected for RNA extraction, protein extraction or organ bath studies.

Real-time Polymerase Chain Reaction

Total RNA was extracted from isolated term fetal rat PAs using RNeasy Mini kit (Quiagen, Mississauga, Canada) and quantified with UV spectrophotometry. The TaqMan One-Step RT-PCR Master Mix reagent kit in combination with predesigned primers TaqMan® Gene Expression Assay (both Applied Biosystems, Foster City, CA) was used to quantify mRNA expression of Kv1.5, Kv2.1 and, BK_{Ca}. Levels of mRNA are normalized to a housekeeping gene (18S rRNA) and expressed as $2^{\Delta\Delta Ct}$ values.[17]

Immunoblotting

Flash frozen 3rd generation PAs were homogenized in RIPA buffer containing antiprotease cocktail (Sigma). Protein concentrations were calculated and equalized before being run on 7.5% SDS-PAGE gels. Protein expression was visualized with rabbit anti-Kv1.5 antibody (1:200, Sigma-Aldrich, Oakville, ON, Canada), rabbit anti-Kv2.1 antibody (1:500, US Biologicals,) and, rabbit anti-BK_{Ca} antibody (1:500, Sigma-Aldrich, Oakville, ON, Canada).

Organ bath studies of fetal rat PAs

Third generation PAs were cut into segment of ≈2 mm length and mounted in microvascular wired-myograph (Danish Myo Technology, Denmark) 6-ml organ baths containing Krebs bicarbonate solution (NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and, glucose 8.3 (mM); pH7.4) aerated with normoxia (21% O_2/5% CO_2/74% N_2) and maintained at 37ºC. Changes in contractile force were recorded with a PowerLab Data Acquisition System (Chart v5.2). Following a 30-min stabilization period, the internal diameter of the vessel segments was set to the experimentally derived optimal resting tension of 20 mmHg. After preconstriction with U46619 (0.1 μM) concentration response curves (CRCs) were created for TEA a potassium-selective ion channel blocker (10 mM), 4-AP a voltage-gated K^+ channel blocker (1mM, 5 mM, 10 mM), Iberiotoxin an inhibitor of BK_{Ca} (200 nM) and, NS1619 a BK_{Ca} channel opener (10^6 -10^{-4} M).

Statistics and drugs

Values are expressed as mean ± SEM. Relaxation responses are expressed as a percentage of the contraction to U46619. Intergroup comparisons were performed with a *t* test. A *p*-value of < 0.05 was considered to be statistically significant. All drugs were soluble in water, except for NS1619, which was dissolved in DMSO.

Results

Expression of potassium channel in term fetal rat pulmonary arteries

The voltage-gated K^+ channels, Kv1.5 and Kv2.1 and the BK_{Ca} channel were all expressed in the PAs of term fetal rat. The relative mRNA expression of Kv1.5 was significantly higher in CDH than in control (2.77 vs. 1.43, p=0.009) (Figure 6.1A). Kv2.1 and BK_{Ca} relative mRNA expression showed no difference between CDH and control.

Western blot showed protein expression of Kv1.5, Kv2.1 or BK_{Ca} in the PAs of term fetal rat (Figure 6.1B). However no significant difference was found between CDH and control group.

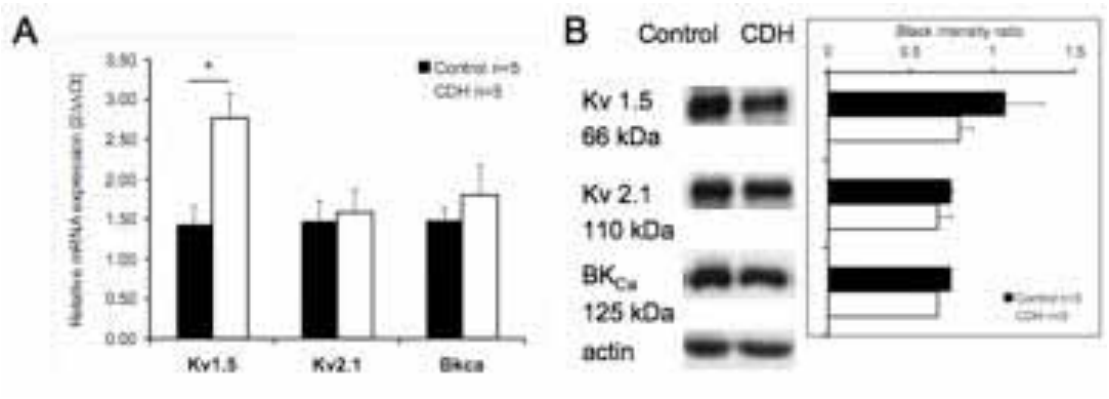

Figure 6.1
A) RT-PCR of potassium channel Kv1.5, KV2.1 and BK_{Ca} in mRNA extracted of pulmonary arteries of nitrofen-induced congenital diaphragmatic hernia (CDH) and control term fetal rat showed expression of all channels. Kv1.5 mRNA was significantly higher in term fetal rat with nitrofen-induced CDH compared to control (2.77 vs. 1.43, p=0.009). Each bar represents mean $2^{\Delta\Delta Ct}$ ± SEM of n=5 PCR with in each reaction at least 3 pulmonary arteries pooled.
B) Protein expression of potassium channel Kv1.5, KV2.1 and BK_{Ca} analyzed by western blot in pulmonary arteries of nitrofen-induced congenital diaphragmatic hernia (n=3, per n number at least 9 PAs are pooled) and control (n=3, per n number at least 9 PAs are pooled) term fetal rat showed no differences between groups.

Function of potassium channels in term fetal rat pulmonary arteries

Opening of the BK_{Ca} channel by NS1619 relaxed the pulmonary artery of term fetal rats by about 50 % of the preconstriction induced by U46619 (Figure 6.2A). The relaxation to NS1619 was significantly less in the PAs of nitrofen-induced CDH fetal rats than in control (for 10^{-4} mol/L NS1619 37% vs. 57%, p=0.046).

Inhibition of the BK_{Ca} channel by Iberiotoxin did not induce vasoconstriction in PAs of CDH fetal rats nor in control. Pretreatment with Iberiotoxin attenuated the NS1619 induced relaxation in both groups (data not shown). Non-specific inhibition of potassium channels by TEA did not result in vasoconstriction of PAs of CDH fetal rats or in control.

Inhibition of the Kv channels by low dose 4-AP (0.1-1mM) led to constriction of PAs, similar in CDH and control fetal term rats (Figure 6.2B).

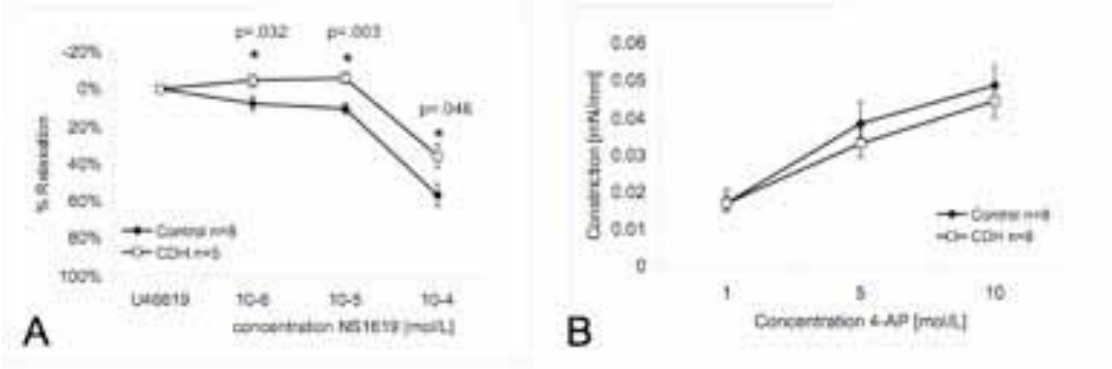

Figure 6.2
A) Concentration response curves of NS1619, a BK_{Ca} opener showed significant relaxation of third generation pulmonary arteries of control (n=8) and CDH (n=5) fetal rats. Relaxation is shown as mean percentage ± SEM of preconstriction to U46619. A significant difference was found between CDH and control PAs for relaxation by NS1619 (10^{-4} mol/L NS1619 37% vs. 57%, p=0.046)
B) Concentration response curves of 4-AP, a Kv channel blocker, showed significant constriction of third generation pulmonary arteries of control (n=8) and CDH (n=8) fetal rats. No difference was found between CDH and control PAs.

Discussion

Neonates with CDH show a maladaptation at birth and are likely to develop PH. The results of this study showed an increased relative mRNA expression of Kv1.5 in PAs of nitrofen-induced CDH term fetal rat compared to control. Furthermore the relaxation to NS1619, a BK_{Ca} channel opener, was significantly attenuated in the CDH group. Potassium channels in the pulmonary vasculature may contribute to normal pulmonary vasodilatation at birth and their impairment may lead to PH in CDH.

Potassium channels are generally expressed in the vascular smooth muscle cells and control vascular tone. We showed that term fetal rat PAs express Kv1.5, Kv2.1 and BK_{Ca} mRNA and protein. The relative mRNA expression of Kv1.5 was significantly higher in PAs of CDH animals. In human adult lung circulation Kv1.5 expression is mainly found in the resistance arteries, where Kv1.5 controls HPV.[18] A higher Kv1.5 expression in CDH fetal rats, compared to controls, might imply an increased sensitivity to hypoxia in CDH PAs with excessive vasoconstriction in response to hypoxia. However, there were no differences in Kv1.5 protein expression between groups. The basis for this discrepancy is unexplained. Furthermore the higher Kv1.5

mRNA expression in CDH has no functional implications on PA constriction since the PA response to the Kv channel antagonist 4-AP was similar between controls and CDH. Nevertheless, in a hypoxia-induced PH model in piglets, constriction to 4-AP is reduced like in adult forms of PH.[19] Beside the differences in species, the postnatal age of these piglets is longer and therefore their pulmonary vascular transition might be more completed increasing the importance of the Kv1.5 channel function.

Contrary to our hypothesis BK_{Ca} expression is similar in PAs of CDH rats and controls. In another study on the expression of K+ channel in the nitrofen rat model, a decreased expression was found for BK_{Ca} RNA by semi-quantitative PCR.[20] Also in an ovine model of neonatal PH, BK_{Ca} mRNA expression was decreased.[21] Both studies used whole lung homogenates instead of the dissected PAs we used, which could explain the difference in results. On the other hand, in our study PAs of CDH fetal rats relaxed significantly less in response to the pharmacological BK_{Ca} opener NS1619. BK_{Ca} is known to be O2 sensitive and mediates membrane hyperpolarization necessary for normal transition at birth.[22] In an ovine model of neonatal PH the contribution of BK_{Ca} to membrane potential and O2 sensitivity is decreased.[23] This is in line with our results, as Iberiotoxin did not cause vasoconstriction on baseline. Therefore, the function of BK_{Ca} channels seems to be impaired in the nitrofen CDH rat model. Also systemic hypertension is associated with deficient functional activity of $BK_{Ca.}$ [24,25]

In conclusion, the results of this study suggest that the function of BK_{Ca} channels in the pulmonary arteries of term fetal rat with nitrofen-induced CDH is impaired. However, importance of BKCa channels in human pulmonary vascular development and in the pathophysiology of PH is still incomplete and mainly based on animal studies. Furthermore the regulation of pulmonary vascular tone in the perinatal period is complex and influenced by several other pathways, like prostacyclin and endothelin. Nevertheless, activation of BK_{Ca} might be considered as a therapeutic target to lower the pulmonary vascular resistance in CDH associated PH.

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Chapter 7

Pulmonary arteries in human congenital diaphragmatic hernia express more contractile protein.

Abstract

Congenital diaphragmatic hernia (CDH) remains one of the major congenital anomalies and significant challenges on the neonatal intensive care. Besides a defect in the diaphragm, the survival of CDH patients is mainly determined by the associated lung hypoplasia and in particular the severity of pulmonary hypertension (PH). Lung hypoplasia is characterized by a smaller pulmonary vascular bed and pulmonary hypertension is related to increased medial wall thickness and extended muscularization of the pulmonary arteries. Both indicate the importance of the pulmonary arteries and especially the pulmonary vascular smooth muscle cell (SMC) in the pathogenesis of CDH associated PH. SMCs exhibit extensive phenotypic diversity and plasticity marked by their expression of contractile proteins.

We hypothesize that the pulmonary artery wall of CDH patients consists of an altered balance of subtypes of SMC. We evaluated the expression of SMC myosin heavy chain (SMMHC), alpha smooth muscle actin (αSMC), and SMC myosin light chain (Myl-9), h-caldesmon and calponin, desmin, and cellular retinoid binding protein (CRBP) by immunohistochemistry on tissue-micro arrays of 24 CDH patients and 24 matched controls.

The pulmonary arteries of CDH patients show a significant higher expression of SMMHC, independent of gestational age, survival, ventilation or use of ECMO. No other differences were noted in the expression of SMC markers between CDH patients and controls. Calponin showed an increasing expression with gestational age and diminished with ECMO treatment.

In conclusion, CDH patients expressed significantly more SMMHC in their pulmonary arteries, suggesting an altered pulmonary SMC subpopulation or maturation to be involved in the pathogenesis of PH associated with CDH.

Introduction

Congenital diaphragmatic hernia (CDH) represents a significant clinical and scientific problem. With an incidence of 1 in approximately 3000 live births, CDH is one of the most common pediatric surgical anomalies.[1] Not closure of the diaphragm is the main concern in treatment of newborns with CDH, the associated lung hypoplasia and pulmonary hypertension in particular form major challenges in neonatal intensive care.[2] Although the etiology and pathogenesis of CDH remain largely unresolved, the morphological abnormalities of the lungs are well described.[3]

A striking feature of the lung morphometry is an abnormal pulmonary vasculature, which shows a reduced volume of the vascular bed, thickening of the arterial media and adventitia, and excessive muscularization of arterioles.[4,5] The pulmonary arteries in CDH fail to undergo appropriate postnatal remodeling, which leads to sustained high vascular resistance and pulmonary hypertension (PH).[6] In turn PH and iatrogenic lung injury induce further aberrant remodeling of the pulmonary arteries.[7] In addition, the pulmonary circulation of CDH patients reacts unpredictable to standard vasodilatatory therapy. For example, only 70% of the patients respond to iNO and the pharmacokinetics of vasodilatory agents, such as the PDE5 inhibitor, Sildenafil are hardly evaluated.[8,9] In other words, the survival of CDH patients is highly determined by its associated pulmonary vascular disease.[6,10,11]

The effector cell of pulmonary vascular function (vasodilatation/vasoconstriction) and structure (remodeling) is the vascular smooth muscle cell (SMC). In the media of the pulmonary artery, a diversity of SMCs exists. SMCs can have a phenotype ranging from synthetic to pure contractile based on their morphology and the expression of cytoskeleton and contractile markers (Figure 7.1).[12,13] The phenotype of the various subpopulation SMC in the pulmonary artery is developmentally regulated and hypothesized to play a role in the vascular remodeling seen in PH.[14] Recently deficiency of contractile proteins in the vascular SMC of a nitrofen-induced CDH model was shown.[15]

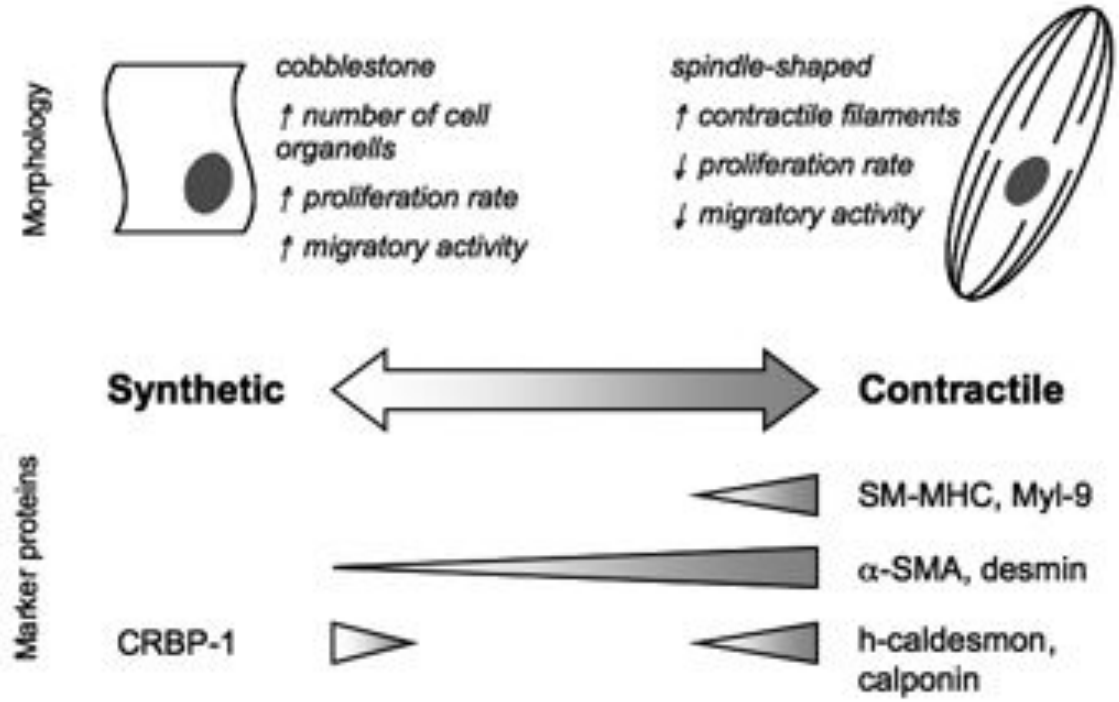

Figure 7.1 Spectrum of smooth muscle cell phenotypes.
SMC myosin heavy chain (SMMHC), alpha smooth muscle actin (αSMC), SMC myosin light chain (Myl-9), cellular retinoid binding protein (CRBP).

As the SMC matures, it expresses more markers of the contractile apparatus, like SMC myosin heavy chain (SMMHC) and SMC myosin light chain (Myl-9). [16,17] Also proteins that are involved in SMC contractility, like h-caldesmon and calponin are expressed in the more mature SMC.[18] Desmin and alpha smooth muscle actin (αSMC) are more general markers of SMCs and their expression increases during maturation.[13] CRBP on the other hand is expressed in undifferentiated, fast growing, immature SMC.[19]

We hypothesize that the pulmonary arteries in patients with CDH consist of an altered SMC phenotype compared to age-matched controls that are possibly involved in the pathogenesis of PH. We therefore examine the immunohistochemical expression of various SMC markers on tissue-micro arrays (TMA). TMA is an efficient high-throughput technology that saves precious tissue and provides highly representative findings.[20]

Material and methods

Human lung tissue was retrieved from the archives of the Department of Pathology of the Erasmus MC, Rotterdam, following approval by the Erasmus MC Medical Ethical Committee. Formalin-fixed and paraffin-embedded lung tissue was available for 54 CDH patients (died between 1989 and 2006). After excluding samples showing severe hemorrhage and/or necrosis, a series of 24 CDH patients was included (mean lung/body weight ratio of term CDH patients =0.0032, normal ratio =0.012). A control group was selected based on match for gestational age (15-40 weeks), death in utero or duration of postnatal survival and, spontaneous breathing and/or ventilation (table 7.1, for demographic information of each patient see appendix). For ECMO treated patients we included control samples matched for gestational and postnatal age. Tissues were re-embedded and processed to create tissue micro array (TMA).

Tissue micro array

Tissue micro array (TMA) were constructed as described by Kononen et al. [21] For each sample, we punched three tissue core biopsies of 1.5 mm in diameter and 3.2 mm in depth from preselected regions to ensure adequate representation of all lung structures. The tissue biopsies from each sample were then placed in linear arrays into empty "recipient" paraffin blocks, one for CDH, and one for control. Tissue cores of adult multi-slides were used as controls, for orientation purpose, and to estimate background labeling for each of the immunohistochemical markers.

Immunohistochemistry

Immunostaining were performed on 4 μm sections of TMAs, mounted on coated slides (Starfrost®, Berlin, Germany) according to standard protocol, using the EnVision™ detection system (DakoCytomation, Glostrup, Denmark) [22]. Primary antibodies used were: SMMS-1 against SMMHC (1:50, DAKO, The Netherlands), αSMA (1:300, Biogenex, San Ramos, CA, USA), Myl-9 against SMC myosin light chain (1:500, Santa Cruz, Heidelberg, Germany), h-caldesmon (1:20, Monosan, the Netherlands), calponin (1: 100, DAKO), desmin (1: 200, Monosan), and CRBP (1:25,

Abcam, Cambridge, UK). For SMMS-1, αSMA, h-caldesmon, and desmin immunostaining antigen retrieval consisted of boiling for 15 minutes in Tris-EDTA buffer (pH 9), remaining antibodies did not require antigen retrieval. Negative controls were performed by omission of the primary antibodies.

The TMA slides for each immunohistochemical marker were blinded examined and of each core the expression of the various SMC markers in the vascular wall was scored as strong (3), clear (2), weak-but-visible (1) or negative (0).

Statistical analysis

The chi-squared test was used for proportions. Differences in SMC marker expression for patients' subgroups (premature, term, and ECMO-treated) were determined by Mann-Whitney rank-sum test. A *p*-value of .05 was considered as statistically significant. All statistics were calculated using SPSS (version 11.0; SPSS Inc., Chicago, IL).

	Developmental stage	n	Gestational age (wk)	Postnatal age	Birth weight (g)
Control	**Premature**	10	31,5 (15-35)	1hr (0-24hr)	2000 (57-2700)
	Term	10	39,5 (38-41)	36 hr (1hr-1wk)	3220 (2490-3950)
	ECMO matched	4	38,5 (34-40)	1wk (24hr-3wk)	2655 (1330-2990)
CDH	**Premature**	10	31,5 (15-36)	1hr (0-48hr)	1032 (30-2515)
	Term	10	39 (37-40)	7hr (1hr-3days)	2835 (2270-3800)
	ECMO treated	4	38 (34-39)	2wk+2days (24hr-5wk)	3000

Table 7.1 Characteristics of CDH patients and controls selected for tissue microarray. Median and range of gestational age, postnatal age, and birth weight of CDH patients and controls divided in premature, term and ECMO-treated subgroups.

Results

To evaluate differences in phenotype of the SMC in the pulmonary artery wall we immunohistochemical stained TMAs of lung tissue of CDH patients and controls for SMMHC (SMMS-1), αSMA, SMC myosin light chain (Myl-9), h-caldesmon, calponin, desmin, and CRBP-1. All markers showed a cytoplasmic staining, specific staining of the positive control (colon or bladder) and no staining of the negative control.

General staining patterns

SMMHC was most prominent in the medial layer of pulmonary arteries of all sizes and some peribronchial cells (Figure 7.2). A very intense staining for αSMA was seen in the medial layer of pulmonary arteries and peribronchial cells (Figure 7.2). Also interstitial cells showed positive staining for αSMA. A less intense staining for myl-9 showed a typical vascular staining pattern of the lung (Figure 7.2). The media of the arteries as well as the endothelium were positively stained. The larger vessels showed a more intense staining compared to the smaller arteries.

The antibody against h-caldesmon showed staining of all pulmonary vessels walls and cells of the bronchial wall (Figure 7.3). Furthermore an intense staining was seen in the interstitium. The endothelium and epithelial cells were negative for h-caldesmon. Calponin was expressed in the SMC of pulmonary arteries and those of the bronchial wall (Figure 7.3). The larger vessels were more intense stained. Some background staining was observed in the interstitium, but no specific expression of calponin was found in the interstitium, endothelium and epithelium.

Immunohistochemistry for desmin showed staining in sporadic cells in the peribronchial area (Figure 7.4). No staining is seen in the pulmonary vessel walls. CRBP showed a very weak staining mainly found in the walls of small pulmonary arteries and peribronchial cells (Figure 7.4).

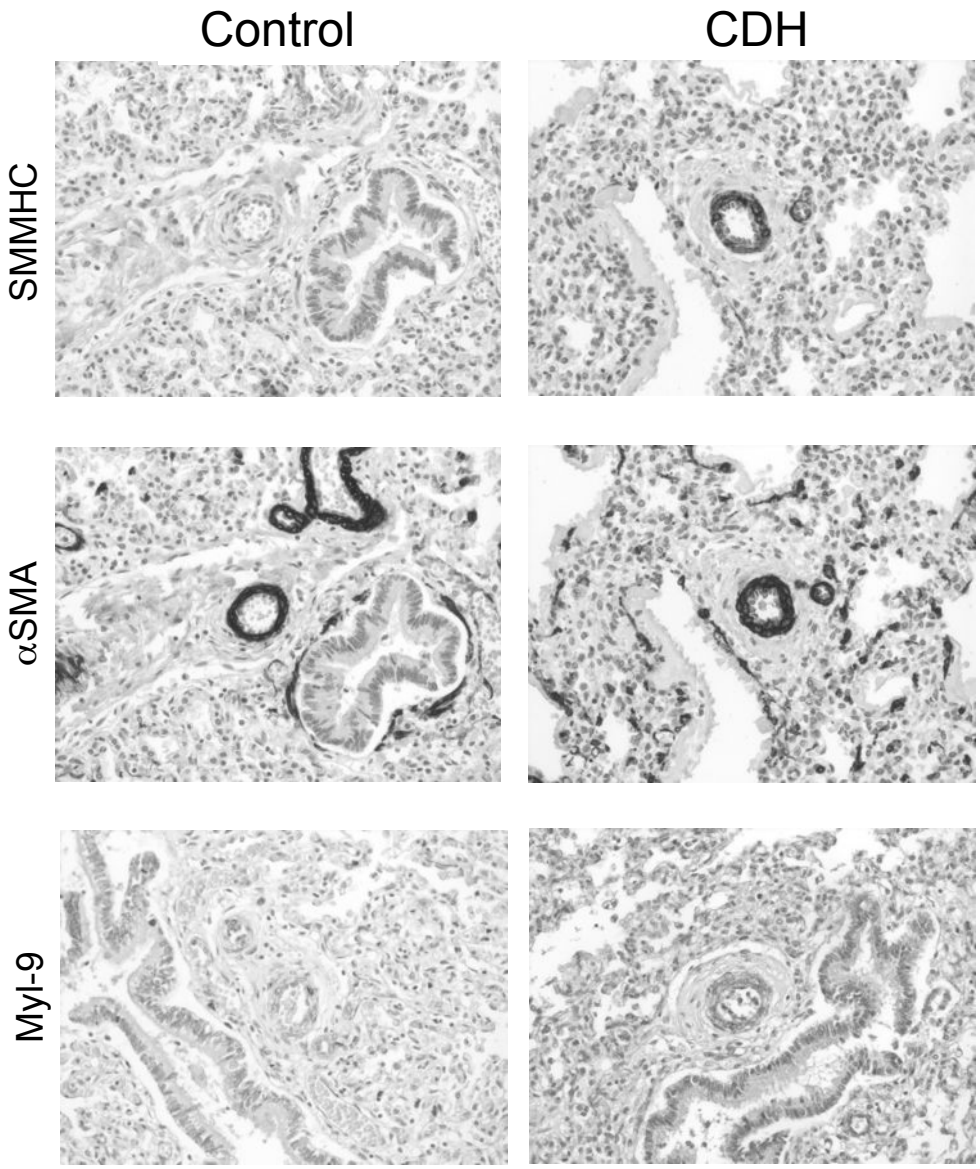

Figure 7.2 (Color figure p 166)
Immunohistochemical staining of SMC myosin heavy chain (SMMHC), alpha smooth muscle actin (αSMC), and SMC myosin light chain (Myl-9) in control and CDH patients' lung tissue of term neonates. Original magnification 400x.

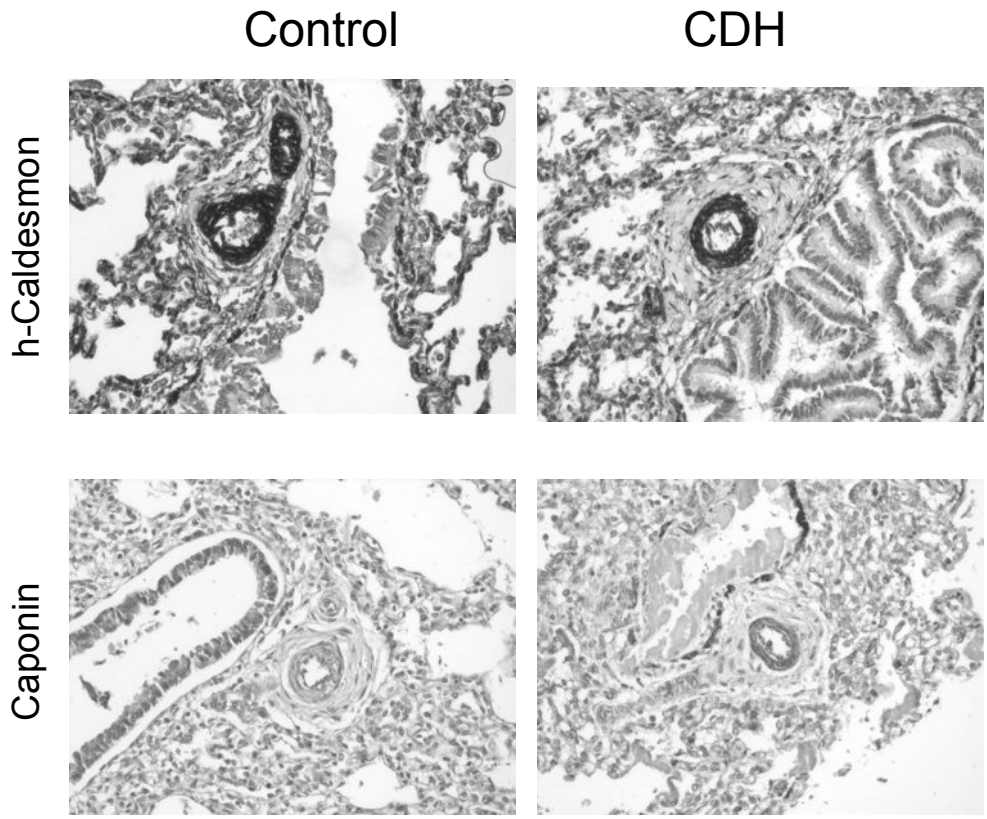

Figure 7.3 (Color figure p 167)
Immunohistochemical staining of h-caldesmon and calponin in control and CDH patients' lung tissue of term neonates. Original magnification 400x.

Expression patterns CDH patients versus control

The TMA represents a heterogenic patient group; therefore we divided the control and CDH patients in three groups; premature (gestational age < 36 weeks), term (gestational age >36 weeks), and ECMO treated (control group matched for postnatal age).

In premature controls we found expression of αSMA, Myl-9, h-caldesmon, and desmin. Compared to control, we found similar expression of αSMA, Myl-9, h-caldesmon, and desmin in the premature CDH group. SMMHC and calponin were significantly higher expressed in the premature CDH patients than in control lung tissue (Table 7.2). Also in term CDH patients the expression of SMMHC was significantly higher than in controls. Expression of the remaining markers was similar for CDH and control in the term group.

Calponin expression increased significantly between premature and term control cases. In the ECMO treated CDH patients the expression of calponin appear to be lost compared to controls with matched postnatal age. However no significance was reached, probably due to small patient numbers. Other markers showed no difference between ECMO treated CDH patients and controls matched for postnatal age. Also when comparing term CDH patients without ECMO treatment to ECMO treated CDH patients no differences in expression pattern was found, except for the diminished expression of calponin.

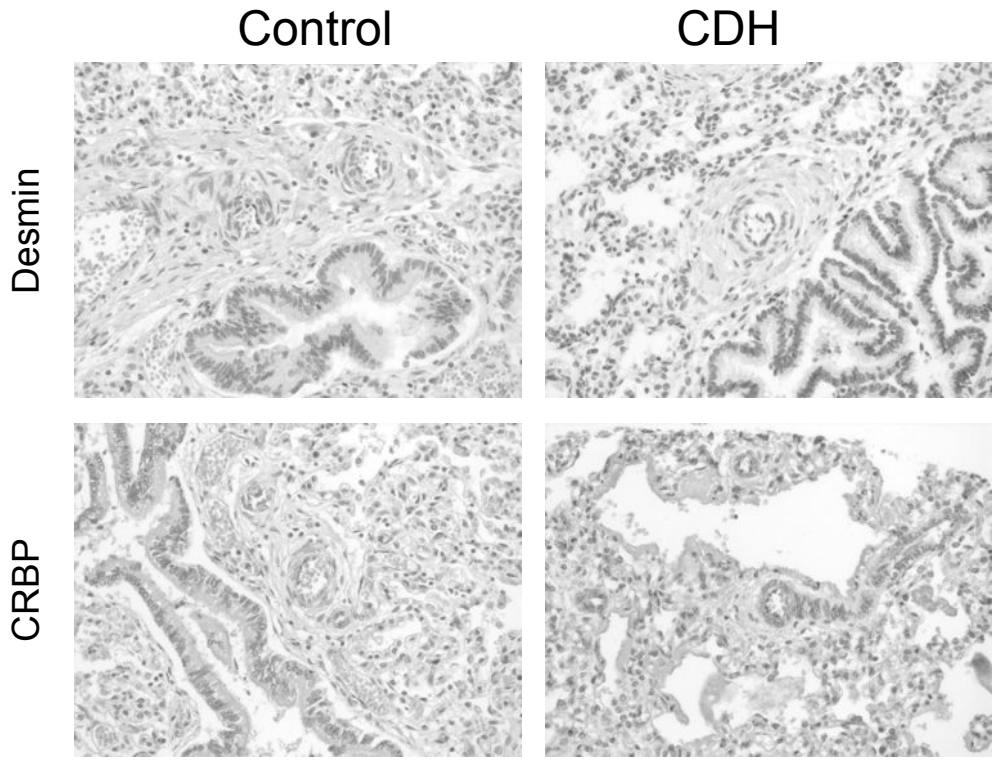

Figure 7.4 (Color figure p 168)
Immunohistochemical staining of desmin and cellular retinoid binding protein (CRBP) in control and CDH patients' lung tissue of term neonates. Original magnification 400x.

Marker	Premature		Term		ECMO treated	
	Control	CDH	Control	CDH	Control	CDH
SMMHC ∗,∗∗	−	+++	+	+++	+	+++
αSMA	+++	+++	+++	+++	+++	+++
Myl-9	++	++	++	++	++	++
Calponin ∗,†	−	+	+	+	+	−
h-Caldesmon	+++	+++	+++	+++	+++	+++
Desmin	+	++	++	++	+/−	+/−
CRBP	−	+/−	−	+/−	−	−

Table 7.2 Summary of immunohistological staining scoring.
SMC myosin heavy chain (SMMHC), alpha smooth muscle actin (αSMC), SMC myosin light chain (Myl-9), h-caldesmon, calponin, desmin, and cellular retinoid binding protein (CRBP) on tissue-micro arrays (TMA) for control and CDH patients divided in premature, term and ECMO treated subgroups. +++ strong, ++ clear, + weak-but-visible or – negative staining. * Significant difference between premature control and premature CDH patients for SMMHC and Calponin expression. ** Significant difference between term control and term CDH patients for SMMHC expression. † Significant difference between premature control and term control for Calponin expression.

Discussion

In this study we evaluated the lungs of CDH patients versus controls for differences in SMC marker expression by using TMAs. It is the first study in human to show phenotypical altered SMCs in the pulmonary artery walls of CDH patients. CDH patients expressed premature SMMHC and calponin in the media of their pulmonary arteries. Moreover, in term CDH patients SMMHC was significantly higher expressed than in control.

The pulmonary artery wall consists of different subpopulations of SMC.[23] These different subpopulations of SMC have marked variation in contractility, proliferation, and matrix-producing capabilities.[24] The distinct roles of these subgroups are believed to be a key determinant of normal and pathological lung development.[25] The spectrum

of SMC phenotypes is determined by protein markers of the contractile apparatus. For example, SMMHC (SM-1 and SM-2) and myosin light chain appear to be distinct marker for contractile SMC.[13] Our results showed significantly increased expression of SMMHC in CDH patients, both premature and at term. In the premature control group no SMMHC staining was found. However, SMMHC type 1 is expressed from 56 days of gestation onwards in human fetal lungs around the airways and in the pulmonary artery wall.[26] In control and nitrofen-induced CDH rat fetus no expression of SMMHC type 2 was shown in the pulmonary vessels.[27] Whether this can be translated into a true more contractile SMC remains speculative. In adult PH, the expression of SMMHC is increased in pulmonary vessels.[28] However, SMC with a synthetic phenotype contributed to the development of specific vascular lesions seen in adult pulmonary hypertension.[25] By contrast, fibroblast migrating to align around the remodeling microvessels also express isoforms of SMMHC, but in the absence of a contractile filament network.[28] Moreover, there is not always a reciprocal relationship between SMC function and differentiation grade.[29] Therefore the increased SMMHC expression in CDH patients cannot be translated directly in functional consequences. Unfortunately our study is limited by the available human material and for obvious reasons functional experiments are unachievable. In literature a number of studies investigate whether or not SMMHC isoform composition is a determinant of contractile behavior. However the SMMHC isoforms SM-1 and SM-2, both detectable by the SMMS-1 antibody we used, are considered not to be related to regulation of vascular tone.[29]

SMMHCs form together with SMC myosin light chains the contractile unit of the SMC. Our results showed no differences between CDH patients and controls for SMC myosin light chain (Myl-9) expression. Nor we did found a difference in Myl-9 expression between premature, term and ECMO-treated patients. This is in contrast with a deficiency in embryonic essential myosin light chain shown in the nitrofen-induced CDH rat model and which led to impaired SMC differentiation.[15] In this teratogenic-based model of CDH a direct effect of nitrofen on pulmonary vascular SMC differentiation cannot be ruled out.

Our results show an increased expression of calponin between premature and term controls. Calponin is involved in SMC contractility and appears normally later in

development of vessels and is correlated with a more mature type of SMC.[30] In a transgenic mouse model of PH, loss of BMPR2 is correlated with reduced calponin expression.[31] However in this PH model it is hypothesized that the SMC dedifferentiate towards a more synthetic phenotype.

Intermediate filament protein desmin is not specific for SMC, but its expression is critical and specific for SMC differentiation.[32] The lung tissue available for this study only showed little desmin expression in the peribronchial region. A similar expression pattern for desmin in human fetal lung was found by Hall et al.[26]

CRBP-1 is involved in retinoic acid (RA) metabolism and is expressed in SMC.[33] Furthermore involvement of the RA pathway is suspected in the pathogenesis of CDH.[34] RA decreases SMC replication and promotes development of a more contractile phenotype.[35,36] The increased SMMHC expression in CDH patients might imply a more contractile SMC population. However no correlation between SMMHC and CRBP expression was found in our study.

In conclusion, we showed that CDH patients expressed premature and significantly more SMMHC in their pulmonary arteries compared to control. However the implications of the expression of a more contractile SMC phenotype in the pulmonary artery wall of CDH patients is not clear. The origin, reaction on proliferative stimuli, and possible involvement in altered vascular tone of this SMC subpopulation needs further investigation using different approaches such as *in vivo* lineage labeling studies and primary cell cultures with *in vitro* pharmacology. Understanding the regulation of vascular SMC differentiation and origin is an important step to elucidated the unpredictable response of the pulmonary circulation on vasodilatatory drugs and the disordered vascular remodeling in case of CDH and other forms of PH.

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Chapter 8

Lysyl oxidase activity is dysregulated during impaired alveolarization of mouse and human lungs.

Abstract

Rationale: Disordered extracellular matrix production is a feature of bronchopulmonary dysplasia (BPD). The basis of this phenomenon is not understood.

Objectives: To assess lysyl oxidase expression and activity in the injured developing lungs of newborn mice and of prematurely-born infants with BPD or at risk or BPD.

Methods: Pulmonary lysyl oxidase and elastin gene and protein expression were assessed in newborn mice breathing 21% or 85% O2, and in patients who died with BPD or at risk of BPD, or control patients. Signaling by TGF-b was pre-emptively blocked in mice exposed to hyperoxia using TGF-b-neutralizing antibodies. Lysyl oxidase promoter activity was assessed using plasmids containing the lox or loxl1 promoters fused upstream of the firefly luciferase gene.

Measurements and Main Results: mRNA and protein levels and activity of lysyl oxidases (Lox, LoxL1, LoxL2) were elevated in the oxygen-injured lungs of newborn mice and infants with BPD or at risk for BPD. In oxygen-injured mouse lungs, increased TGF-b signaling drove aberrant lox, but not loxl1 or loxl2 expression. Lox expression was also increased in oxygeninjured fibroblasts and pulmonary artery smooth muscle cells.

Conclusions: Lysyl oxidase expression and activity are dysregulated in BPD in injured developing mouse lungs and in prematurely-born infants. In developing mouse lungs, aberrant TGF-b signaling dysregulated lysyl oxidase expression. These data support the postulate that excessive stabilization of the extracellular matrix by excessive lysyl oxidase activity might impede the normal matrix remodeling that is required for pulmonary alveolarization and thereby contribute to the pathological pulmonary features of BPD.

Lysyl Oxidase Activity Is Dysregulated during Impaired Alveolarization of Mouse and Human Lungs

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***Rationale*: Disordered extracellular matrix production is a feature of bronchopulmonary dysplasia (BPD). The basis of this phenomenon is not understood.**

***Objectives*: To assess lysyl oxidase expression and activity in the injured developing lungs of newborn mice and of prematurely born infants with BPD or at risk for BPD.**

***Methods*: Pulmonary lysyl oxidase and elastin gene and protein expression were assessed in newborn mice breathing 21 or 85% oxygen, in patients who died with BPD or were at risk for BPD, and in control patients. Signaling by transforming growth factor (TGF-β) was preemptively blocked in mice exposed to hyperoxia using TGF-β–neutralizing antibodies. Lysyl oxidase promoter activity was assessed using plasmids containing the *lox* or *loxl1* promoters fused upstream of the firefly luciferase gene.**

***Measurements and Main Results*: mRNA and protein levels and activity of lysyl oxidases (Lox, LoxL1, LoxL2) were elevated in the oxygen-injured lungs of newborn mice and infants with BPD or at risk for BPD. In oxygen-injured mouse lungs, increased TGF-β signaling drove aberrant *lox*, but not *loxl1* or *loxl2*, expression. Lox expression was also increased in oxygen-injured fibroblasts and pulmonary artery smooth muscle cells.**

***Conclusions*: Lysyl oxidase expression and activity are dysregulated in BPD in injured developing mouse lungs and in prematurely born infants. In developing mouse lungs, aberrant TGF-β signaling dysregulated lysyl oxidase expression. These data support the postulate that excessive stabilization of the extracellular matrix by excessive lysyl oxidase activity might impede the normal matrix remodeling that is required for pulmonary alveolarization and thereby contribute to the pathological pulmonary features of BPD.**

Keywords: lung development; septation; TGF-β; transforming growth factor

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The formation of extracellular matrix structures is perturbed in bronchopulmonary dysplasia (BPD), which is believed to contribute to the arrested alveolarization seen in "new BPD."

What This Study Adds to the Field

The expression and activity of lysyl oxidases, which regulate collagen and elastin cross-linking, are dysregulated in BPD in mice and humans. These data could suggest that overstabilization of the extracellular matrix may impede normal matrix remodeling, thereby creating a less plastic lung structure.

Bronchopulmonary dysplasia (BPD) is a consequence of prolonged mechanical ventilation of premature infants with supplemental oxygen. The syndrome was first described by Northway and colleagues in 1967 (1) and remains a significant complication of premature birth, affecting nearly 10,000 newborns annually in the United States. BPD causes long-term respiratory consequences that reach beyond childhood (2). Today, BPD—which is also called "new BPD" or chronic lung disease of early infancy—is believed to result from an arrest of late lung development that is characterized by alveolar simplification (3, 4) and an arrest of microvascular development (5, 6) or microvascular dysangiogenesis (7, 8). Thus, BPD leads to a long-term reduction in the total number of alveoli, and hence, reduces the surface area available for gas exchange.

The pathogenesis of new BPD is not fully understood; however, several lines of evidence indicate that a severely perturbed extracellular matrix (ECM) metabolism contributes to this disorder. For example, an accumulation of elastic fibers has been observed in patients with classic BPD (9, 10), and secondary collagen fibers in the saccular wall of patients who died with new BPD are "thickened, tortuous, and disorganized" (11). Excessive production and accumulation of elastin has also been reported in animal models of BPD. For example, in the injured lungs of premature lambs, increased deposition of elastic fibers has been associated with thickened walls of the terminal respiratory units (12). In addition, in the injured lungs of mouse pups with BPD, increased elastin synthesis has also been reported (13, 14). These observations are important, because the ECM plays a pivotal role in late lung development, and any perturbations to the formation and remodeling of matrix structures would affect the development of the immature lung (15).

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Although many studies have examined the synthesis and production of ECM molecules in BPD, few studies to date have addressed the subsequent post-translational processing of these molecules, or the remodeling of the matrix itself (11, 12). Therefore, in this study the expression and activity of lysyl oxidases, which play a key role in regulating the stability of the ECM, have been examined in an animal model of BPD, as well as in ventilated human neonates who have died either with BPD or at risk for BPD.

Five lysyl oxidases have been described: the archetypical member, Lox, and four closely related, or Lox-like (LoxL) members, named LoxL1–LoxL4 (16, 17). All lysyl oxidases catalyze the oxidative deamination of lysine and hydroxylysine residues to peptidyl α-aminoadipic-δ-semialdehydes, generating reactive semialdehydes that condense to form intramolecular and intermolecular covalent cross-links in elastin and collagen molecules. This process drives fiber maturation and thus imparts structural stability to the ECM (16, 17).

In this study it was hypothesized that lysyl oxidase expression and activity are dysregulated in BPD. The data reported here demonstrate that lysyl oxidase expression and activity are indeed up-regulated in the injured lungs of newborn mice and human infants, and that this up-regulation is driven, in part, by the excessive transforming growth factor (TGF)-β signaling associated with oxygen injury to the lung. It is proposed that the persistence and overabundance of elastin fibers and the formation of thickened, tortuous, and irregularly distributed collagen fibers observed in BPD may result from "overstabilization" of these important matrix molecules through excessive lysyl oxidase activity, making them resistant to normal remodeling processes in the developing lung. This "locked" lung structure may well underlie the arrested development of immature lungs and lead to the impaired septation and vascularization observed in BPD.

METHODS

Animal and Tissue Treatment

The animal ethics authority of the government of the State of Hessen and the Subcommittee for Research Animal Studies of the Massachusetts General Hospital approved all animal procedures. The newborn mouse model of BPD used in this study, in which newborn mice are exposed to normoxic (21% O_2) or hyperoxic (85% O_2) gas mixtures for the first 28 days after birth, has been defined and characterized by others (14, 18, 19) and described previously by our own laboratory (20, 21). Mice were killed at days 1, 7, 14, 21, and 28 after birth for analysis, which spans the period of late lung development in mice. The processing of lung tissue from this model for RNA and protein analysis, as well as immunohistology, has been described previously (20, 21), as has the inhibition of TGF-β signaling in mouse pup lungs with a TGF-β1,2,3–neutralizing IgG_1 antibody (1D11; Genzyme, Cambridge, MA) (22). In these studies, an isotype-matched nonimmune IgG_1 antibody (MOPC21; Sigma, St. Louis, MO) did not modulate pulmonary TGF-β signaling.

Human Tissue

Human material was used with the approval of the Human Subjects Review Committees of the Erasmus University Medical Centre and the University of Giessen Lung Center, which use human research protection principles espoused in the Declaration of Helsinki. Neonatal human lung tissue was retrieved from archived autopsy material at the Erasmus University Medical Centre and the University of Giessen Lung Center. The clinical characteristics of the patients from which the specimens were derived are presented in Table 1 (two groups of control patients) and Table 2 (patients with BPD or at risk for developing BPD, called the "BPD group"). Two groups of control patients were used: control group 1, which included patients with the gestational age at birth and birth weight matched to the BPD group; and control group 2, which included patients with the chronological age at death matched to the chronological age at death of the BPD group. Tissue samples were collected at autopsy, within 24 hours of death. The average time

TABLE 1. CLINICAL CHARACTERISTICS OF CONTROL PATIENTS

Patient (Material)*	Birth Weight, g	M/F	Gestational Age, wk	Chron. Age at Death, d	Combined Lung Weight, g	Days FI_{O_2} >0.50	Days Mechanical Ventilation	Cause of Death/Autopsy Diagnosis and Medication
Control group 1, matched to BPD group (Table 2) for birth weight and gestational age at birth								
1 (h, r)	954	M	26	<1	14.6	0	0	Intracranial hemorrhage
2 (h, r)	914	M	27	<1	15.5	0	0	DiGeorge syndrome (22q11.2 deletion); intrauterine infection
3 (r)	1,210	M	28	<1	ND	0	0	Hypoxic-ischemic encephalopathy
4 (h, r)	826	M	29	<1	ND	0	0	Hydrocephalus, Arnold Chiari malformation
5 (r)	852	M	27	<1	24.0	0	0	Hypoxic-ischemic encephalopathy
6 (h, r)	995	M	27	<1	19.0	0	0	Placental abruption
7 (h, r)	758	M	26	<1	17.0	0	0	Intrauterine infection
Median	914		27					
Mean ± SE	929 ± 55		27 ± 0.4					
Control group 2, age matched to BPD group (Table 2) for chronological age at death								
8 (h)	2,334	M	33	<1	ND	0	0	Intrauterine death, no congenital abnormalities evident
9 (h)	1,904	F	34	<1	34.0	0	0	Intrauterine death (chorioamnionitis)
10 (r)	1,625	M	33	3	ND	0	0	Congenital heart malformation. Drugs: atropine, prostaglandin A1
11 (h, r)	2,350	M	35	<1	30.0	0	<1 h	Perinatal asphyxia. Drugs: atropine, adrenaline
12 (h)	2,040	M	33	<1	ND	0	<1 h	Multiple congential anomalies (VACTERL). Drugs: adrenaline
13 (r)	1,740	F	34	<1	ND	0	0	Intrauterine death (ventriculomegaly)
14 (h, r)	1,800	M	32	5	ND	0	5	Meningoencephalitis
15 (h, r)	1,190	M	31	<1	ND	0	0	Placental abruption
Median	1,852		33					
Mean ± SE	1,873 ± 135		33 ± 0.44					

Definition of abbreviations: BPD = bronchopulmonary dysplasia; Chron. = chronological; F = female; M = male; ND = not determined; VACTERL = vertebra/anus/cardiac/trachea/esophagus/radius/renal/limb anomalies.

* Material includes RNA (r) and histological sections (h).

TABLE 2. CLINICAL CHARACTERISTICS OF PATIENTS WITH BPD OR AT RISK FOR BPD

Patient (Material)*	Birth weight, g	M/F	Gestational age, wk	Chron. age at death, d	Combined lung weight, g	Days F_{IO_2} >0.5	Days Mechanical Ventilation†	Cause of Death/Microbiology/Autopsy Diagnosis/Drugs‡
16 (h)	650	M	27	9	33.3	9	9 (c, hf)	BPD, IRDS, sepsis. Drugs: surfactant, inotropes, tobramycin, vancomycin, cortisone
17 (r)§	720	M	29	62	59.0	13	62 (c, hf)	BPD, IRDS, *Staphylococcus aureus* sepsis. Drugs: surfactant, inotropes, tobramycin, flucloxacillin, cortisone
18 (h, r)	1055	M	32	18	ND	18	18 (c)	BPD, ventricular septal defect, Edwards syndrome (trisomy 18). Drugs: inotropes
19 (h, r)	825	F	27	6	35.0	6	6 (c, hf)	BPD, IRDS, bronchopneumonia, intracranial hemorrhage. Drugs: surfactant, inotropes, tobramycin, penicillin
20 (h)	920	M	26	19	ND	17	19 (c, hf)	BPD, retinopathy, patent ductus arteriosus; *Streptococcus*, *Staphylococcus* and *Ureaplasma* infection. Drugs: surfactant, indomethacin, dexamethasone, inotropes, vancomycin, penicillin, erythromycin
21 (h)§	650	M	27	32	44.0	16	32 (c)	BPD, IRDS, *Staphylococcus epidermidis* infection. Drugs: surfactant, dexamethasone, inotropes, penicillin, vancomycin
22 (r)§	835	M	26	65	ND	27	65 (c, hf)	BPD, cerebral bleeding, ductus arteriosus. Drugs: surfactant, inotropes, dexamethasone, theophylline
23 (h, r)§	930	M	26	99	186.0	98	99 (c, hf)	BPD, IRDS, pneumothorax, subependymal hemorrhage. Drugs: surfactant, inotropes, dexamethasone, tobramycin, penicillin, amphotericin
24 (h, r)§	1250	F	28	34	86.0	34	34 (c, hf)	BPD, IRDS, *Staphylococcus epidermidis* sepsis. Drugs: surfactant, furosemide, amoxicillin, erythromycin
25 (r)§	1220	M	31	35	95.0	35	35 (c, hf)	BPD, IRDS, right ventricular hypertrophy, anemia, rickets. Drugs: furosemide, amoxicillin, vancomycin
Median	878		27	33		18	33	
Mean ± SE	906 ± 68		28 ± 0.8	38 ± 8		27 ± 8	38 ± 9	
P value vs. CTRL1‖	0.111 (NS)		0.403 (NS)	0.016				
P value vs. CTRL2‖	<0.001		<0.001	0.949 (NS)				

Definition of abbreviations: BPD = bronchopulmonary dysplasia; Chron. = chronological; CTRL = control; IRDS = infant respiratory distress syndrome; F = female; M = male; ND = not determined; NS = not significant.

* Material includes RNA (r) and histological sections (h).

† c, conventional ventilation; hf, high-frequency ventilation.

‡ Inotropes include dopamine, dobutamine, and adrenaline.

§ Patients have clinically defined BPD.

‖ By paired Student *t*-test, compared with the control 1 (CTRL1) and control 2 (CTRL2) groups in Table 1. In the case of chronological age at death, the postmenstrual ages at death, rounded to the nearest full week, were compared.

between death and tissue processing was similar in all three patient groups.

Cell Culture

The NIH/3T3 fibroblast-like cell line and primary human fibroblasts and primary human pulmonary artery smooth muscle cells (paSMC) were cultured as described previously (20, 23). The human fibroblast cell line HFL1 was passaged as recommended by the American Type Culture Collection (ATCC). Gas tension in the culture media was monitored daily. When the headspace gas contained 21% oxygen, the cell culture media exhibited a Po_2 of 142.4 ± 4.9 mm Hg for NIH/3T3 cells and 137.4 ± 8.3 mm Hg for paSMC. With the hyperoxic 85% oxygen-containing headspace gas, the cell culture media exhibited a Po_2 of 506.3 ± 16.7 mm Hg for NIH/3T3 cells and 466.9 ± 29.4 mm Hg for paSMC over the experimental time course (maximum 96 h). The pH and Pco_2 values did not appreciably change over the experimental time course.

Immunohistochemistry

Hematoxylin staining, Hart's elastin staining, picrosirius red, and Masson's trichrome staining for collagen, and staining for the expression of lysyl oxidase isoforms was performed on 3-μm tissue sections as described previously (20), with goat anti-Lox (SC-32409; blocking peptide SC-32409P), goat anti-LoxL1 (SC-48720; blocking peptide SC-48720P), goat anti-LoxL2 (SC-48723; blocking peptide SC-487243), and goat anti-LoxL3 (SC-48728; blocking peptide SC-48728P). All antibodies were used at 1:50 dilution, and were from Santa Cruz (San Francisco, CA). In the antibody blocking studies, the antibodies were preadsorbed with a 10-fold molar excess of the peptides described above, for 30 minutes at room temperature, before application to lung sections. Immune complexes were visualized with a Histostain *Plus* Kit (Zymed, San Francisco, CA).

Analysis of Gene Expression

Total RNA (collected from whole mouse lungs as described above or from peripheral regions of human lungs) was screened by semiquantitative or quantitative real-time reverse transcription (RT) polymerase chain reaction (PCR) with the primers listed in Table 3, as described previously (20). For quantification of relative mRNA expression by semiquantitative RT-PCR, band intensities from specific samples were normalized for loading using the constitutively expressed *hspa8* or *gapdh* band amplified from the same sample. Densitometric analysis of amplicon bands generated in the linear range of product amplification was performed using a GS-800 model densitometer with Quantity One software (both from Bio-Rad Laboratories, Munich, Germany). For real-time PCR, changes in mRNA expression were evident comparing ΔCt values, and using the *hmbs* gene as an internal control (23).

Protein Isolation and Immunoblotting

Protein was collected, transferred to charged membranes, and immunoblots were probed as described previously (20) with the antibodies

TABLE 3. PRIMERS USED FOR REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION

Gene	Forward Primer	Reverse Primer	Amplicon Size, bp	Number of Cycles	Annealing Temperature, °C
Semiquantitative real-time RT-PCR					
Mouse					
eln	5′-GGAGTTCCCGGTGGAGTCTATT-3′	5′-ACCAGGAATGCCACCAACACCTG-3′	1,079	25	60.0
emilin1	5′-AACCGTGTCTCCACTCATGA-3′	5′-GGTGTCCAGTCGTTCGCAGA-3′	1,470	26	60.0
fbln5	5′-CTAGATATTGATGAATGCCG-3′	5′-TGCCTCTGAAGTTGATGACA-3′	1,180	26	60.0
gapdh	5′-AACTTTGGCATTGTGGAAGG-3′	5′-ACACATTGGGGGTAGGAACA-3′	222	25	60.0
lox	5′-ATGCGTTTCGCCTGGGCTGTGC-3′	5′-CTAATACGGTGAAATTGTGCAGCC-3′	630	31	60.0
loxl1	5′-AGCTTGCTCAACTCGGGCTCTGAG-3′	5′-TCAGGACTGGACGATTTTGCAG-3′	1,668	32	57.5
loxl2	5′-GGTGCTGAAGAATGGAGAATGGG-3′	5′-TTACTGTACAGAGAGCTGGTTATTT-3′	1,295	32	57.5
loxl3	5′-ATGAGAGCTGTCAGTGTGTGG-3′	5′-GCACCGAACTTCACTCAAGTGG -3′	1,132	32	57.0
loxl4	5′-ATGATGTGGCCCCAACCACC-3′	5′-TCAGATCAAGTTGTTCCTGAGTCGCT-3′	2,273	32	57.0
Human					
hspa8	5′-TGGGTGGAGAAGATTTTGAC-3′	5′-ACCACAGGGAGGAGCTCCAC-3′	1,219	24	60.0
lox	5′-CCTGCTGATCCGCGACAACC-3′	5′-CCTGAGGCATACGCATGATGTCC-3′	937	31	60.0
loxl1	5′-CCGGACCTCAGCGCTCCGAGAGTAGC-3′	5′-CCACGTTGTTGGTGAAGTCAGACTCC-3′	1,031	31	60.0
loxl2	5′-GGTCTGCAGAGAGCTGGGCTTTGG-3′	5′-GGCAGTCGATGTCATGGCGGTACATG-3′	1,000	31	60.0
loxl3	5′-GGCTTCCTGACGGCTGGTCGCAAG-3′	5′-GTGCTGAGTGCAGCAACAGATCTGATG-3′	1,034	31	60.0
loxl4	5′-GCACAGAGAGCTCCTTGGACCAG-3′	5′-GCACAGAGAGCTCCTTGGACCAG-3′	1,284	31	60.0
Quantitative real-time RT-PCR					
Mouse					
hmbs	5′-GGTCCAGAAGATGACCCACA-3′	5′-AAGCTGCCGTGCAACATCCA-3′	120	—	—
Lox	5′-GCACTGCACACACACAGGGA-3′	5′-TTAGTGTAGTCTGATTCAGG-3′	120	—	—
Human					
hmbs	5′-TTGCTGCTGTCCAGTGCCTA-3′	5′-AGATGAAGCCCCCACATACT-3′	130	—	—
lox	5′-TGCCAGTGGATTGATATTAC-3′	5′-TACGGTGAAATTGTGCAGCC-3′	130	—	—
loxl1	5′-CTGCTATGACACCTACAATG-3′	5′-TGTGTAGTGAATGTTGCATC-3′	120	—	—
loxl2	5′-TCGGCCTCAGCCGCGCAGAC-3′	5′-CCTCCATGCTGTGGTAGTGC-3′	120	—	—
loxl3	5′-ATCACGGATGTGAAGCCAGG-3′	5′-TGAAGGCATCACCAATGTGG-3′	120	—	—
loxl4	5′-AAGTGGCAGAGTCAGATTTC-3′	5′-TGTTCCTGAGACGCTGTTCC-3′	110	—	—

Definition of abbreviation: RT-PCR = reverse transcriptase–polymerase chain reaction.

used for immunohistochemistry (1:750), as well as goat anti-LoxL4 (SC-48732; Santa Cruz, 1:500) and mouse α-tubulin (SC-58667; Santa Cruz, 1:2,000). Immune complexes were detected using peroxidase-conjugated secondary antibodies: rabbit anti-mouse (R&D Systems, Wiesbaden, Germany; 1:2,500) and donkey anti-goat (Santa Cruz, 1:1,000). Densitometric analysis of bands was performed as described above.

Elastin and Desmosine Measurements

Soluble elastin levels were determined using the FASTIN elastin assay (BioColor, County Antrim, UK) as per the manufacturer's recommendations. Desmosine was measured using the procedures of Starcher and colleagues (24) as modified by Hornstra and colleagues (25). The levels of these ECM components were related to the total protein levels in the samples.

Lysyl Oxidase Activity Assay

Lysyl oxidase activity is reported as β-aminopropionitrile-sensitive activity assessed using Amplex Red (Invitrogen, Karlsruhe, Germany) detection and 1,5-diaminopentane (Sigma, Taufkirchen, Germany) as substrate, as described previously (26).

Luciferase-based Promoter Reporter Assay

The construction of luciferase-linked promoter reporter plasmids containing the −2,073/+1434 proximal region of the murine *lox* gene or the −712/−1 proximal region of the human *loxl1* gene in pGL2 (Promega, Madison, WI) has already been described (27, 28), as has the construction of a luciferase-linked promoter reporter plasmid containing 1,547 bp of 5′ flanking sequence from the human *lox* gene with a pGL3 backbone (29).

Statistical Treatment of Data

Unless otherwise indicated, data are presented as mean ± SD. Differences between groups were analyzed by analysis of variance with the Student-Newman-Keuls *post hoc* test for multiple comparisons, or by Student *t*-test, with P values less than 0.05 regarded as significant.

RESULTS

Elastin and Desmosine Metabolism Is Perturbed in Oxygen-injured Mouse Lungs

Neonatal mice breathing 21% oxygen from Postnatal Day (P)1 exhibited typical elastin deposition in developing alveoli that appeared to be condensed into punctate foci in the tips of developing septa at days P7 and P28 (Figure 1A, *arrows*). In contrast, mice exposed to 85% oxygen from P1 for similar durations exhibited disorganized elastin fiber networks in the developing septa. Changes in septal collagen distribution were not evident by Masson's trichrome staining (Figures 1Ba and 1Bb), but septal collagen abundance appeared to be increased, or collagen fibers were thickened, in lungs from 85% oxygen-exposed mice examined by picrosirius red staining under polarized light (Figures 1Bc and 1Bd). Soluble elastin protein levels were comparable in normoxia- and hyperoxia-exposed pups at P14, but were elevated at P28 in hyperoxia-exposed pups (Figure 1C). Furthermore, levels of desmosine, a marker of elastin cross-linking by lysyl oxidases, were elevated in the lungs of hyperoxia-exposed pups both at P14 and P28 (Figure 1D). Although the expression of elastin (*eln*) mRNA normally peaked by P21, it remained high at P28 in mice breathing 85% oxygen (Figure 1E). In addition to elastin, levels of mRNA encoding fibulin-5 and emilin-1, two important elastic fiber components, also appeared to be elevated in oxygen-injured lungs of mouse pups (Figure 1E), indicating that in addition to elastin, the expression of other components of the elastic fibers are affected in lungs exposed to 85% oxygen. Together, these data indicate that elastin metabolism is dysregulated in the 85%

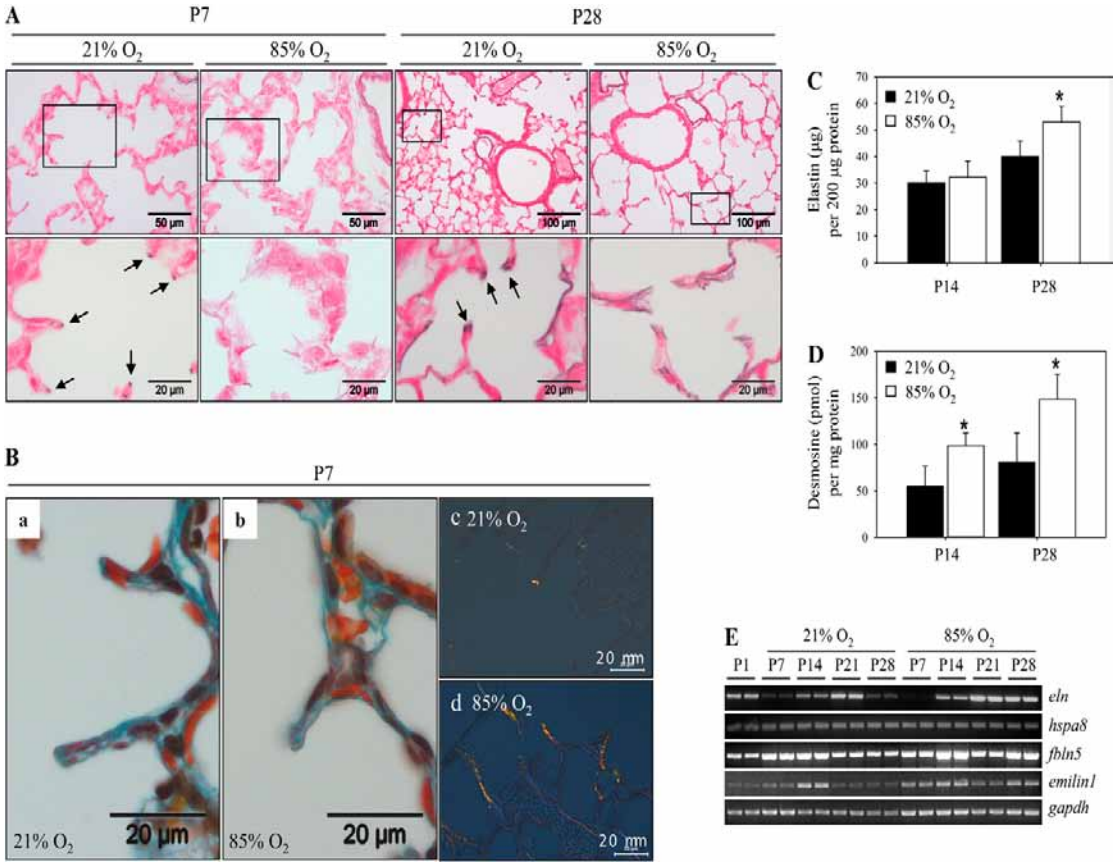

Figure 1. Elastin production and cross-linking are dysregulated in the injured developing mouse lung. (*A*) Hart's stain for elastin in air-exposed mouse pup lungs, indicating punctate elastin foci (*arrows*) in the developing septa at Postnatal Day (P)7 and P28 in the lungs of pups exposed to 21% O_2 that are absent in the lungs of pups exposed to 85% O_2. (*B*) Assessment of collagen morphology by Masson's trichrome stain (*a* and *b*) in the developing septa of P7 mouse pups exposed to 21% O_2 or 85% O_2. Collagen was also assessed by picrosirius red staining observed under polarized light in mouse pups exposed to (*c*) 21% O_2 or (*d*) 85% O_2. Both (*C*) soluble elastin and (*D*) desmosine were elevated in 85% O_2-exposed pups (*open bars*) compared with 21% O_2-exposed pups (*solid bars*) at P28 (n = 5). (*E*) Expression of elastin (the *eln* gene), fibulin-5 (the *fbln5* gene), and emilin-1 (the *emilin1* gene) mRNA monitored by semiquantitative reverse transcriptase–polymerase chain reaction in the first month of postnatal life of pups exposed to 21% O_2 or 85% O_2. The constitutively expressed *hspa8* and *gapdh* genes served as controls for loading equivalence. *$P < 0.01$.

oxygen-injured developing mouse lung, and that this dysregulation is associated with altered elastin turnover or cross-linking, as revealed by elevated desmosine levels.

Lysyl Oxidase Expression and Activity Are Elevated in Oxygen-injured Mouse Lungs

The elevated desmosine levels in the 85% oxygen-exposed mouse pup lungs (Figure 1D) suggested that hyperoxia disrupts the mechanisms responsible for ECM maturation. Because elastin cross-linking is primarily performed by lysyl oxidases, the expression and function of these enzymes were examined in the injured developing lung. Lox was weakly expressed at P1 in the air-breathing mouse pup lung, and was not detected at all between P7 and P28 (Figure 2). In contrast, pronounced *lox* gene expression (Figure 2A; quantified in Figure 2B) and Lox protein expression (Figure 2C; quantified in Figure 2D) was observed in the lungs of hyperoxia-exposed mouse pups between P7 and P28. The expression of *loxl1* and *loxl2* mRNA was progressively increased between P7 and P28 in normoxia-exposed pups, and although a similar trend was observed in hyperoxia-exposed pups, *loxl1* and *loxl2* mRNA expression was higher in hyperoxia-exposed pups at P21 and P28 compared with age-matched normoxia-exposed pups (Figures 1A and 1B). This trend in expression was confirmed at the protein level for LoxL2 (Figures 2C and 2D). Strong LoxL1 protein expression was observed in hyperoxia-exposed pups throughout the period P7 to P28 compared with relatively weak expression in normoxia-treated pups over the same time-frame. No appreciable changes in the trends in *loxl3* and *loxl4* gene expression (Figure 2A) or LoxL3 or LoxL4 protein expression (Figure 2C) were evident in normoxia- versus hyperoxia-exposed pups over the period P7 to P28. Consistent with the trend of increased expression of some lysyl oxidases in oxygen-injured mouse pup lungs, lysyl oxidase activity was increased in the lungs of P14 and P28 hyperoxia-exposed pups compared with age-matched litter mates (Figure 2E).

The most pronounced changes in lysyl oxidase expression in the lungs of hyperoxia-exposed pups were seen on P21 and P28. Therefore, P21 mouse lungs were stained for Lox, LoxL1, LoxL2, and LoxL3 to assess expression and localization of lysyl oxidases in the developing septa (Figure 3). No immunoreactivity was observed for LoxL4 (indeed, LoxL4 appears to have

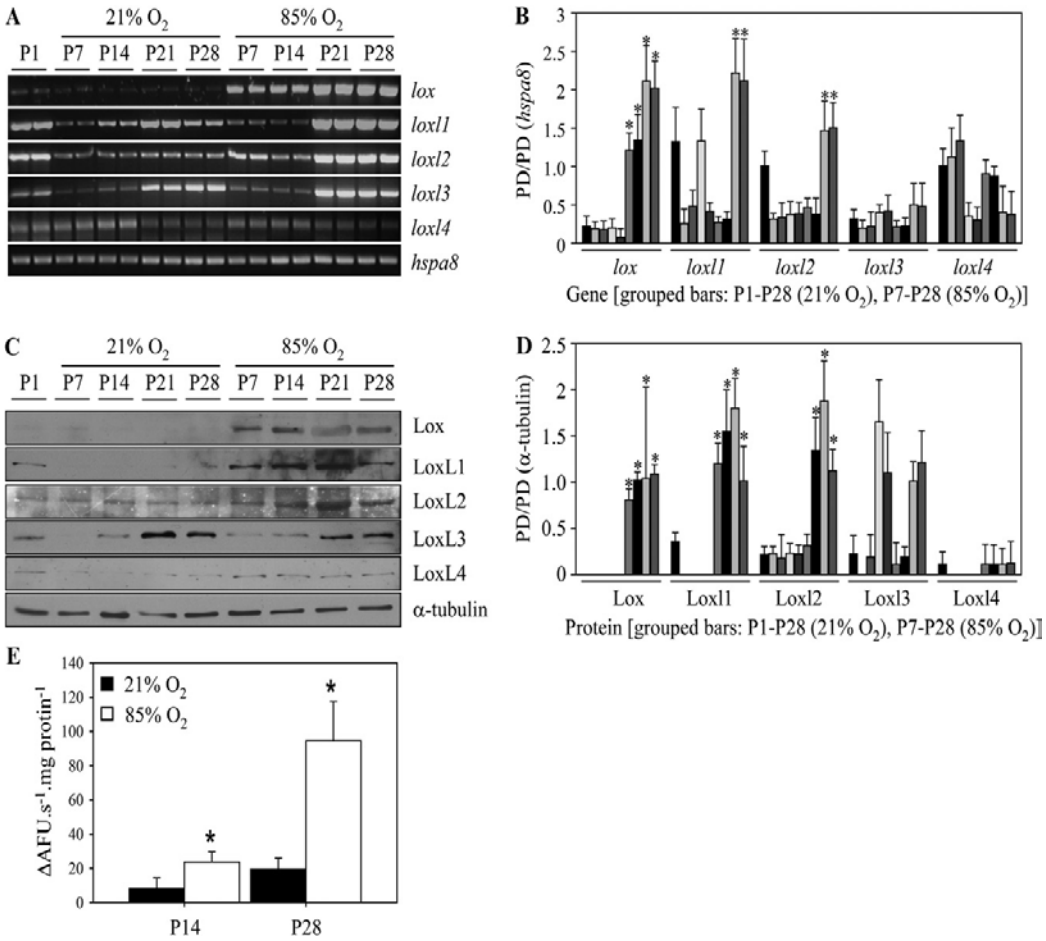

Figure 2. Lysyl oxidase mRNA and protein expression is dysregulated in the injured developing mouse lung. (*A*) Expression of lysyl oxidase mRNA was monitored by semiquantitative reverse transcriptase–polymerase chain reaction where the constitutively expressed *hspa8* gene served as a loading control. These data were quantified by densitometric analysis in *B*. (*C*) Expression of lysyl oxidase protein was monitored by immunoblot, using constitutively expressed α-tubulin as a loading control. These data were quantified by densitometric analysis in *D*. (*E*) Lysyl oxidase activity, assessed by an Amplex Red–based assay, was elevated in whole lung extracts from 85% O_2-exposed pups (*open bars*) compared with 21% O_2-exposed pups (*solid bars*) at P14 and P28. * $P < 0.01$ (n = 4 for *A* and *C*, and n = 5 for *E*). AFU = arbitrary fluorescence units.

the lowest expression of all lysyl oxidases in the lung; Figures 2A and 2B), and these images have therefore been omitted. Staining specificity was confirmed by preadsorption of antibodies with a competing peptide (Figure 3; *right column*). Consistent with the mRNA and protein expression data (Figure 2), pronounced immunoreactivity for Lox, LoxL1, and LoxL2 was observed in the developing alveolar septa of hyperoxia-exposed pups, whereas immunoreactivity was less pronounced for the normoxia-treated group (Figure 3; *central* vs. *left column*), and no differences in staining intensity or localization for LoxL3 were observed between the normoxia- and hyperoxia-treated groups.

Lysyl Oxidases Are Induced by Transforming Growth Factor-β and Hyperoxia

Although these studies indicate that lysyl oxidase expression and activity are increased in the hyperoxic mouse pup lung, the mechanisms regulating these enzymes are unknown. TGF-β has been broadly implicated in regulating the expression and activity of lysyl oxidases (30, 31), and TGF-β signaling is dynamically regulated during mouse and human lung alveolarization (32). Furthermore, TGF-β signaling is abnormally increased in oxygen-injured mouse lungs (20, 22). Therefore, the effects of TGF-β, hyperoxia, and combinations thereof, on the expression of lysyl oxidases in fibroblasts (both mouse fibroblast-like NIH/3T3 cells and primary human lung fibroblasts) and paSMC were assessed, because fibroblasts and smooth muscle cells are the primary lysyl oxidase–producing cells (33). In mouse fibroblast-like NIH/3T3 cells, the expression of the *lox* gene was not detected under baseline conditions in a 21% oxygen environment. However, exposure of these cells to 85% oxygen increased *lox* mRNA expression, as did stimulation of NIH/3T3 cells with 2 ng/ml TGF-β (20, 23). A combination of 85% oxygen and TGF-β had a synergistic effect, causing pronounced *lox* mRNA expression (Figure 4A). In contrast, hyperoxia alone was unable to drive *loxl1* gene expression in NIH/3T3 cells; however, stimulation with 2 ng/ml TGF-β increased *loxl1* gene expression in NIH/3T3 cells, and this induction was increased when cells were maintained in an 85% oxygen environment (Figure 4A). No *loxl2* gene mRNA was detected in NIH/3T3 cells, and neither 85% oxygen nor TGF-β had any impact on *loxl3* and *loxl4* gene expression by NIH/3T3 cells

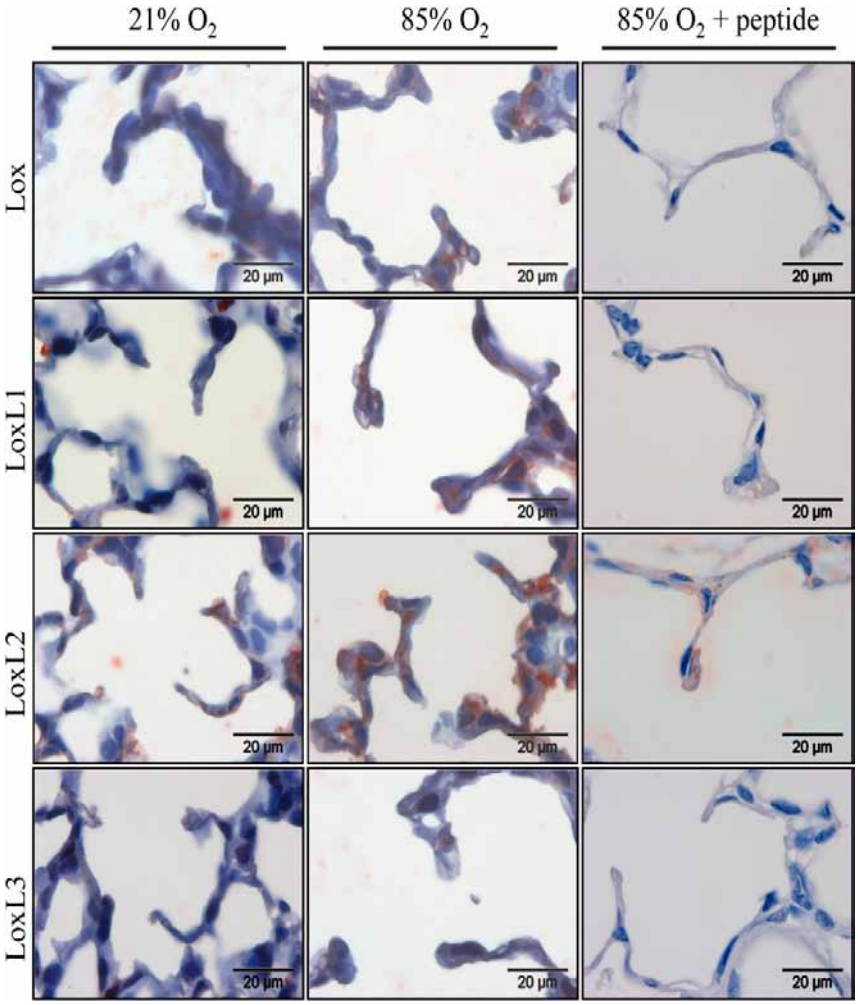

Figure 3. Lysyl oxidase protein expression is dysregulated in the septa of oxygen-injured mouse pup lungs. Increased staining intensity is observed for Lox, LoxL1, and LoxL2 in the septa of mice exposed to 85% O_2 compared with 21% O_2-exposed mouse pups at P14. Antibody specificity was confirmed by preadsorption of antibodies with a competing peptide, which had been used as the immunogen for antibody generation. Results for LoxL4 are omitted as no immunoreactivity was observed with the anti-LoxL4 antibody on mouse lung sections.

(Figure 4A). Identical trends were observed in primary human lung fibroblasts (data not shown).

In human paSMC, 2 ng/ml TGF-β increased mRNA expression of the *lox* gene, both in 21% oxygen and 85% oxygen environments (Figure 4B). However, no synergistic effect of 85% oxygen and TGF-β was observed, as had been observed in NIH/3T3 cells and human fibroblasts. Neither 85% oxygen nor TGF-β, or combinations thereof, impacted the expression of *loxl1*, *loxl2*, *loxl3*, or *loxl4* (Figure 4B). Taken together, these data indicate that *lox* and *loxl1* can be induced in fibroblasts by both hyperoxia and TGF-β, and that these stimuli can act synergistically. In contrast, TGF-β, but not hyperoxia alone, could drive *lox* expression in paSMC.

To further explore the mechanism of hyperoxia-induced *lox* and *loxl1* gene expression, the NIH/3T3 mouse fibroblast cell line and the HFL1 human fibroblast cell line were transfected with luciferase-linked promoter reporter plasmids, in which the *lox* and *loxl1* promoter regions were located upstream of a firefly luciferase gene, and activity could be assessed in the cells by measuring the expression of firefly luciferase. Consistent with the NIH/3T3 cell data presented in Figure 4A, TGF-β stimulated *lox* promoter activity, and this effect was enhanced in an 85% oxygen environment (Figure 5A, *solid bars*). Similarly, TGF-β also stimulated *loxl1* promoter activity in NIH/3T3 cells (Figure 5A, *open bars*); however, stimulation with TGF-β in an 85% oxygen environment did not have an additive effect on *loxl1* promoter activity (Figure 5A, *open bars*). This contrasts with data presented in Figure 4A, however, might be explained by the use of a human *loxl1* promoter reporter construct in a mouse fibroblast-like cell line. Therefore, studies were also performed in the transfected human HFL1 fibroblast cell line, where TGF-β (2 ng/ml) could stimulate *loxl1* promoter activity, and this effect was enhanced in an 85% oxygen environment (Figure 5A; *left panel*, *open bar*).

Incubation of NIH/3T3 cells harboring a murine *lox* luciferase-linked promoter reporter plasmid in an 85% oxygen environment caused a fivefold increase in *lox* promoter activity without addition of exogenous TGF-β (Figure 5B). This effect was abrogated when a TGF-β–neutralizing antibody was added to the cell-culture medium, suggesting that hyperoxia drove autocrine stimulation of the *lox* promoter through TGF-β production by NIH/3T3 cells. No autocrine activation of the human *loxl1* promoter was observed in NIH/3T3 cells. Interestingly, although a hyperoxia environment was able to augment *loxl1* promoter activity in response to exogenously applied TGF-β (Figure 5A, *right panel*), no autocrine activation of the human *loxl1* promoter was observed in HFL1 cells either (Figure 5B, *right panel*). This probably indicates that although the *loxl1* promoter can be activated by exogenously applied TGF-β, the levels of TGF-β or the increased activity of the

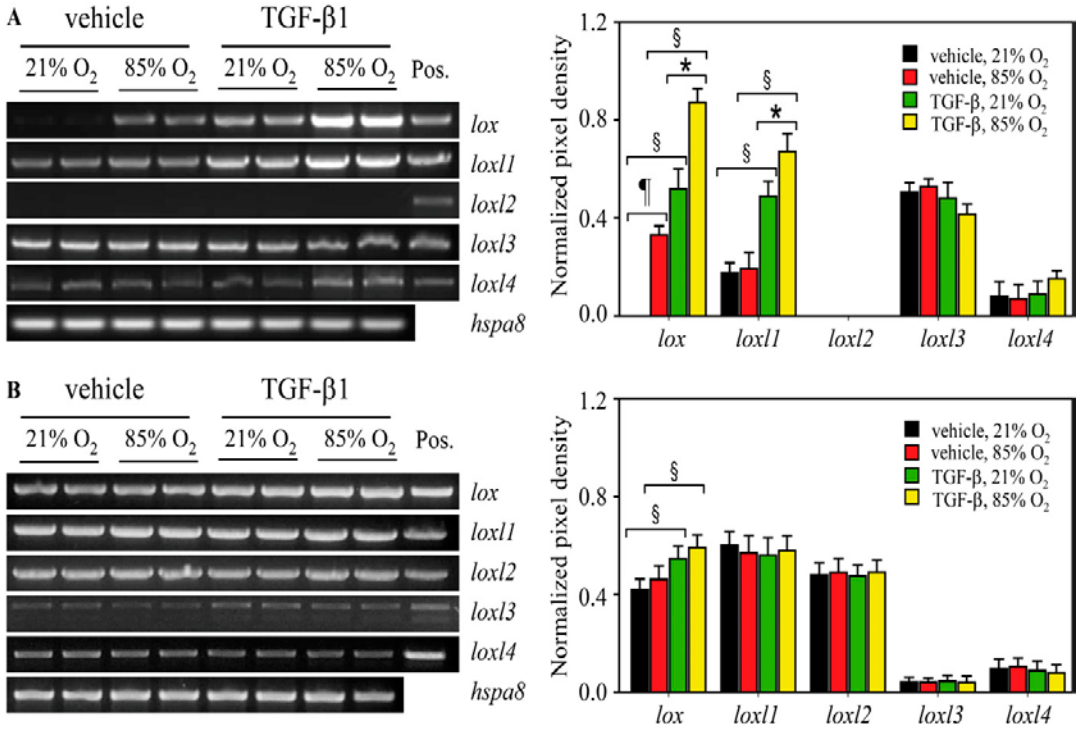

Figure 4. Lysyl oxidase mRNA expression can be modulated *in vitro* by oxygen and transforming growth factor (TGF)-β. (*A*) Murine NIH/3T3 fibroblast-like cells and (*B*) human pulmonary artery smooth muscle cells were maintained under hyperoxic (85% O_2) or normoxic (21% O_2) conditions for 24 hours, before addition of TGF-β (2 ng/ml) for an additional 24 hours, after which mRNA was isolated and assessed for lysyl oxidase gene expression. Whole-lung mRNA from mouse or human lungs, respectively, served as a positive control for the polymerase chain reactions (PCR) (Pos.), whereas expression of the *hspa8* gene was used as a loading control. The PCR amplicons derived from two separate cell cultures are represented for each condition. For quantification, densitometric data for amplicons derived from six different cell cultures per condition were averaged; ¶ $P < 0.01$ comparing 85% O_2 versus 21% O_2 exposures in the presence of vehicle; § $P < 0.01$ comparing TGF-β–stimulated versus unstimulated cells with 21% oxygen or 85% oxygen exposure; * $P < 0.01$ comparing 85% O_2 versus 21% O_2 exposures 24 hours after TGF-β stimulation.

TGF-β pathways caused by exposure to hyperoxia are not sufficient to activate the *loxl1* promoter. Taken together, these data suggest that increased oxygen tension augments Lox, and perhaps LoxL1 production by lung fibroblasts, through induction of the *lox* and *loxl1* promoter activity by TGF-β.

Attenuating TGF-β Signaling *In Vivo* Prevents Induction of *lox* Expression by Hyperoxia

Baseline TGF-β signaling is increased in the lungs of mouse pups exposed to 85% oxygen (20). To explore whether hyperoxia induced abnormal lysyl oxidase expression in the lungs of mouse pups through TGF-β–dependent signaling mechanisms, pups were treated with TGF-β1,2,3–neutralizing antibodies before exposure to 85% oxygen. This has previously been demonstrated to dampen TGF-β signaling in the lungs of 85% oxygen-exposed pups, to improve elastin biogenesis and assembly, and to limit the deleterious effects of hyperoxia on alveologenesis and vasculogenesis (22). Treatment of pups with TGF-β1,2,3–neutralizing antibodies before exposure to 85% oxygen reduced pulmonary *lox* mRNA expression in the lung, as assessed by semiquantitative RT-PCR (Figure 6A), as well as quantitative real-time RT-PCR (Figure 6B). No effects on the mRNA levels of *loxl1*, *loxl2*, *loxl3*, or *loxl4* were observed (data not shown). Moreover, Lox protein immunoreactivity in the lungs of pups exposed to 85% oxygen appeared to be less intense in lung tissue from pups treated with TGF-β1,2,3–neutralizing antibodies, compared with tissue from pups treated with an isotype-matched nonimmune control antibody (Figure 6C). Additionally, improved elastin deposition was evident in the lungs of mouse pups exposed to hyperoxia that were treated with TGF-β1,2,3–neutralizing antibodies (Figure 6D).

Lox and LoxL1 Expression Is Increased in Human Infants with BPD or at Risk for BPD

The data presented above suggest that lysyl oxidase expression is increased in the injured developing mouse pup lung. To assess whether the expression of lysyl oxidases is similarly modulated in the injured developing lung of humans, the immunoreactivity and mRNA levels of lysyl oxidases were examined in 10 prematurely born infants who died either with BPD or had been at risk for the development of BPD (Table 2; the "BPD group"). The BPD group probably represents a mixture of new BPD and classic BPD. The use of surfactant in 8 out of 10 patients places these patients in the post-surfactant era, and the extremely low birth weight (median, 878 g) and gestational age at birth (27 wk) argue in favor of new BPD, although some degree of fibrosis can be observed in some patients, which is more characteristic of classic BPD. Two groups of control patients were used, which included either seven patients with the gestational age at birth and birth weight matched to the BPD group (control group 1; Table 1), or eight patients with the

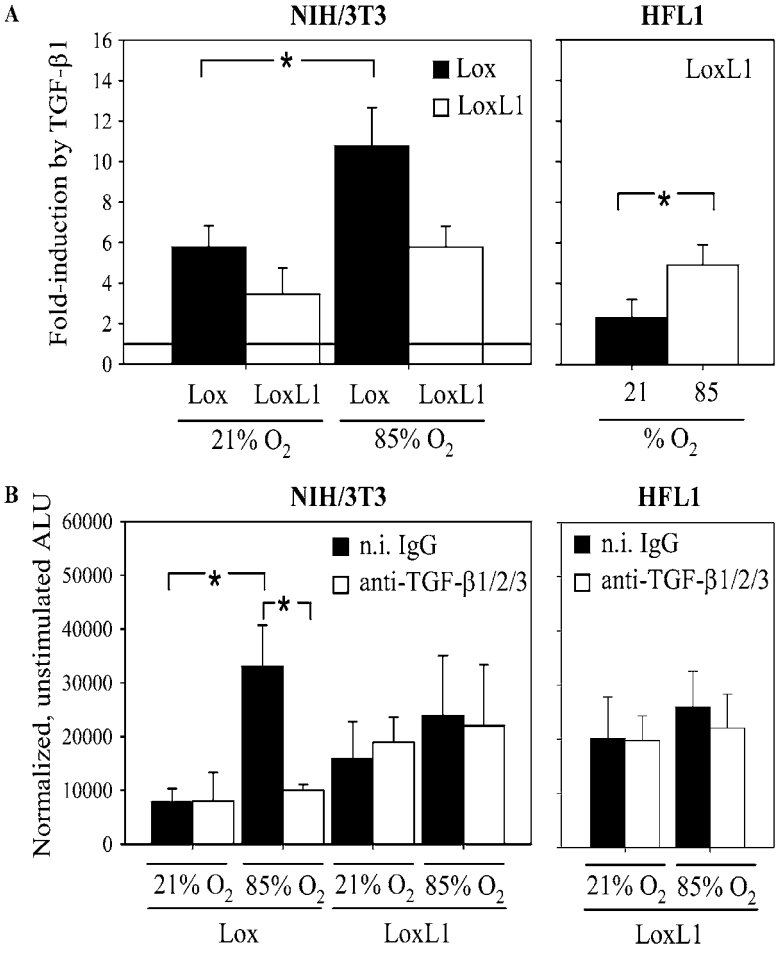

Figure 5. Hyperoxia induced autocrine activation of lysyl oxidase promoters in NIH/3T3 through transforming growth factor (TGF)-β. (*A*) Murine NIH/3T3 fibroblast-like cells and human HFL1 fibroblast-like cells were transfected with plasmid constructs in which the mouse *lox* or human *loxl1* promoters had been inserted, upstream of a firefly luciferase gene. The cells were maintained under hyperoxic (85% O_2) or normoxic (21% O_2) conditions for 24 hours, before addition of TGF-β (2 ng/ml) for an additional 12 hours, after which cell extracts were assessed for firefly luciferase activity. To control for the effects of ligand stimulation and hyperoxia on the baseline transcriptional activity of cells, values were normalized for the transcriptional activity of the pGL3-control vector (20). (*B*) Murine NIH/3T3 fibroblast-like cells and human HFL1 fibroblast-like cells were treated as described in *A*, except that medium was supplemented with nonimmune IgG or anti–TGF-β1,2,3–neutralizing antibodies over the entire time course of the experiment (10 μg/ml). **P* < 0.01 (n = 5). ALU = arbitrary luminescence units; n.i. = nonimmune.

chronological age at death matched to the chronological age at death of the BPD group (control group 2; Table 1).

Lung sections from control patients (Figures 7A, 7B, 7E, and 7F) exhibit normal lung expansion with loose extracellular matrix in the interalveolar septa without inflammatory cells. The epithelial lining of the alveoli contains a single cell layer without epithelial defects, and hyaline membranes are not evident. In contrast, unequal lung expansion and broadened intraalveolar septa are evident in lung sections from patients with BPD (Figures 7C, 7D, 7G, and 7H), with desquamated epithelial lining and casts evident in the larger airways, along with eosinophilic infiltrates and an increased abundance of neutrophilic cells in the intraalveolar septa.

Pronounced immunoreactivity was observed for LoxL1 in the smooth muscle layer of blood vessels in control infants (age-matched for chronological age at death with the BPD group) who died without apparent lung disease (Figures 7A and 7B) and in patients with BPD (Figures 7C and 7D). LoxL1 immunoreactivity was also observed in the alveolar tissue of patients with BPD (Figures 7C and 7D) but not those of control patients (Figures 7A and 7B). A similar pattern of staining for Lox was observed. Interestingly, although LoxL1 appeared to stain the entire vascular smooth muscle layer, Lox expression appeared to be confined to the inner smooth muscle layer (Figure 7B vs. Figures 7F and 7H). Lox staining was evident exclusively in the pulmonary vascular smooth muscle of control patients (Figures 7E and 7F), whereas lung sections from the group of patients with BPD revealed additional staining in cells within the alveolar lumen and in cells lining the alveolar walls. The source of Lox and LoxL1 in the alveolar lumen and cells lining the alveolar walls has not been clarified. Both Lox and LoxL1, which are secreted enzymes, may be derived from interstitial fibroblasts, or from the lung epithelium itself, because Lox and LoxL1 have now been detected in prostate, mammary, and retinal epithelial cells (34–36).

The antibody specificity was confirmed by preadsorption of anti-Lox and anti-LoxL1 antibodies with a competing peptide (Figures 7I and 7J). No changes in the immunoreactivity of LoxL1, LoxL2, LoxL3, or LoxL4 were seen in lung sections from any patients with BPD compared with control patients (data not shown).

A more quantitative assessment of lysyl oxidase expression was obtained by screening whole-lung RNA from patients with BPD and control patients by quantitative real-time RT-PCR. Consistent with the immunohistochemical data, an increase in lung *lox* mRNA expression levels was seen in patients with BPD versus both the control group with the gestational age at birth and birth weight matched to the BPD group (control group 1), and the control group with the chronological age at death matched to the chronological age at death of the BPD group (control group 2) (Figure 7K). In contrast, lung *loxl1* mRNA expression levels were increased in patients with BPD compared with the control group 2, but not when compared with control group 1 (Figure 7K). The mRNA expression levels

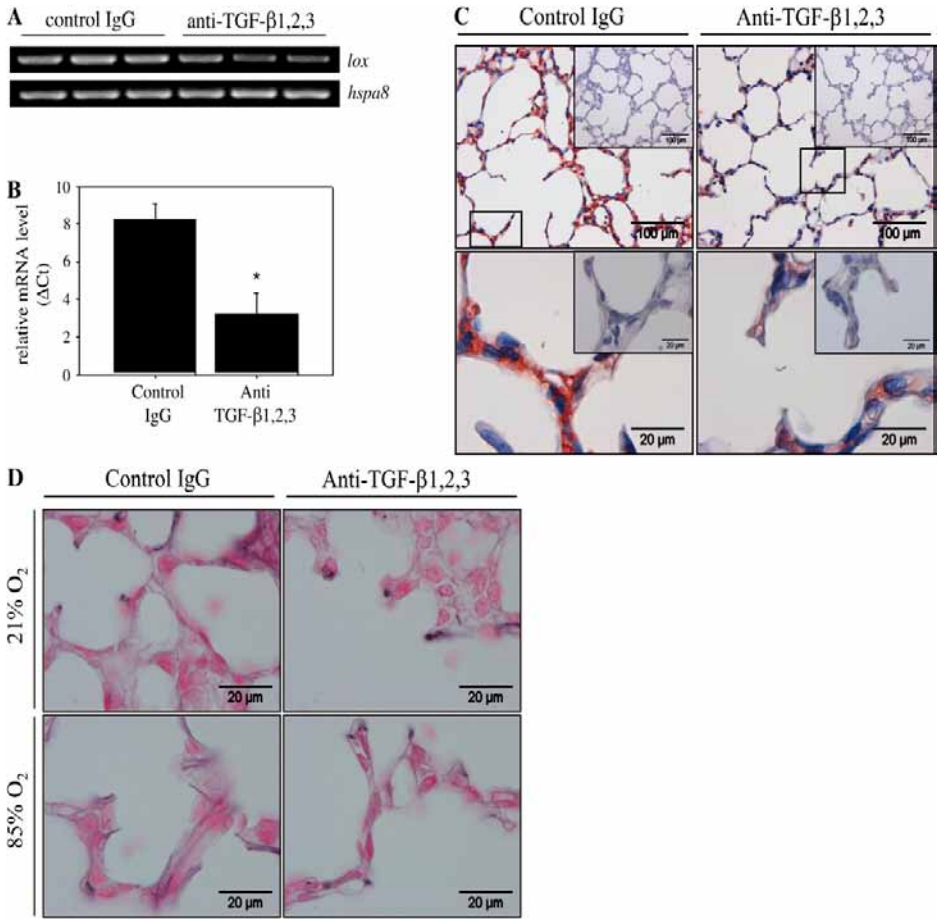

Figure 6. Treatment of neonatal mice with transforming growth factor (TGF)-β1,2,3–neutralizing antibodies suppressed the induction of *lox* gene expression by hyperoxia. Neonatal mice were treated either with control IgG or anti–TGF-β1,2,3–neutralizing antibodies before exposure to hyperoxic (85% O_2) or normoxic (21% O_2) conditions for 10 days, and then killed. The expression of lysyl oxidases was assessed in mRNA from mouse lungs by (*A*) semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR), where expression of the *hspa8* gene was used as a loading control; or (*B*) real-time quantitative RT-PCR (n = 5); or (*C*) by immunohistochemistry. Antibody specificity was confirmed by preadsorption of antibodies with a competing peptide, which had been used as the immunogen for antibody generation (*insets*). Additionally, (*D*) lung sections from mice were screened for assessment of elastin deposition in the developing septa by Hart's elastin stain. $*P < 0.01$.

for *loxl2*, *loxl3*, and *loxl4* were unchanged between the BPD and both control groups. As 6 out of 10 patients with BPD or at risk for developing BPD received corticosteroids, these two subgroups of patients (patients 17, 22, and 23 vs. patients 18, 19, 24, and 25; Table 2) were assessed for differences in *lox* mRNA expression. This comparison is relevant as corticosteroids have powerful effect on gene expression. No difference in the expression level of any lysyl oxidase mRNA was observed between the corticoid-treated and untreated groups of patients (results not shown).

DISCUSSION

The production and remodeling of the ECM are critical processes required for the secondary septation of the developing lung and the production and maturation of functional pulmonary gas exchange units (37, 38). The pathogenic mechanisms that result in the perturbed deposition and remodeling of the ECM observed in important developmental lung diseases such as BPD (12, 15) are poorly understood. Although several studies performed to date have examined the production of ECM molecules in BPD, few have addressed the subsequent processing of these molecules, which can facilitate the remodeling of the matrix itself. Therefore, in this study, the expression and activity of lysyl oxidases, a family of enzymes that play a crucial role in regulating the stability of the ECM, were examined in an animal model of BPD, and in prematurely born infants who have died with BPD or at risk for BPD.

The data presented here demonstrate the up-regulation of three elastin/collagen cross-linking enzymes (Lox, LoxL1, and LoxL2) in newborn animals with hyperoxic lung injury and dysregulated ECM development, and up-regulation of at least two elastin/collagen cross-linking enzymes (Lox and LoxL1) in patients with BPD. Interestingly, two of these enzymes, Lox and LoxL1, have already been identified as being temporally regulated during normal mouse lung development (39). Additionally, both $lox^{-/-}$ (25, 40) and $loxl1^{-/-}$ (41) mouse pups exhibit disturbed respiratory tract development, and although $lox^{-/-}$ embryos develop to term, they die within hours of birth with evidence of pronounced cardiopulmonary

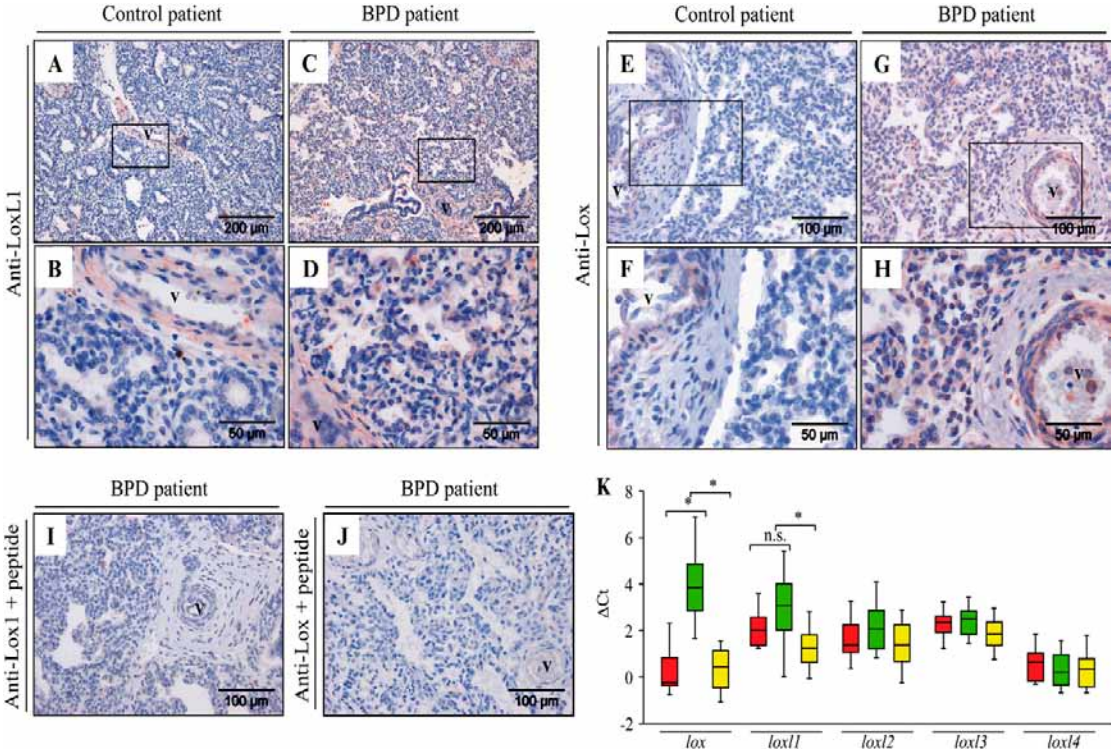

Figure 7. The expression of Lox and LoxL1 was elevated in the lungs of neonatal patients who died with BPD or were at risk for BPD. The expression of lysyl oxidases was assessed in the lungs of patients with BPD or at risk for BPD, as well as in control lungs. A low-power (200-μm scale) view of representative sections from (*A*) patients in control group 2 (the histopathology of six patients [Table 1] was examined; in this case, sections from patient 11 are illustrated) and from (*C*) patients in the BPD group (the histopathology of seven patients [Table 2] was examined; in this case, sections from patient 24 are illustrated), stained for LoxL1. (*B, D*) High-power views (50-μm scale) are also illustrated for LoxL1 staining in the same sections (the magnified area is demarcated in the low-power view by a *black box*). Representative medium-power views (100-μm scale) are illustrated for the same two patients, stained for Lox (*E, F*), with the corresponding high-power views (50-μm scale) illustrated in *F* and *H* (the magnified area is demarcated in the low-power view by a *black box*). (*I, J*) Antibody specificity was confirmed by preadsorption of antibodies with a competing peptide, which had been used as the immunogen for antibody generation, before staining a section of lung tissue from the same patient with BPD. Staining was consistently more intense in patients with BPD, and the sections illustrated are representative of the trends observed in a total of five patients assessed per group (as indicated in Tables 1 and 2). The expression of lysyl oxidase mRNA was also assessed in mRNA isolated from the lungs of seven patients in control group 1 (*red*), seven patients with BPD or at risk for BPD (*green*) and five patients in control group 2 (*yellow*) by quantitative real-time reverse transcriptase–polymerase chain reaction (*K*). The *bars* represent the data range and the *boxes* represent lower and upper quartiles. The *line* within the quartile box indicates the median; $*P < 0.01$.

failure (25, 40). These data suggest a key role for this family of enzymes in the development of the respiratory tract. Additional studies in these knockout mice have revealed key roles for lysyl oxidases in imparting tensile strength to connective tissue, because they exhibit pathologies consistent with a loss of structural stability of the ECM. For example, soon after parturition, $lox^{-/-}$ pups exhibit ruptured arterial aneurysms and ruptured diaphragms (25), whereas adult $loxl1^{-/-}$ mice develop pelvic organ and rectal prolapse and excessive skin laxity (41). In addition to the induction of lysyl oxidase gene expression by hyperoxia reported in this study, mechanical ventilation of mice with room air or with 40% oxygen gas mixtures for 8 hours was also able to increase *lox*, but not *loxl1* mRNA expression (13); however, a similar modulation of lysyl oxidase gene expression has not been observed in premature lambs mechanically ventilated with high ($F_IO_2 \approx 0.26$) levels of oxygen (42). Variable responses of elastin gene expression to oxygen levels has also been reported. Although a mild increase in *eln* mRNA levels was observed in response to 85% oxygen exposure of mice in this study, Bruce and colleagues (43) reported a decrease in elastin gene expression in the lungs of rats exposed to 95% oxygen between P4 and P14. This paradox might be explained by the higher oxygen concentrations used in the rat model, or by the very different sensitivities to high oxygen concentrations displayed by mammals, even between different mouse strains (44).

The transcriptional regulation of lysyl oxidases is poorly described; however, several growth factors are known to drive lysyl oxidase expression (16, 17). Notable among these are members of the TGF-β family of peptide growth factors, which are also key regulators of late lung development (45). Recently, excessive TGF-β activity has been identified as a key pathogenic factor in animal models of BPD (20, 22, 46), which suggested that dysregulated TGF-β signaling might underlie the aberrant expression of lysyl oxidases in BPD. Indeed, dampening of TGF-β signaling in the newborn lung injury model used in this study reduced the expression of the *lox* gene, although expression of other members of the lysyl oxidase family was unaffected. Thus, in the case of oxygen injury to the

lungs of mouse pups, elevated Lox levels are attributable to increased TGF-β signaling in the lungs of affected animals, which most likely drives Lox production by lung fibroblasts. Along these lines, elevated levels of TGF-β1 have also been detected in bronchoalveolar lavage fluids of ventilated neonates who went on to develop new BPD, versus ventilated neonates who did not (47). This observation might suggest that elevated *lox* mRNA levels seen in infants who died with BPD or were at risk for BPD may be attributable to elevated TGF-β levels in these neonates.

The increased expression of lysyl oxidase family members reported here is consistent with the increased levels of tissue desmosine, an enzymatic product of lysyl oxidase, that are observed in animal models of BPD (this study and Reference 12). Patients who develop BPD exhibit increased urinary desmosine levels (48), which have been attributed to increased elastin degradation due to proteolysis in association with infection and prolonged exposure of infants to hyperoxia (49). However, data presented here suggest that the increased desmosine levels may be due, at least in part, to an overactive cross-linking system in affected lungs, which could promote excessive cross-linking of elastin and collagen fibers, perhaps resulting in the accumulation of elastic fibers in peripheral lung tissue (9, 10). Such mechanisms could give rise to the "thickened, tortuous and disorganized" collagen fibers that are often observed in patients who have died with BPD (11). Such disorganized collagen fibrils have been observed in the skin of patients with amyotrophic lateral sclerosis who have excessive lysyl oxidase activity. In such patients, the thickness of collagen fibers directly correlated with the number of stable collagen cross-links (50), further supporting the notion that elevated lysyl oxidase activity might generate the thickened collagen fibers observed in the lungs of patients with BPD. This idea is also supported by the observation that elastic fibers in the lungs of $lox^{-/-}$ pups are thinner than in wild-type age-matched pups, and that collagen fibers in $lox^{-/-}$ pups were dispersed and loose (51), contrasting with the thickened, tightly bundled fibers observed in patients with BPD (11). Clearly, other mechanisms of elastin fiber assembly may also be affected in BPD. Mechanical ventilation of mice with 40% oxygen gas mixtures for 8 hours decreased the expression of fibulin-5 and emilin-1 (13), which are critical players in elastin fiber dynamics. In this study, both fibulin-5 and emilin-1 expression appeared to be dysregulated at the mRNA level, with increased mRNA abundance evident in oxygen-injured lungs. Dysregulated expression levels of both molecules would most likely affect elastin fiber assembly, and suggests an avenue independent of lysyl oxidases, which may impact elastin fiber formation and maintenance in oxygen-injured lungs.

Changes in lysyl oxidase activity and expression have not been causally implicated in the development of BPD in this study. The early perinatal death of the $lox^{-/-}$ knockout animals prevents their use in studies of postnatal lung injury. However, with the data presented in mind, the authors would like to propose a novel hypothesis of how disordered elastin and collagen cross-linking and deposition might inhibit alveolarization in the injured developing lung. It is generally believed that as the lung develops, a continuous cycle of ECM deposition and remodeling occurs: matrix scaffolding must be erected to support the developing structures of the lung and in some locations the matrix must be removed and/or otherwise remodeled to permit extension or reshaping of existing structures (15). The authors propose that excessive elastin and collagen cross-linking by overabundant lysyl oxidases might overstabilize elastin and collagen and make them chemically resistant to the normal proteolytic and other remodeling events that are required for successful ECM remodeling and alveolar and microvascular development.

This hypothesis is supported by studies in which it has been demonstrated that the degree of elastin and collagen cross-linking determines the susceptibility of ECM fibers to proteolytic degradation, and hence, remodeling. Disruption of elastin cross-linking through dietary copper restriction (which down-regulates the activity of Cu^{2+}-dependent lysyl oxidases) reduced the accumulation of elastin in the aorta of experimental animals (52), which was directly attributed to increased proteolytic degradation of elastin molecules in copper-restricted animals, because elastin had become more susceptible to proteolysis as a result of reduced cross-linking (52). Indeed, the inability to cross-link tropoelastin renders this molecule sensitive to proteolysis by trypsin-like proteases (53). Conversely, hyper-crosslinking of collagen fibers in tissue-engineered arteries through stimulation of lysyl oxidase activity increased the stability of collagen fibers to proteolysis (54). Thus, elevated lysyl oxidase activity observed in animal models of BPD—and in patients with BPD—may generate excessively cross-linked elastin and collagen fibers that are resistant to proteolysis and degradation-dependent remodeling.

Clearly, the impact of an overstabilized matrix would not be limited to the division of the alveolar septa, but could also extend to pulmonary vascular development. In addition to decreased alveolar septation, patients with BPD exhibit pronounced disruption of pulmonary vascular development (5–8). An inability to remodel the vascular matrix, as might occur in an overstabilized vascular matrix, would also have implications for vascular development. Remodeling of the ECM by proteases promotes cell migration, which is critical for vascular development, and matrix-bound growth factors that are released during vessel matrix remodeling, promoting proper angiogenesis by regulating endothelial migration and growth (reviewed in Reference 55). These processes would be impeded by an overstabilized ECM. Furthermore, Lox has already been implicated in several vascular pathologies (56) in which, when overexpressed, Lox can promote local neointimal thickening and vessel muscularization. These observations are interesting, because in addition to dysregulated angiogenesis, the pulmonary vasculature in patients with BPD undergoes remodeling that includes medial hypertrophy (thus, vessel thickening) and muscularization of small arteries.

Thus, it is proposed here that as a consequence of up-regulated expression of ECM cross-linking enzymes such as lysyl oxidases, the ECM is less plastic and resists remodeling that facilitates lung growth. This may, at least in part, underlie the impaired septation and vascular development seen in patients with BPD and animal models of BPD.

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Chapter 9

Congenital diaphragmatic hernia; a pulmonary vascular point of view.

General discussion

Introduction

Congenital diaphragmatic hernia (CDH) remains one of the most intricate disorders to face the pediatric surgeon and neonatologist. With an incidence of approximately 1 in 2500 live births, CDH is a relative common congenital disorder and represents a major cause of perinatal morbidity and mortality.[1,2] CDH is not merely a defect in the diaphragm, but is a disease characterized by "compromised lung" growth *in utero* and frequently associated with various anomalies, which in some cases are diagnosed as a defined syndrome.[3,4] The etiology of CDH is still largely unknown, although our knowledge of genetic and environmental factors involved in CDH has extended remarkable the pas few years, for reviews see Klaassens et al., Beurkens et al.[5,6]
The lack of properly designed RCT's with enough power is an important shortcoming to guide optimal patient care. In studies aimed to evaluate the role and significance of therapies such as ECMO and iNO, CDH patients are included as a subgroup.[7,8] However, none of these studies had the primary aim to investigate CDH patients as a target population.

Probability of survival is determined mainly by the degree of lung hypoplasia and the severity of pulmonary hypertension (PH). Therefore therapy is primarily focused on the associated pulmonary pathophysiology, which surpasses the need to close the diaphragmatic defect. Only few patients die due to an absolute shortage of lung parenchyma. Yet, PH in CDH patients is often resistant to our modern treatment modalities, including inhaled nitric oxide (iNO).[9,10] As a consequence, PH of the newborn, which is intimately tied to defects in pulmonary vascular development and a disordered process of pulmonary vascular remodeling (in total pulmonary vascular "disease") determines the significant morbidity and mortality in infants with CDH.

The focus of this thesis is to understand pulmonary vascular "disease" associated with CDH. In this chapter we discuss the following major issues and speculate on the remaining research questions and clinical implications:

1) Derailment of pulmonary vascular development in CDH
2) Imbalance of pulmonary vascular tone in CDH
3) Structural remodeling of the pulmonary arterial wall in CDH

Pulmonary vascular development

Morphology

The vascular network of the lung is established from 5 weeks of gestation onwards and continues to develop postnatal up to 2 years of age.[11] This network is composed of endothelial cells, enclosed by vascular smooth muscle cells, pericytes, and other mesenchymal cells. The conducting airways and vessels branch together and all pre-acinar vessels are present by the 17th week of gestation. At the level of the alveoli (intra-acinar) the vessels branch into an extensive capillary network, a process referred to as microvascular maturation.[12] Although originally considered as passive "followers" of airway branching new insights focus on the determining role of pulmonary vascular development guiding airway differentiation especially in the hypoxic environment of the fetus and normal alveolarization postnatal.[13,14]

In CDH aberrant development of the pulmonary vasculature occurs at three different stages (Figure 9.1).

Prenatal a smaller number and generation of airways and adjacent arteries with excessive muscularization of the small pulmonary arteries is observed.[15-17] Secondly, CDH patients fail to undergo appropriate transition at birth. The normal postnatal remodeling of the pulmonary arteries does not occur and increased thickness of the adventitial and media layer remains.[18] Sustained high pulmonary vascular resistance and lung injury induced by mechanical ventilation, high FiO_2, and eventually infection may lead to further medial hypertrophy and thickening of pulmonary veins in newborns with CDH. As PH persists, complex lesions of the intima are seen. The

pathology of established stages of PH in newborns with CDH show similarities to the end stage PH in adults.[18-20]

Although the altered morphology of the pulmonary arteries in CDH is well described, less is known about the molecular mechanisms that are involved in normal and pathological pulmonary vascular development and remodeling. Detailed investigation of the role of inflammatory cytokines as in adult PH is not available so far in newborn PH. Moreover even rather basic information such as the proliferation/ apoptosis ratio of the pulmonary vasculature is lacking.

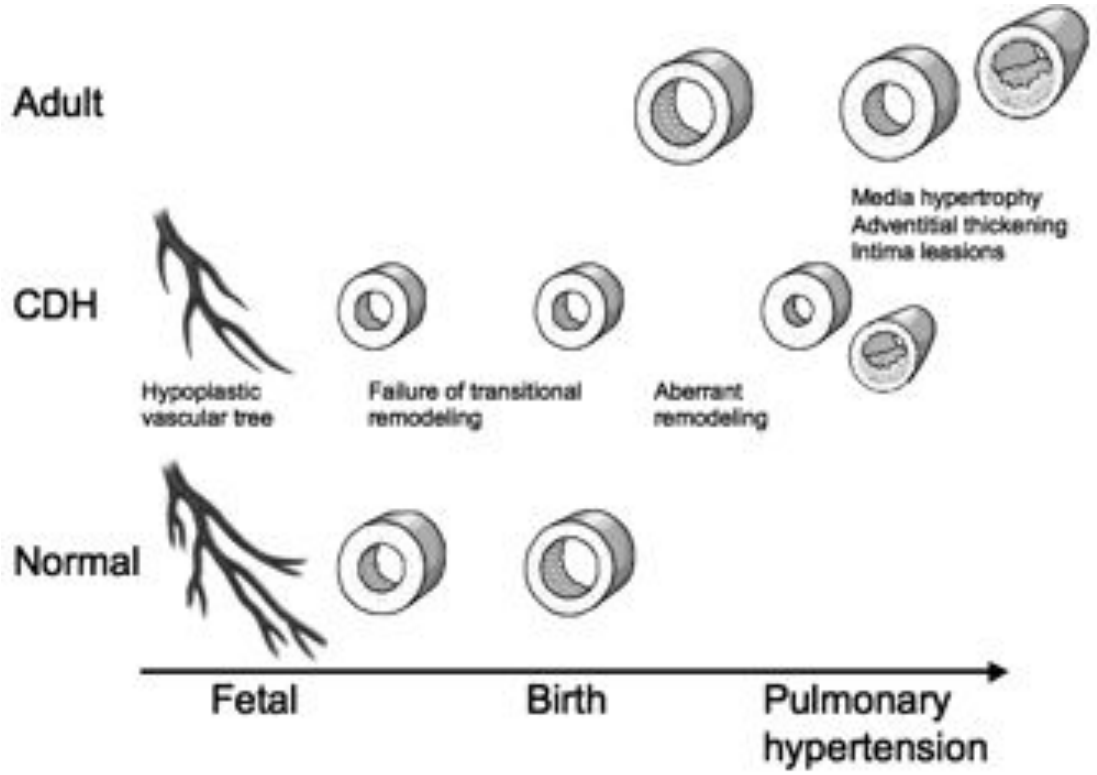

Figure 9.1 (Color figure page 174)
Aberrant development of the pulmonary vasculature in CDH.
(Figure was produced using Servier Medical Art)

Molecular mechanisms

Since vascularization of the developing lung is very complex it must be tightly controlled. Although much has been learned regarding the mechanisms involved in lung bud formation and airway branching, our understanding of factors controlling pulmonary vascular development is more recent.[21] Vascular development is controlled by three different families of endothelial derived growth factors: VEGF and its

different isoforms angiopoietins and ephrins.[22] Furthermore, hypoxia inducible factors (HIF) are known to act as important transcription factors in pulmonary vascular development.[23,24]

By far, the most investigated growth factor is VEGF and its different isoforms, which together with its receptors is well known to be critical for vascular development. [25] Although evidence is scarce, there appears to be no critical role for VEGF in the early pathogenesis of CDH, as no difference in VEGF expression is shown in human lung tissue (**Chapter 4**). However, in lung explants of nitrofen exposed rat fetus, VEGF expression is reduced and exogenous VEGF can improve growth of lung explants.[26,27] Furthermore, the increased lung growth in tracheal occlusion studies in animals is paired with increased VEGF-A expression.[28] Whether a causal relationship exists or just an epiphenomenon remains to be elucidated. In the process of microvascularization during the alveolarization period of lung development, VEGF also plays a considerable role.[29] Nevertheless, data on VEGF expression in newborns with CDH is contradicting; one study showed an increased VEGF protein expression [30], one showed a decreased VEGF mRNA expression (**Chapter 4**), and one did not find any difference in VEGF protein expression in human newborns with CDH. [31] The very precise and coordinated regulation of VEGF in normal angiogenesis, which depends on oxygen tension, other growth factors, and receptor availability probably, explains the contradicting results in this very heterogeneous patient group. Also in adult PH, VEGF may have a dual role, with early protection followed by a potentially pathogenic induction of pulmonary vascular remodeling.[32,33]

Next, Angiopoietins (Ang) and their corresponding tyrosine kinase receptors (Tie) are necessary for maturation of the pulmonary vasculature by mediating endothelial cell survival and recruitment of vascular smooth muscle cells.[34,35] One study in nitrofen-induced lung hypoplasia in murine fetuses showed increased Ang-1 protein expression prenatal.[36] However, in extremely premature infants, who developed BPD, decreased Ang1 inhibits pulmonary angiogenesis.[37] Also in the lungs of patients with various forms of PH Ang-1 and Tie2 expression are strongly upregulated and associated with SMC hyperplasia.[38,39] So, Ang-1 is necessary for stabilization and

maturation of the developing vascular network, but overexpression is associated with early arrest of vascular development or aberrant vascular remodeling.

Other endothelial derived angiogenic growth factors that are suggested to play a role in pulmonary vascular development are Ephrins and their receptors. They are activated by VEGF and expressed in human fetal pulmonary arteries.[40,41] Ephrins control cellular migration and spatial organization of arteries, capillaries and venes.[42] Their role in the altered pulmonary vascular development in CDH remains to be investigated.

Angiogenic growth factors like, VEGF, Angiopoietins, and Ephrins are activated by hypoxia-inducible factor α (HIFα), one of the most important transcription factors involved in angiogenesis.[43] The three homologues of HIFα are expressed early in the developing lung and HIF-2α even shows a developmental regulated expression in the human lung (**Chapter 3**).[44] Stabilization of HIF during early murine lung development leads to hypervascularization and stimulation of HIF in the primate model of BPD leads to enhances angiogenesis in the alveolar stage.[45,46] Evaluation of HIF-1α expression in lungs of CDH patients revealed differences in expression patterns.[31] Furthermore downregulation of HIF-2α might play a role in the decreased VEGF expression in CDH patients (**Chapter 4**). Moreover, in adult forms of PH HIF-2α plays a critical role in pulmonary vascular remodeling.[47] The results of our studies in combination with the current literature on the role of HIF in lung and vascular development are highly suggestive for involvement of the HIF pathway in the pathophysiology of CDH.

So far, stimulation of angiogenic growth factors, like VEGF in combination with Ang-1 and enhancement of HIF signaling by PHD inhibition in experimental non-CDH animal models has shown to preserve pulmonary vascular development. [29,46] Both are suggested as new therapeutic avenues, however major gaps in our understanding remain before translation of angiogenic growth factors modulation can be applied safely in a clinical setting.

Although, our understanding of the molecular mechanisms controlling pulmonary vascular development has grown rapidly in the past decade, the challenge now is to integrate the knowledge on the involved transcription and growth factors to improve our understanding of the aberrant vascular development in CDH.
Therefore we need insight in,

1) growth factor availability and the coordinated presence of their receptors,
2) downstream transcription targets of these growth factors,
3) their role in lung injury, and
4) the relative combination of growth factors for appropriate balance between angiogenesis and vessel maturation.

Pulmonary vascular tone in CDH: a matter of balance

Besides studies investigating the morphological abnormalities of pulmonary vascular development in CDH, other studies focus on the presumed altered pulmonary vascular reactivity. Every clinician, who takes care of CDH patients, knows the frustration of being unable to predict the pulmonary vascular response to the instituted therapy.
The fact that only 30% of high-risk newborns with CDH response to inhaled NO and the scarcity of pharmacokinetic data of vasodilators as Sildenafil, Bosentan etc. adds to the usual "trial and error" aspect of therapy. Even the effect of ECMO, although debated, is not really understood. In principle venous/venous-ECMO supplying the pulmonary vasculature with oxygenated blood would be the ideal mode to modulate pulmonary vascular tone. However, in clinical practice many failures have been observed. The inability to "open" the pulmonary vessels under these circumstances has guided many institutions to use venous/arterial ECMO as their primary mode of treatment when ECMO is offered. It cannot be ruled out that the diminished flow to the pulmonary vasculature in itself has an effect on the behavior of these vessels.
Normally at birth oxygenation, shear stress and respiration causes a drop in pulmonary vascular resistance, allowing the pulmonary circulation to abruptly accommodate 100% of the cardiac output.[48] Failure of the pulmonary circulation to achieve and sustain this pressure drop leads to PH of the newborn, as is seen in CDH.

Besides these physiological stimuli, an impaired release of vasodilators together with an increase of vasoconstrictor production and enhanced myogenic tone of the vascular smooth muscle cells (SMC) are considered potential mechanisms leading to the development of PH in CDH.[49]

Thus far, nitric oxide (NO) and prostacyclin (PgI_2) are considered the key players involved in the transition at birth and the most potent vasodilators of the pulmonary vasculature.[50] Laboratory studies designed to characterize the NO-pathway in animal-models of CDH have yielded conflicting results. In the nitrofen-induced rat model, endothelial nitric oxide synthase (eNOS) expression and activity was decreased.[51,52] However in the surgical lamb model no reduction in eNOS expression or activity was demonstrated.[53,54]

More downstream in the NO pathway, PDE5 inhibition has become available as a therapeutic option in the treatment of PH. In CDH animal model studies no difference in PDE5 expression or activity was found (**Chapter 5**).[54,55] However, most data on PDE5 expression and activity are derived from studies in animal models of PH other than CDH and showed an increased expression of PDE5.[56,57] In newborns with CDH associated PH, Sildenafil, a PDE5 inhibitor improved oxygenation and cardiovascular function.[58,59] Also combination therapy of Sildenafil with iNO increases the efficacy.[60] Recently, i.v. Sildenafil was well tolerated by newborns with PH without CDH and higher infusion doses (1.6 mg/kg/24hr) had an acute and sustained improvement in oxygenation.[61] However, no randomized controlled trials are available and the pharmacokinetics of Sildenafil in a group of CDH patients following ECMO is highly variable.[62]

Another vascular tone modulator that gains perspective in the treatment of PH in newborns with CDH is the prostaglandin family. Studies on the prostaglandin pathway in animal models are limited and showed higher level of prostacyclin (PGI_2) in nitrofen-induced CDH.[63] PGI_2 is a potent vasodilator of the pulmonary circulation and case reports described successful use in the treatment of newborns with PH.[64-66] However, only case reports and small studies are available for this group of drugs in CDH patients.

On the vasoconstrictor site of the pulmonary vascular tone research has been focused on the role of Endothelin (ET-1). Although ET-1 causes an intense vasoconstriction in vitro, its effects in the intact pulmonary circulation are complex and depending on the presence and distribution of its receptors. Stimulation of the ET-B receptor produces a vasodilator response, but ET-1 is more likely to cause vasoconstriction via the ET-A receptor. Several studies in animal models of CDH showed increased expression and activity of ET-1 and its ET-A receptor in CDH.[67-69] Also in newborns with PH ET-1 plasma levels were elevated.[70] Both, non-selective ET receptor antagonists, like Bosentan as well as, specific ET-A receptor antagonists, like Sitaxsentan and Ambrisentan may improve PH.[71-73] However no randomized controlled trials have been carried out to test the effects of ET receptor antagonists in newborns with PH.

In summary: more has to be learned about the specific reaction pattern of the pulmonary vasculature in CDH. The obvious difficulty of obtaining human material and subsequent evaluation using a combined approach of in-vitro pharmacology and agent and receptor density studies hampers progress in our level of knowledge. Till that time the early identification of the patient who will not respond as well as the choice of the appropriate drug or combination of drugs to decrease pulmonary vascular resistance remains one of the major challenges in clinical practice.

PH in newborns is essentially reversible, however a significant number of patients with PH and CDH become refractory to pulmonary vasodilator therapy. Therefore, the focus has recently moved to the altered responsiveness of the vascular smooth muscle cell (SMC), such as enhanced myogenic tone. Finally, the balance between vasodilators and vasoconstrictors results in a state of SMC polarization, which in turn determines the phosphorylation/ dephosphorylation status of myosin light chain (MLC), the conductor of SMC contraction.[74]

The key players in SMC polarization are potassium channels. There are various families of potassium channels, which might play different roles in the pulmonary vascular transition at birth and in the development of PH in CDH.[75] In the nitrofen rat model we showed an attenuated vasodilator response to a BK_{Ca} agonist in the CDH

group. (**Chapter 6**) However no pharmacological modulation of potassium channels has yet become available as possible treatment of PH. [76]
Next, the phosphorylation of the MLC depends on SMC Ca^{2+} sensitivity, which is influenced by RhoA and the Rho-kinase pathway. Although the function or possible dysfunction of the Rho-kinase pathway in the pulmonary vasculature in CDH is unknown, a Rho-kinase inhibitor, Fasudil, showed promising results in the treatment of PH in several animal studies.[77,78] To date the clinical use of Rho-kinase inhibitors is still experimental.[79]

In summary, there is a need for a better understanding of the physiology of the developing pulmonary circulation. Especially regarding, 1) alter vascular tone, reactivity and function in animal models of CDH, taking in account that these studies are often conducted in early stages of pulmonary transition. The real PH treatment problem in newborns with CDH usually presents after an initial period of relative stability and is influenced by mechanical ventilation, shear stress, and hyperoxic exposure, 2) new treatment strategies and targets, including modulation of SMC reactivity by, for example Fasudil, and 3) the effect of pulmonary vascular tone modulators on the potential lung growth and pulmonary vascular maturation. New treatments will preferentially target the long-term control of vascular remodeling rather than vasoconstriction. In newborns, the aim must be to act as quickly and as effectively as possible to "re-track" pulmonary arterial remodeling along the normal pathway. For example, early and prolonged iNO therapy prevented pulmonary vascular remodeling and partially improved lung structure at least in a BPD neonatal rat model. [80] In addition, international collaboration between centers is highly important to enhance further research and to accomplish randomized-controlled trials of promising therapeutic agents in this particular patient group.[81]

Structural remodeling of the pulmonary arterial wall

Also in CDH patients a significant part of lung growth and pulmonary vascular remodeling at the alveolar level occurs postnatal.[82] In newborns with PH, abnormal vascular remodeling begins at or soon after birth. The abnormal shaped peripheral pulmonary arteries are composed of SMC that show hyperplasia and hypertrophy. Furthermore, in contrast to normal postnatal vascular remodeling, the SMC myofilament concentration is abnormally high and the SMC deposit excessive collagen and elastin around themselves.[83,84] These changes appear to fix the vessel in an incompletely dilated state with a reduced lumen (Figure 9.2).[85-87]

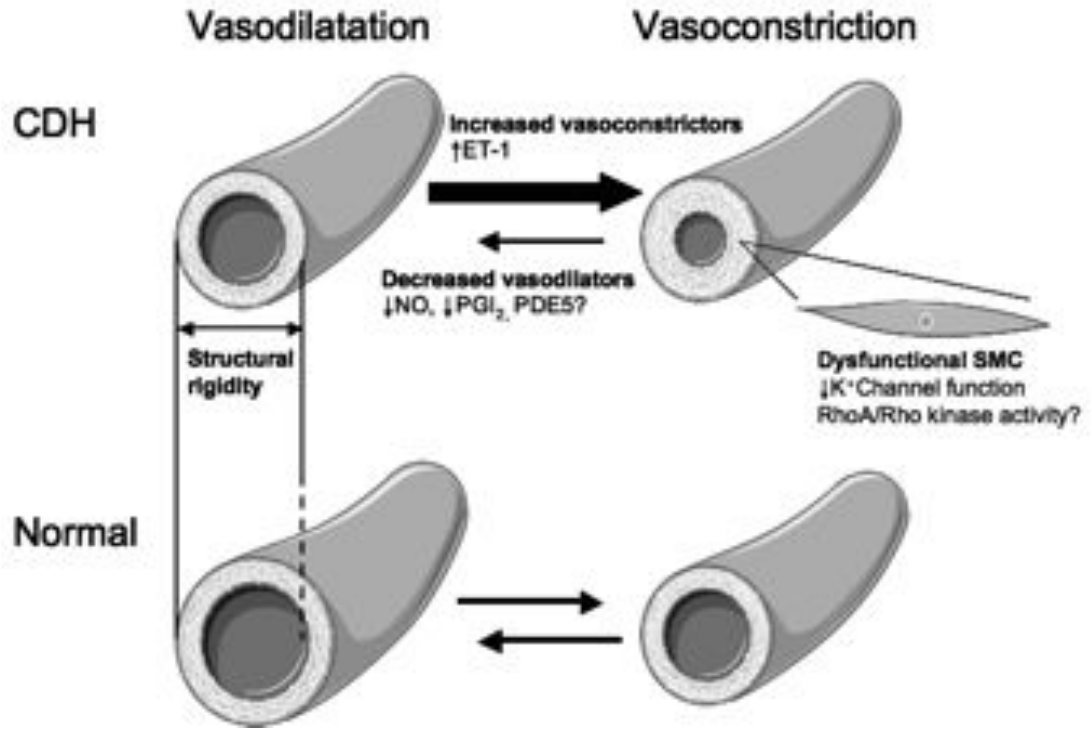

Figure 9.2 (Color figure page 175)
Pulmonary vascular tone in CDH is characterized by increased vasoconstrictors, decreased vasodilators, and dysfunctional SMC. The aberrant vascular development appears to fix the vessels in an incomplete dilated state. ET-1; endothelin, NO; nitric oxide, PGI_2; prostacyclin, PDE5; phosphodiesterase 5.
(Figure was produced using Servier Medical Art)

Altered SMC function is implicated in the pathogenic vascular remodeling and one level of regulation is the SMC phenotypic modulation. The pulmonary arterial vessel wall exists of different populations SMC, which exhibit various phenotypes, ranging from synthetic to purely contractile.[88] Actin and myosin, the two major contractile proteins are believed to determine the SMC phenotype and their expression is developmentally regulated.[89] During phenotypic modulation, SMC's change their morphology, cell function, and biochemical characteristics.[90] In the nitrofen-induced CDH model and in CDH patients, myosin isoforms are increased suggesting a more contractile phenotype that may be responsible for the increased vascular rigidity and decreased compliance (**Chapter 7**).[91]

Also the extracellular matrix (ECM) plays a role during postnatal microvascularization of the lung and vascular remodeling induced by PH and lung injury.[92] ECM molecules and ECM remodeling by proteases promotes cell migration and play a key role in regulating angiogenesis.[93] Metalloproteinase's (MMPs) and their inhibitors (TIMPs) are involved in ECM turnover and showed different patterning in pulmonary arteries of CDH patients.[94] However, in the nitrofen-induced CDH model conflicting results revealed either no differences in MMPs and TIMPs expression or increased MMP activity.[95] Therefore, no conclusive remarks can be made about the involvement of MMPs and TIMPs in the pathogenesis of CDH associated abnormal vascular remodeling.

A more isolated form of altered postnatal vascular remodeling is BPD; a significant complication of premature birth. Moreover, one-third of newborns with CDH will develop BPD, despite being born at term.[96] In **chapter 8** we propose that in a BPD mice model and in a series of 10 BPD patients, as a consequence of upregulated expression of ECM crosslinking enzymes, such as lysyl oxidases, the ECM is "over-stabilized" and resists remodeling that facilitates normal lung growth and microvascular development. These pathways have not been investigated in CDH so far.

Overall we postulate that a more contractile SMC phenotype and an over-stabilized ECM hinder normal postnatal vascular remodeling in CDH. However, considerable work is needed to address the presumed altered SMC biology and ECM modulation in CDH. Additional in vitro studies on primary pulmonary vascular SMC cultures of CDH models or deceased patients are needed to determine the phenotypic spectrum of the pulmonary arterial wall in CDH. For clinical practice the functional implications of this altered SMC population on pulmonary vascular reactivity is an important target for future research. For possible future therapeutic studies we need more insight which pathways are involved in the phenotypic modulation of vascular SMC. In vivo lineage labeling studies can elucidate if the vascular SMC population in CDH comes from another lineage and might add to a different view on the pathogenesis of PH in CDH.

Concerning the ECM in the pulmonary vasculature in CDH, we need to investigate 1) the composition at various levels of the vascular tree, 2) where and how its function is impaired, and 3) the possibilities to interfere therapeutically with the modulation of the ECM.

Perspectives

Recently, inflammatory processes, like recruitment of macrophages, T and B lymphocytes, and increased circulating chemokines and cytokines, are increasingly recognized as major pathogenic component of pulmonary vascular remodeling in adult forms of PH.[97] The recognition of inflammatory mediators offers potential specific targets for lung injury prevention and anti-PH therapy. In adult forms of PH this has led to clinical trials investigating, for example, the use of tyrosine kinase inhibitors. Likewise, the use of scavengers of reactive oxygen species is proposed in the therapy of PH.[98]

Another therapeutic modality of growing interest is the use of mesenchymal progenitor cells to promote vascularization and prevent or regenerate the loss of the pulmonary microvasculature. Mesenchymal progenitor cell interventions are based on the paracrine effects (i.e. increased VEGF expression) and could be augmented by

gene therapy approaches (i.e. eNOS overexpression).[99] Future research into the stimulation of mesenchymal progenitor cells already stationed in the lung will aid to identify more mesenchymal progenitor cell derived factors and new therapeutic targets. Studies are needed to fill gaps in our understanding of the mechanistic role and the potential efficacy of mesenchymal progenitor cells in pulmonary vascular diseases in general and in CDH in particular. At present studies are underway to study homing of stem cells in animal models of CDH mainly the toxicology based nitrofen based rat model.

Concluding remarks

Taking all together, the studies in this thesis confirm the importance of pulmonary vascular "disease" in CDH. Pulmonary vascular disease in CDH has its origin in lung development and leads to impaired vascular function and structural different pulmonary arteries (Figure 3).

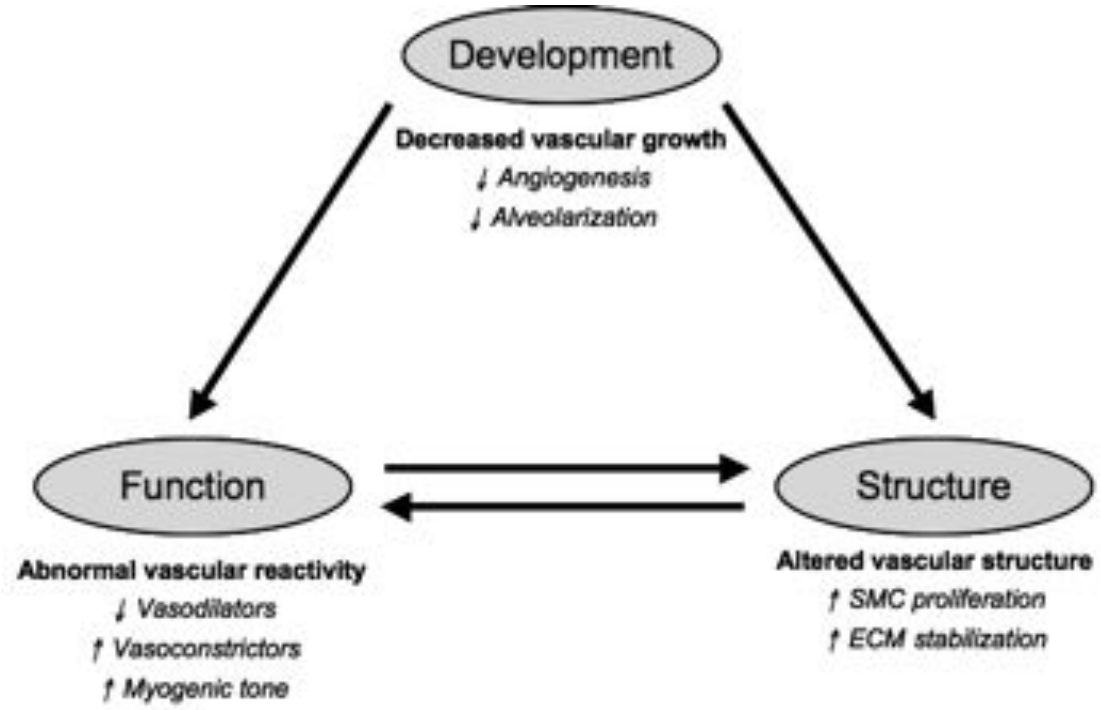

Figure 9.3
Pulmonary vascular disease in congenital diaphragmatic hernia
(Figure was produced using Servier Medical Art)

The impaired vascular function is characterized by an imbalance between vasodilators and vasoconstrictors with an enhanced SMC reactivity. The structural different vascular wall is formed by proliferative SMC and an increased ECM stability. The structural rigidity of the vascular wall limits the margin of the vessels to dilated. This in turn increases vascular resistance, hence PH and leads to structural remodeling of the vessel wall. The effects of shear forces, hyperoxia and inflammatory mediators in the evolution of fixed pulmonary hypertension needs further evaluation. In other words, a vicious circle has started and leads to the death of another CDH patient. Elucidation of the factors responsible for the decreased vascular development, altered vascular responsiveness and abnormal postnatal vascular remodeling will result in:

1) additional therapeutic strategies to enhance postnatal pulmonary vascular development
2) prevent further harm by lung injury, and
3) retain or restore proper pulmonary vascular function in newborns with CDH.

We have to keep in mind that our assumptions on working mechanisms of the drugs used today are based on in-vitro models using cell lines or vascular rings and /or animal models of pulmonary hypertension with or without diaphragmatic defect. The identification of the patient who needs new therapeutic approaches to modulate the vascular tone at an early stage is clearly needed. Today many of the drugs are used as a last resort in an “altered host” due to the negative effects of artificial ventilation, hyperoxia of inspiratory air, tissue hypoxia and combination of drugs with partly identified modes of action and uncertainty of drugs-drug interactions in these critically ill newborns.

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Summary/ Samenvatting

Summary

Congenital diaphragmatic hernia (CDH) is a severe birth defect, characterized by a defect of the diaphragm, lung hypoplasia, and pulmonary hypertension (PH). With an incidence of approximately 1 per 2500 life births CDH is a relative common anomaly. CDH is a cause of neonatal PH that lacks a proven and efficient therapy although a variety of treatment modalities have been proposed. As such CDH remains a significant challenge for the pediatric surgeon and neonatologist.

In **chapter 1** we reviewed the main pathways in the regulation of pulmonary vascular tone related to the pathophysiology of neonatal PH and evaluated therapeutic targets within these pathways.

Chapter 2 gives an outline of the thesis. Pathophysiology of the pulmonary vasculature plays an important yet underestimated role in the mortality and morbidity of newborns with CDH. Studying pulmonary vascular changes will provide targets for innovative treatment strategies.

In **chapter 3** the expression of various angiogenesis-related factors were evaluated in a series of normal human lungs from 13.5 weeks of gestation onwards till term. Hypoxia inducible factors (HIF) appear to be key molecules for angiogenesis. However, documentation of qualitative and quantitative expression of HIF in normal human lung development was limited. Therefore, we evaluated expression of HIF-1α, HIF-2α, and HIF-3α along with von Hippel-Lindau protein, VEGF-A and its receptor, VEGFR-2 by quantitative PCR. Both HIF-2α and VEGF-A mRNA expression showed increasing trend during gestation. Furthermore, localization of HIF-1α and HIF-2α was evaluated by immunohistochemistry and this revealed that while HIF-1α is expressed in epithelial cells only, HIF-2α is expressed in both epithelial and mesenchymal cells.

These results in normal human lungs were used as baseline data for the study in **chapter 4** in which expression of these angiogenic factors were evaluated in lungs of human CDH cases. VEGF-A showed a decreased mRNA expression in the alveolar stage of the lungs of 4 CDH patients, which might be related to a decreasing trend of HIF-2α mRNA expression. In addition, immunohistochemistry in 16 CDH cases showed no differences in expression of PHD3, a posttranscriptional modulator of HIF. This suggests that CDH patients are potentially responsive to stimulation of the HIF pathway through inhibition of PHD3.

These findings support the role of the HIF-VEGF pathway in pulmonary vascular development and airway branching morphogenesis in the human lung and their possible role in the pathogenesis of CDH.

Chapter 5 describes the expression and function of various phosphodiesterases (PDE) in the pulmonary arteries of nitrofen-induced CDH fetal rats and controls. Quantitative PCR showed expression of several PDE isoforms, but revealed no differences in expression between CDH and control rats. In vascular ring studies, vasodilatation of pulmonary arteries was induced by PDE2, 3, 4, 5 antagonists. Only diminished inhibition of PDE2 dilated pulmonary arteries of CDH fetal rat was found compared to controls. As in our study, the most potent and clinical most important is PDE5 antagonist, Sildenafil. As a series of supportive experiments, we showed that a hyperoxic environment attenuates vasodilatation by PDE5 inhibition.

In **Chapter 6** we investigated the expression and function of potassium channels in the pulmonary arteries of nitrofen-induced CDH fetal rats and controls. Quantitative PCR revealed a higher expression of Kv1.5 in PAs of CDH rats without any implications in the functional vascular ring studies. No differences in mRNA or protein expression were found for Kv2.1 or BK_{Ca}, an important K^+ channel in the pulmonary vascular transition at birth. BK_{Ca} agonist NS1619 showed less vasodilatation of PAs of CDH rats compared to controls. Activation of BKca channels is considered to enhance pulmonary vasodilatation in CDH.

Chapter 7 presents a pilot study on the immunohistochemical expression of smooth muscle cell (SMC) markers on a tissue array of a series of 24 CDH patients and matched controls. SMC myosin heavy chain is significantly more expressed in the CDH group. We postulate the possibility of a difference in SMC subpopulation or maturation, involved in the pathogenesis of PH in CDH.

To evaluate the disordered extracellular matrix production in postnatal injured developing lung we used a different disease model in **chapter 8.** In a mice model of bronchopulmonary dysplasia (BPD) and in prematurely born infants with BPD, we demonstrated that mRNA and protein levels and activity of lysyl oxidases were elevated. In developing mouse lungs, aberrant TGF-β signaling dysregulated lysyl oxidase expression. We postulate that excessive stabilization of the extracellular matrix by excessive lysyl oxidase activity might impede the normal extracellular matrix remodeling that is required for pulmonary alveolarization and microvascular development in lung maturation.

The last chapter contains the general discussion of the results of studies presented in this thesis; remaining gaps in our understanding of the working mechanism of phenotypic changes and response to common used drugs and areas for further research in pulmonary vascular “disease” associated with CDH.

In conclusion, pulmonary vascular “disease” is a key determinant of morbidity and mortality in CDH. It exists of decreased prenatal vascular development, an altered vascular responsiveness, and a disordered process of vascular remodeling. The effect of potential “injurious” therapies such as artificial ventilation with high inspiratory oxygen fractions adds to the evolution of persistent pulmonary hypertension resistant to our current treatment modalities. Better insight in pulmonary vascular “disease” associated with CDH will guide future therapeutic approaches.

Samenvatting

Congenitale hernia diafragmatica (CDH) is een ernstig aangeboren afwijking, die gekarakteriseerd wordt door een defect in het middenrif, long hypoplasie en pulmonale hypertensie (PH). CDH is een relatief veelvoorkomende anomalie met een incidentie van een op de 2500 levend geborenen. Omdat CDH, als oorzaak van neonatale PH geen efficiënte therapie kent, blijft CDH een bijzondere uitdaging voor de neonatologen en kinderchirurgen.

Een algemene inleiding in de moleculaire regulatie van de pulmonale vaattonus wordt gegeven in **hoofdstuk 1**. Het beschrijft een uiteenzetting van de huidige therapeutische behandelingsopties voor PH bij pasgeborenen in relatie tot de pathofysiologie.

Hoofdstuk 2 geeft kort de inhoud van het proefschrift weer. De pathofysiologie van de longvasculatuur speelt een belangrijke, maar nog ondergewaardeerde rol in de mortaliteit en morbiditeit van pasgeborenen met CDH. Het onderzoeken van deze vasculaire afwijkingen zal leiden tot nieuwe innovatieve behandelmethodes.

In **hoofdstuk 3** werd de expressie onderzocht van diverse angiogenese-gerelateerde factoren in een serie gezonde humane longen vanaf 13,5 weken tot aan het eind van de zwangerschap. Hypoxia Inducible Factors (HIF's) bleken sleutelmoleculen te zijn voor angiogenese. De kwalitatieve en kwantitatieve expressie van HIF's in de normale longontwikkeling bij humane foetussen is echter beperkt gedocumenteerd. Daarom werd met behulp van kwantitatieve PCR de expressie van HIF-1α, HIF-2α, en HIF-3α geëvalueerd, alsmede de expressie van Von Hippel-Lindau proteïne, VEGF-A en diens receptor VEGFR-2. Zowel de mRNA expressie van HIF-2α als die van VEGF-A vertoonde een stijgende trend bij een toename van de zwangerschapsduur. Vervolgens keken we met behulp van immunohistochemisch onderzoek naar de lokalisatie van HIF-1α en HIF-2α expressie. Het bleek dat HIF-1α alleen maar in epitheelcellen tot expressie kwam, terwijl HIF-2α zowel in epitheel cellen als in mesenchymale cellen te zien was.

De bevindingen in de normale longontwikkeling bij de mens diende als uitgangspunt voor het onderzoek dat in **hoofdstuk 4** wordt beschreven. Hierin werd de expressie van deze angiogenese-gerelateerde factoren in de longen van overleden CDH patiënten bekeken. De mRNA expressie van VEGF-A was verlaagd in 4 CDH patiënten, waarbij de longen zich in de alveolarisatie fase van longontwikkeling bevonden. Deze bevinding is mogelijk gerelateerd aan de dalende trend van HIF-2α mRNA expressie in deze longen. Aanvullend werden er immunohistochemisch kleuringen verricht in 16 CDH patiënten en deze toonden geen verschil in PHD3 expressie ten opzichte van normale humane longen. PHD3 reguleert de posttranscriptionele expressie van HIF's en zou daarom kunnen worden aangemerkt als mogelijke therapeutisch optie om HIF gestimuleerde pulmonale vaatgroei te bevorderen.

Deze bevindingen ondersteunen de theorie dat de HIF-VEGF pathway een rol speelt bij de vaatontwikkeling en morfogenese van de luchtwegen in humane longontwikkeling en mogelijk betrokken is bij de pathogenese van CDH.

Hoofdstuk 5 beschrijft de expressie en functie van verschillende phosphodiesterases (PDEs) in de pulmonaal arteriën van foetale ratten met nitrofen-geïnduceerde CDH. Kwantitatieve PCR detecteerde de meeste PDEs, maar liet geen verschil in mRNA expressie zien tussen CDH en controle ratten. In functionele ring experimenten, veroorzaakte de antagonisten van PDE 2, 3, 4 en 5 een vaatverwijding van de pulmonaal arterie. Alleen de vaatverwijding na inhibitie van PDE 2 was beduidend minder in de CDH groep. Ook in onze studie veroorzaakte de PDE 5 antagonist, Sildenafil de sterkste vaatverwijding en is daarom klinisch het belangrijkst. In een serie extra experimenten toonden we aan dat de vaatverwijding door PDE 5 inhibitie negatief beïnvloed wordt door een hoog zuurstof gehalte.

In **hoofdstuk 6** hebben we de expressie en functie van kaliumkanalen in pulmonaal arteriën van foetale ratten met nitrofen-geïnduceerde CDH onderzocht. De mRNA expressie van Kv1.5 was hoger in de pulmonaal arteriën van CDH ratten, maar dit had geen functionele gevolgen in de ring studies. Er werd geen verschil gevonden in de

mRNA of eiwit expressie van de andere onderzochte kaliumkanalen, Kv2.1 en BK_{Ca}. BK_{Ca} is een belangrijk kaliumkanaal dat betrokken is bij het verlagen van de pulmonale vaatweerstand in de perinatal periode. NS1619, een BK_{Ca} agonist, veroorzaakte een beduidend mindere vaatverwijding in de pulmonaal arteriën van CDH ratten. De activatie van dit kanaal kan daarom wellicht overwogen worden als therapeutisch optie voor pulmonale vaatverwijding in CDH patiënten.

Hoofdstuk 7 beschrijft een voorbereidende studie betreffende het expressiepatroon van eiwitten die kenmerkend zijn voor vasculaire gladde spiercellen. Middels immunohistochemisch onderzoek van een tissue micro array (TMA), welke longweefsel van 24 CDH patiënten bevat, toonden we aan dat myosin heavy chain meer tot expressie komt in de gladde spiercel van CDH patiënten dan in de controlegroep. We speculeren dat een andere subpopulatie van gladde spiercellen of een verandering in maturatie van de gladde spiercellen in de pulmonale vaatwand een rol speelt in de pathogenese van CDH.

Om de invloed van postnatale longschade op de ongeorganiseerde extracellulaire matrix productie te kunnen onderzoeken, hebben we in **hoofdstuk 8** gebruik gemaakt van een ander ziekte model. Zowel in een muis model met bronchopulmonale dysplasie (BPD) als prematuren met BPD toonden we een verhoogde expressie (mRNA en eiwit) en activiteit van lysyl oxidases aan. In ontwikkelende muis longen veroorzaakten afwijkende TGF-β signalen de ontregeling van de lysyl oxidase expressie. We poneren de hypothese dat door een verhoogde lysyl oxidase activiteit, de extracellulaire matrix te stabiel is om een normale herschikking van de matrix mogelijk te maken, welke nodig is voor alveolarisatie en microvascularisatie.

In het laatste hoofdstuk wordt een algemene discussie gevoerd over de resultaten van de studies uit dit proefschrift, de huidige kennis met zijn omissies betreffende pulmonale vasculaire afwijkingen met betrekking tot het ziektebeeld CDH en de mogelijkheden voor toekomstig onderzoek.

De conclusie luidt dat de pulmonale vasculaire afwijkingen bepalend zijn voor de morbiditeit en mortaliteit van CDH patiënten. Deze afwijkingen bestaan uit beperkte prenatale vaatontwikkeling, een veranderde vaatreactiviteit en een ongeorganiseerd proces van postnatale vaatmodulatie. In combinatie met aanvullende longschade leidt dit tot een persisterende PH, welke uiteindelijk niet meer reageert op de huidige behandelingsopties. Een beter inzicht in de pulmonale vasculaire afwijkingen die gepaard gaan met CDH zal leiden tot nieuwe therapeutisch mogelijkheden.

Appendix

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Ongelooflijk, maar waar mijn proefschrift is klaar. Tijd voor een dankwoord aan allen, die mij gesteund en geholpen hebben en in het bijzonder aan:

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Curriculum Vitae

Irene van der Horst was born on 7th of February 1977 in 's-Hertogenbosch, the Netherlands. After graduating from the Stedelijk Gymnasium in 's-Hertogenbosch in 1995, she studied psychology at the University of Maastricht. In 1997 she started her medical training at the University of Maastricht. After obtaining her medical degree in 2003, she worked as a resident at the Pediatric Surgery department of the Erasmus MC-Sophia Children's Hospital in Rotterdam (Prof. Dr. F.W.J. Hazebroek). From 2004 onwards, she was a research physician at the department of Pediatric Surgical Intensive Care of the Erasmus MC-Sophia Childern's Hospital in Rotterdam (Prof. Dr. D. Tibboel), working on the research presented in this thesis. As a part of this research project she worked from January 2006 to December 2006 at the Vascular Biology Group at the University of Alberta, Edmonton, Canada (Dr. B. Thébaud). In 2008 she interrupted her PhD training to work as a resident at the department of Surgery of the Amphia Hospital in Breda. In July 2010 she will start her training in radiology at the department of Radiology at St. Catharina Hospital in Eindhoven (Dr. A.V. Tielbeek).

She lives together with Jules Sneeboer; they have two lovely children Margot (2007) and Arthur (2010).

Table 7.1 Characteristics of CDH patients and controls selected for tissuemicroarray.

Patient no	Group	GA	Birth weight (g)	Postnatal age (hr)	Ventilation	ECMO	Lung/body ratio
1	Control	39	3190	24	No	No	Unknown
2	Control	41	3250	18	Yes	No	Unknown
3	Control	40	2500	3	Yes	No	0.0110
4	Control	41	3775	168	Yes	No	Unknown
5	Control	40	3950	72	Yes	No	Unknown
6	Control	39	2490	3	Unknown	No	Unknown
7	Control	38	3110	119	Yes	No	Unknown
8	Control	40	3700	72	No	No	Unknown
9	Control	38	3035	1	No	No	Unknown
10	Control	38	3710	48	Yes	No	0.0198
11	Control	35	2000	24	Yes	No	Unknown
12	Control	35	2380		Unknown	No	Unknown
13	Control	35	2390	1	No	No	Unknown
14	Control	34	2000	24	Yes	No	Unknown
15	Control	35	2700	7	Yes	No	Unknown
16	Control	29	1480	1	Yes	No	0.0110
17	Control	23	361	0	No	No	Unknown
18	Control	21		0	No	No	0.0210
19	Control	19	189	0	No	No	Unknown
20	Control	15	57	0	No	No	Unknown
21	Control	40	2910	3 wks	Yes	No	Unknown
22	Control	40	2400	3 wks	Yes	No	Unknown
23	Control	37	2990	119	Yes	No	Unknown
24	Control	34	1330	24	No	No	Unknown
25	CDH	40	2800	24	Yes	No	0.0016
26	CDH	40	3800	3	Yes	No	Unknown
27	CDH	40	3225	7	Yes	No	Unknown
28	CDH	40	2765	2	No	No	0.0014
29	CDH	39	2000	72	Yes	No	0.0050
30	CDH	39	2270		No	No	Unknown
31	CDH	38	2900	24	Yes	No	0.0013
32	CDH	38	2870	1	Yes	No	0.0055
33	CDH	37		6	Yes	No	Unknown
34	CDH	37		22	Yes	No	Unknown
35	CDH	36	1800	48	Yes	No	Unknown
36	CDH	36	2300	6	Yes	No	Unknown
37	CDH	36	2515		Yes	No	0.0009
38	CDH	34	1250	5	Yes	No	0.0053
39	CDH	34	2235	1	Yes	No	0.0004
40	CDH	29	813	1	Yes	No	0.0090
41	CDH	23	344	0	No	No	0.0100
42	CDH	21	416	0	No	No	0.0017
43	CDH	19	116	0	No	No	0.0155
44	CDH	15	30	0	No	No	Unknown
45	CDH	39	3000	2 wks + 2 days	Yes	Yes	Unknown
46	CDH	39		2 wks + 2 days	Yes	Yes	Unknown
47	CDH	37	3000	5 wks	Yes	Yes	0.0081
48	CDH	34		24	Yes	Yes	Unknown

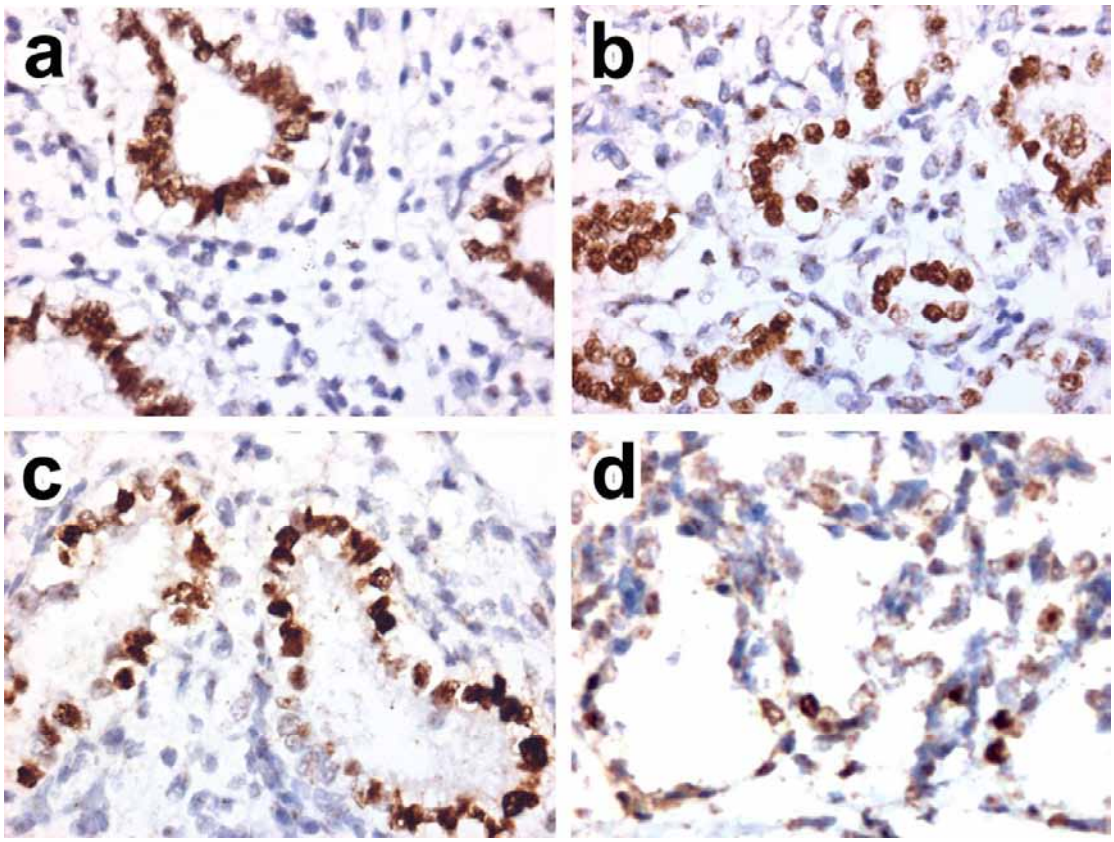

Figure 3.3 HIF-1α immunostaining (See page 49)
Strong nuclear expression of HIF-1α in airway epithelium of human fetal lung at different gestational ages; (a) 16 wk, (b) 21 wk, (c) 27 wk, and (d) 31 wk. In Figure 3d staining is somewhat weaker than in the others. (Magnification, X 400)

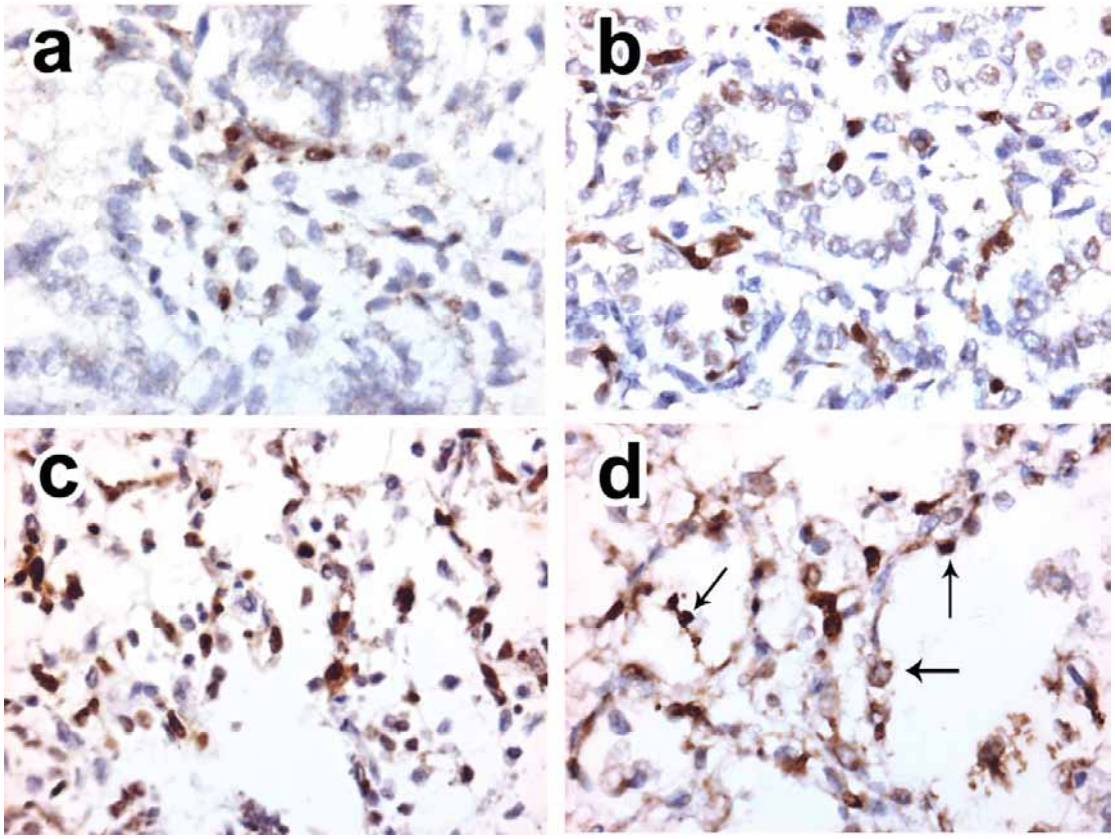

Figure 3.4 HIF-2α immunostaining (See page 50)
Predominant nuclear reactivity of HIF-2α in the interstitium at early stages of human fetal lung development; (a) 16 wk, (b) 21 wk. Later on in gestation, HIF-2α expression was also detected in alveolar-lining cells; (c) 27 wk, and (d) 33 wk. Arrows indicate HIF-2α reactivity in alveolar-lining cells. (Magnification, X 400)

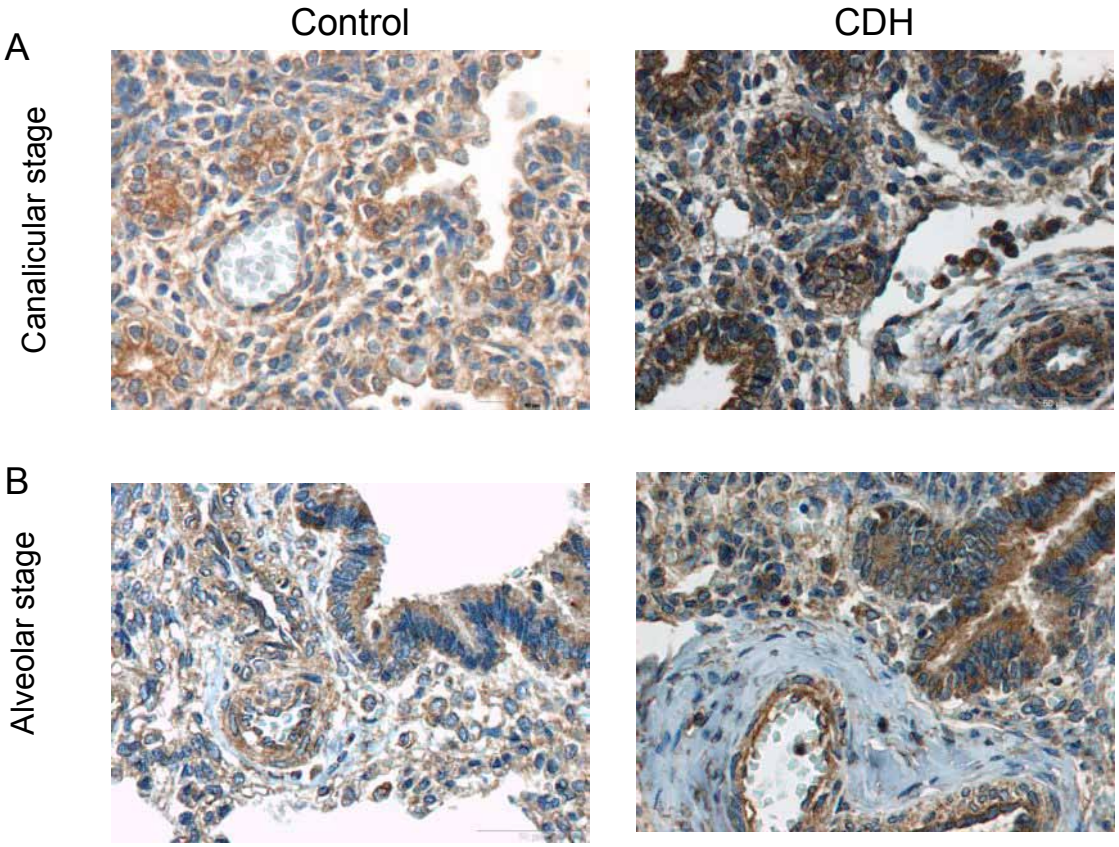

Figure 4.3 (See page 64)
Immunostaining with anti-prolyl hydroxylase 3 (PHD3) of congenital diaphragmatic hernia (CDH) and control lung tissue in A) the canalicular and B) alveolar stage of lung development showed positive expression of PHD3 in all cell types of the lung, except for the arterial adventitia. All cases scored a clear to strong staining and no differences were found between CDH patients and controls for neither developmental stage. (Original magnification 400x)

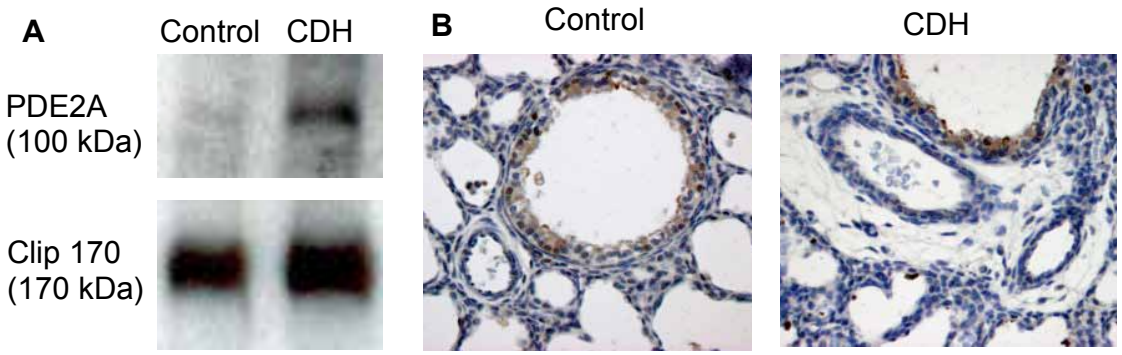

Figure 5.4 (See page 79)
A) Western blot of protein extracted form whole lung showed lower PDE2 expression in CDH (n=3) fetal rat compared to control (n=3). Clip-170 was used as protein loading control.
B) Immunohistochemistry showed expression of PDE2 mainly in bronchial epithelium, arterial media and type 2 pneumocytes.

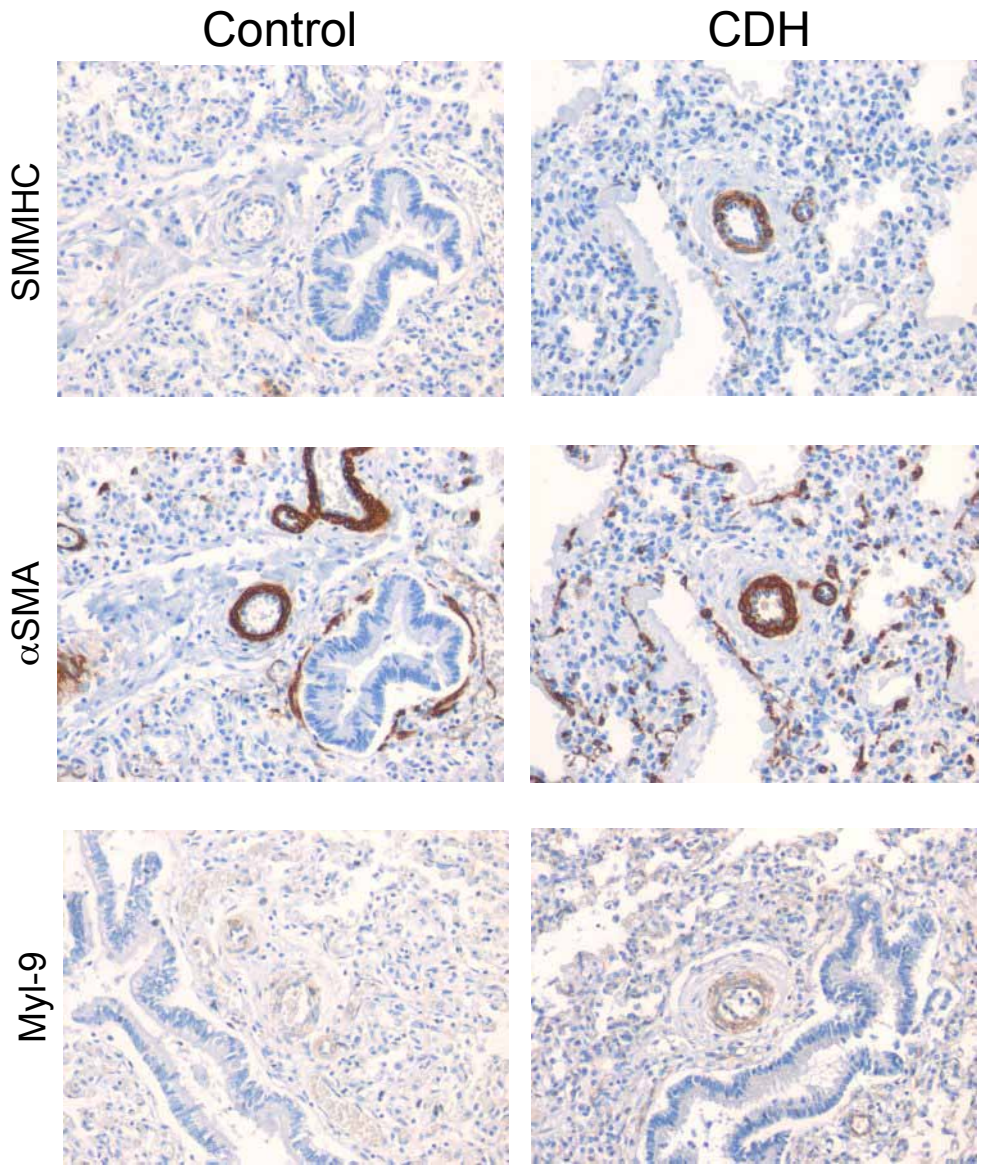

Figure 7.2 (See page 102)
Immunohistochemical staining against SMC myosin heavy chain (SMMHC), alpha smooth muscle actin (αSMC), and SMC myosin light chain (Myl-9) in control and CDH patients' lung tissue of term neonates. Original magnification 400x

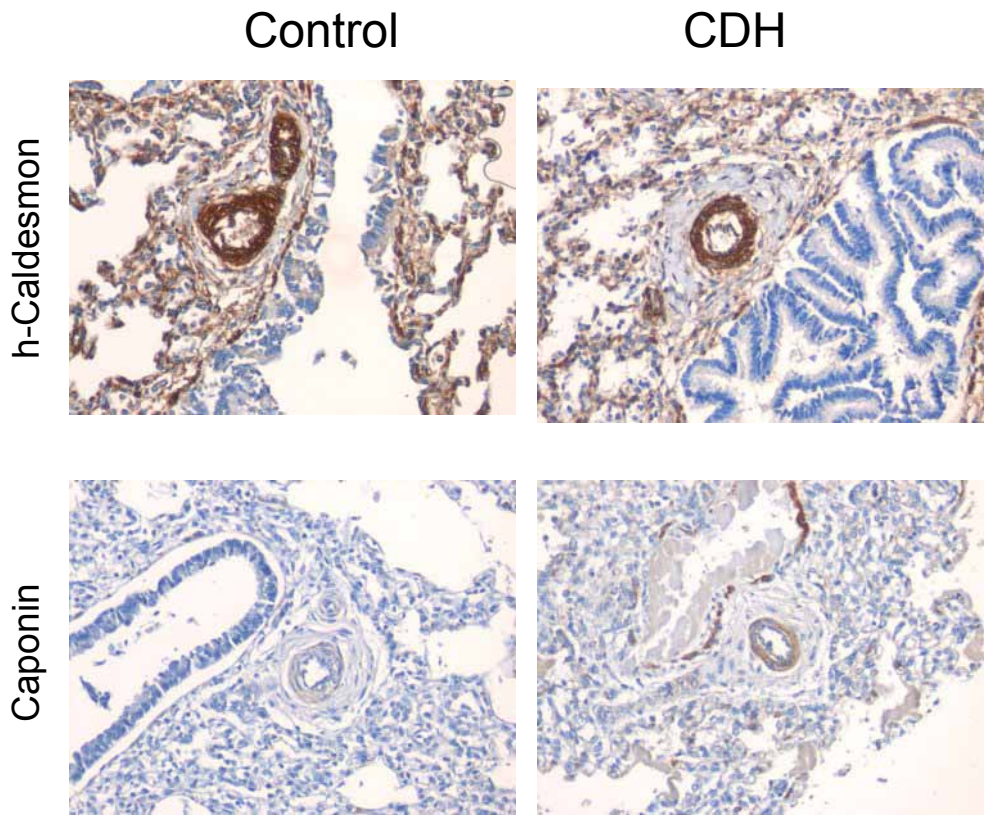

Figure 7.3 (See page 103)
Immunohistochemical staining of h-caldesmon and calponin in control and CDH patients' lung tissue of a term neonates. Original magnification 400x.

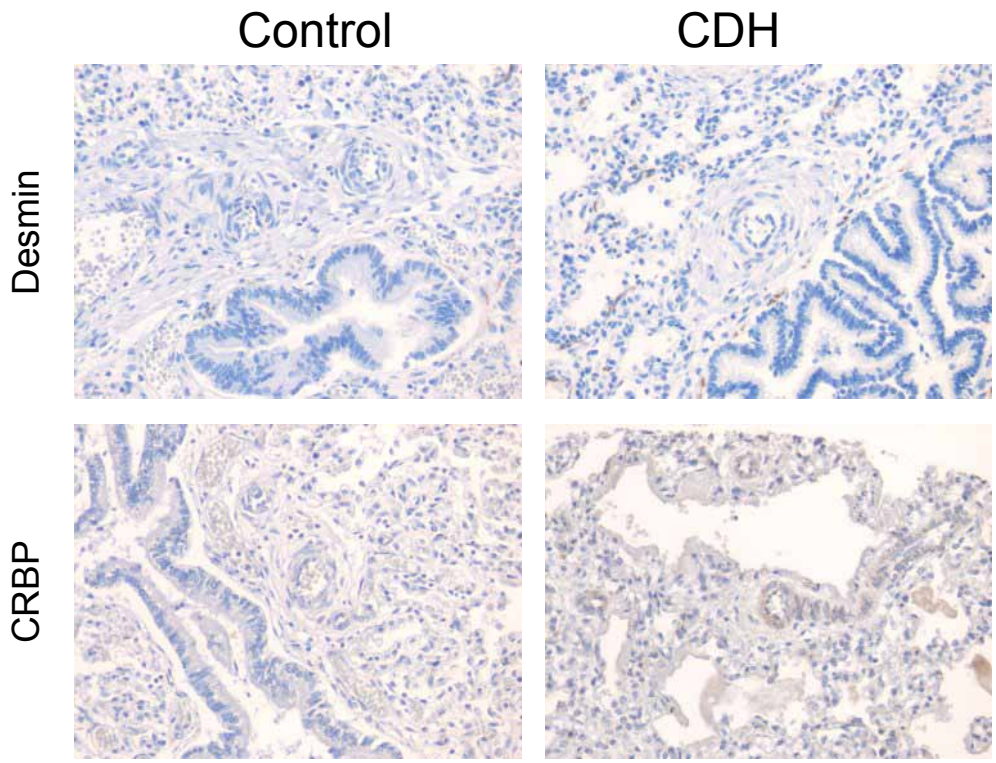

Figure 7.4 (See page 104)
Immunohistochemical staining of desmin and cellular retinoid binding protein (CRBP) in control and CDH patients' lung tissue of term neonates. Original magnification 400x.

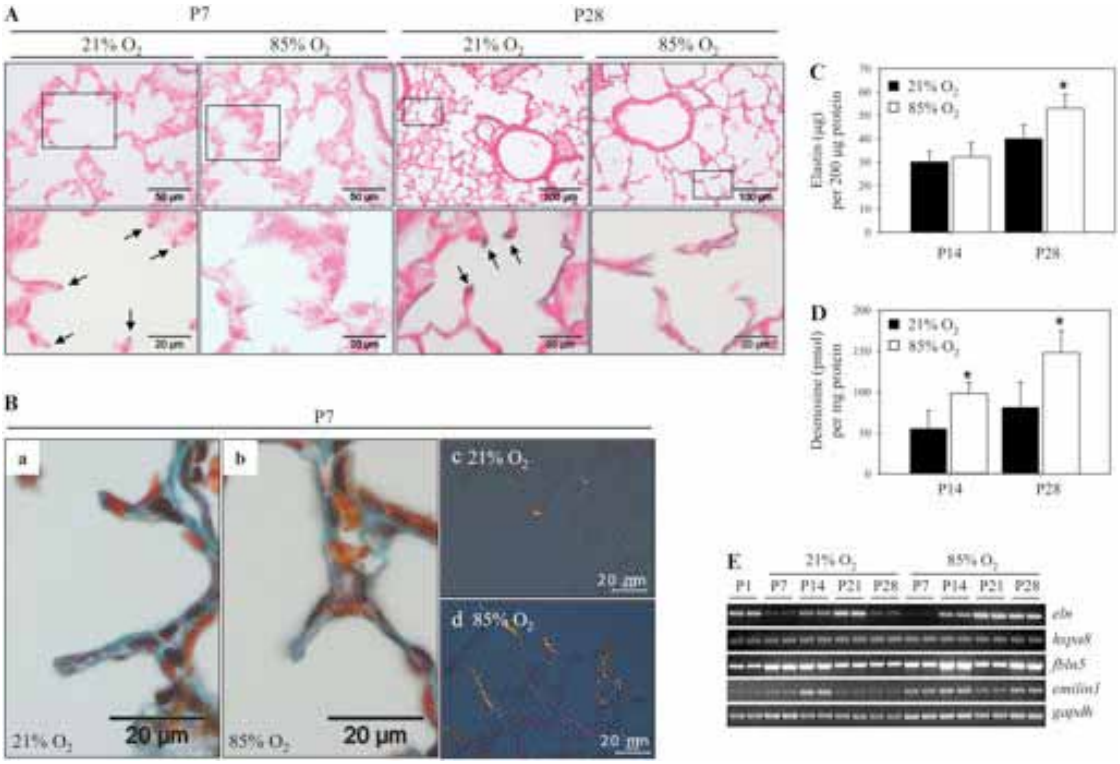

Figure 8.1 (See page 117) Elastin production and cross-linking are dysregulated in the injured developing mouse lung. (*A*) Hart's stain for elastin in air-exposed mouse pup lungs, indicating punctate elastin foci (*arrows*) in the developing septa at Postnatal Day (P)7 and P28 in the lungs of pups exposed to 21% O_2 that are absent in the lungs of pups exposed to 85% O_2. (*B*) Assessment of collagen morphology by Masson's trichrome stain (*a* and *b*) in the developing septa of P7 mouse pups exposed to 21% O_2 or 85% O_2. Collagen was also assessed by picrosirius red staining observed under polarized light in mouse pups exposed to (*c*) 21% O_2 or (*d*) 85% O_2. Both (*C*) soluble elastin and (*D*) desmosine were elevated in 85% O_2-exposed pups (*open bars*) compared with 21% O_2-exposed pups (*solid bars*) at P28 (n = 5). (*E*) Expression of elastin (the *eln* gene), fibulin-5 (the *fbln5* gene), and emilin-1 (the *emilin1* gene) mRNA monitored by semiquantitative reverse transcriptase–polymerase chain reaction in the first month of postnatal life of pups exposed to 21% O_2 or 85% O_2. The constitutively expressed *hspa8* and *gapdh* genes served as controls for loading equivalence. **P* < 0.01.

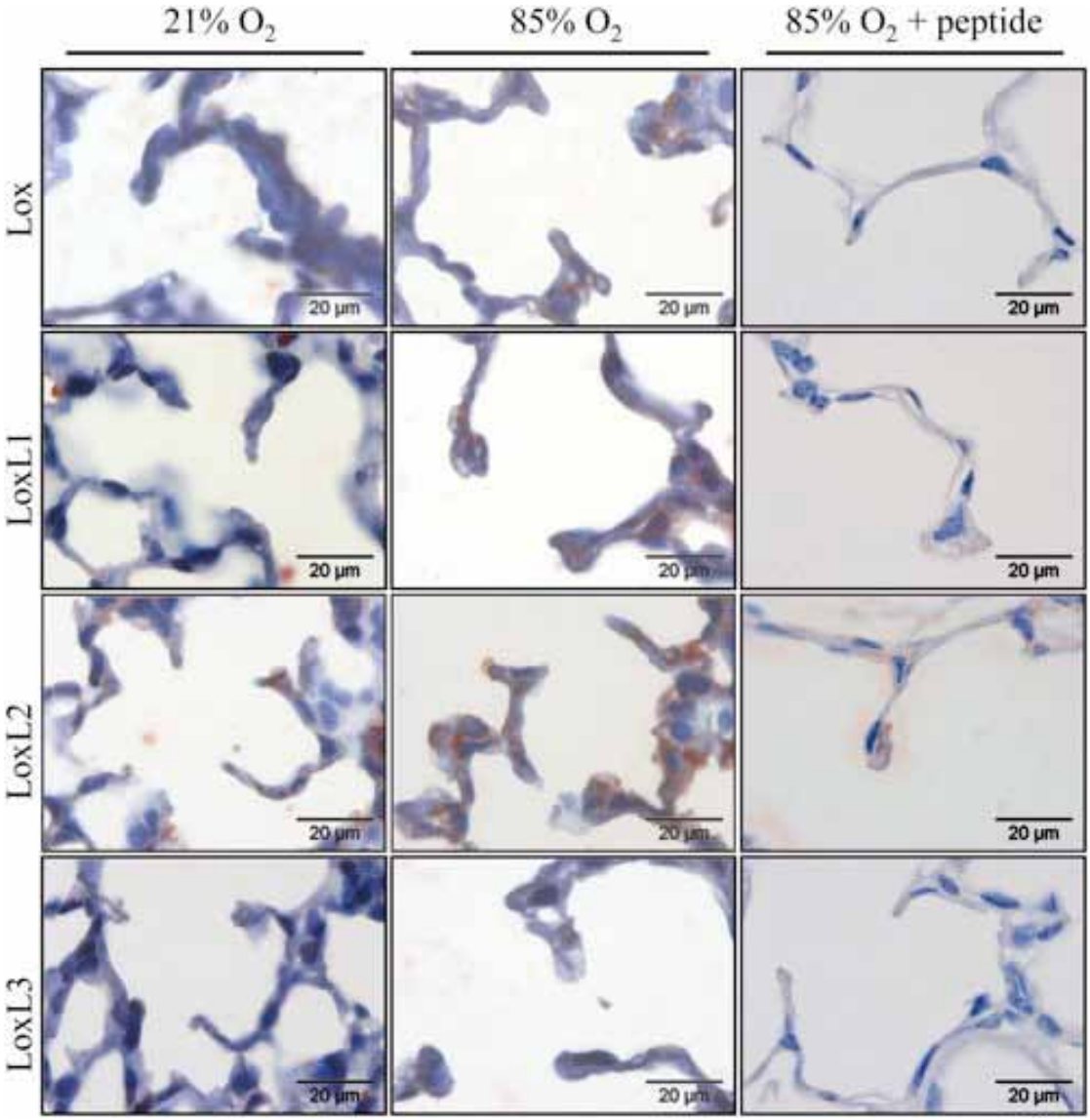

Figure 8.3 (See page 119) Lysyl oxidase protein expression is dysregulated in the septa of oxygen-injured mouse pup lungs. Increased staining intensity is observed for Lox, LoxL1, and LoxL2 in the septa of mice exposed to 85% O_2 compared with 21% O_2-exposed mouse pups at P14. Antibody specificity was confirmed by preadsorption of antibodies with a competing peptide, which had been used as the immunogen for antibody generation. Results for LoxL4 are omitted as no immunoreactivity was observed with the anti-LoxL4 antibody on mouse lung sections.

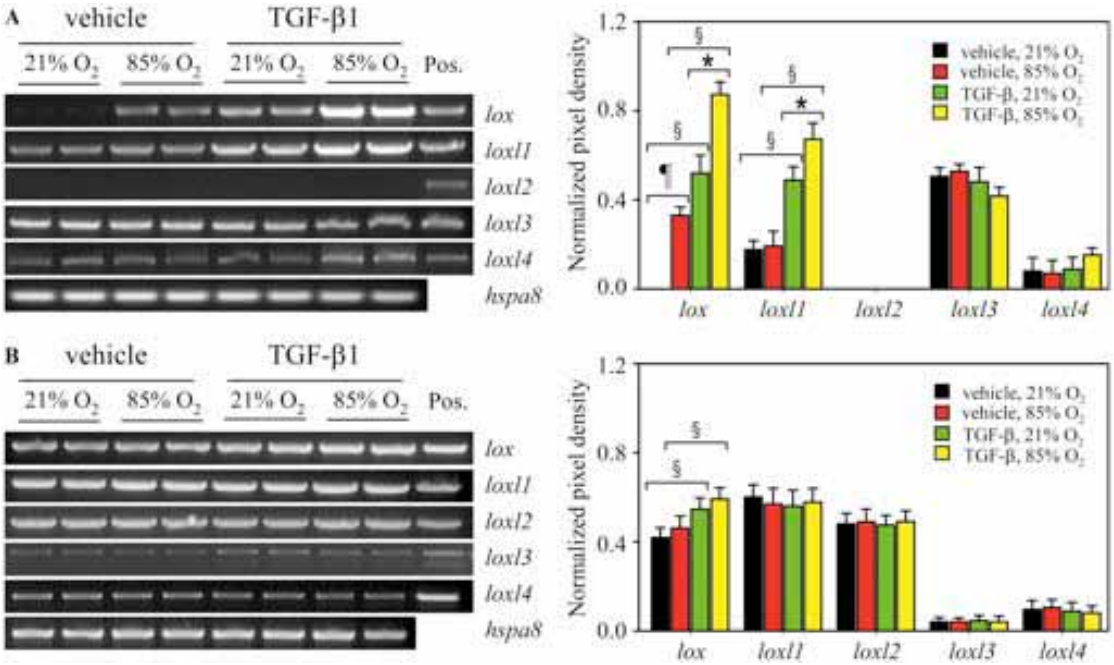

Figure 8.4 (See page 120) Lysyl oxidase mRNA expression can be modulated *in vitro* by oxygen and transforming growth factor (TGF)-β. (*A*) Murine NIH/3T3 fibroblast-like cells and (*B*) human pulmonary artery smooth muscle cells were maintained under hyperoxic (85% O_2) or normoxic (21% O_2) conditions for 24 hours, before addition of TGF-β (2 ng/ml) for an additional 24 hours, after which mRNA was isolated and assessed for lysyl oxidase gene expression. Whole-lung mRNA from mouse or human lungs, respectively, served as a positive control for the polymerase chain reactions (PCR) (Pos.), whereas expression of the *hspa8* gene was used as a loading control. The PCR amplicons derived from two separate cell cultures are represented for each condition. For quantification, densitometric data for amplicons derived from six different cell cultures per condition were averaged; ¶ $P < 0.01$ comparing 85% O_2 versus 21% O_2 exposures in the presence of vehicle; § $P < 0.01$ comparing TGF-β–stimulated versus unstimulated cells with 21% oxygen or 85% oxygen exposure; * $P < 0.01$ comparing 85% O_2 versus 21% O_2 exposures 24 hours after TGF-β stimulation.

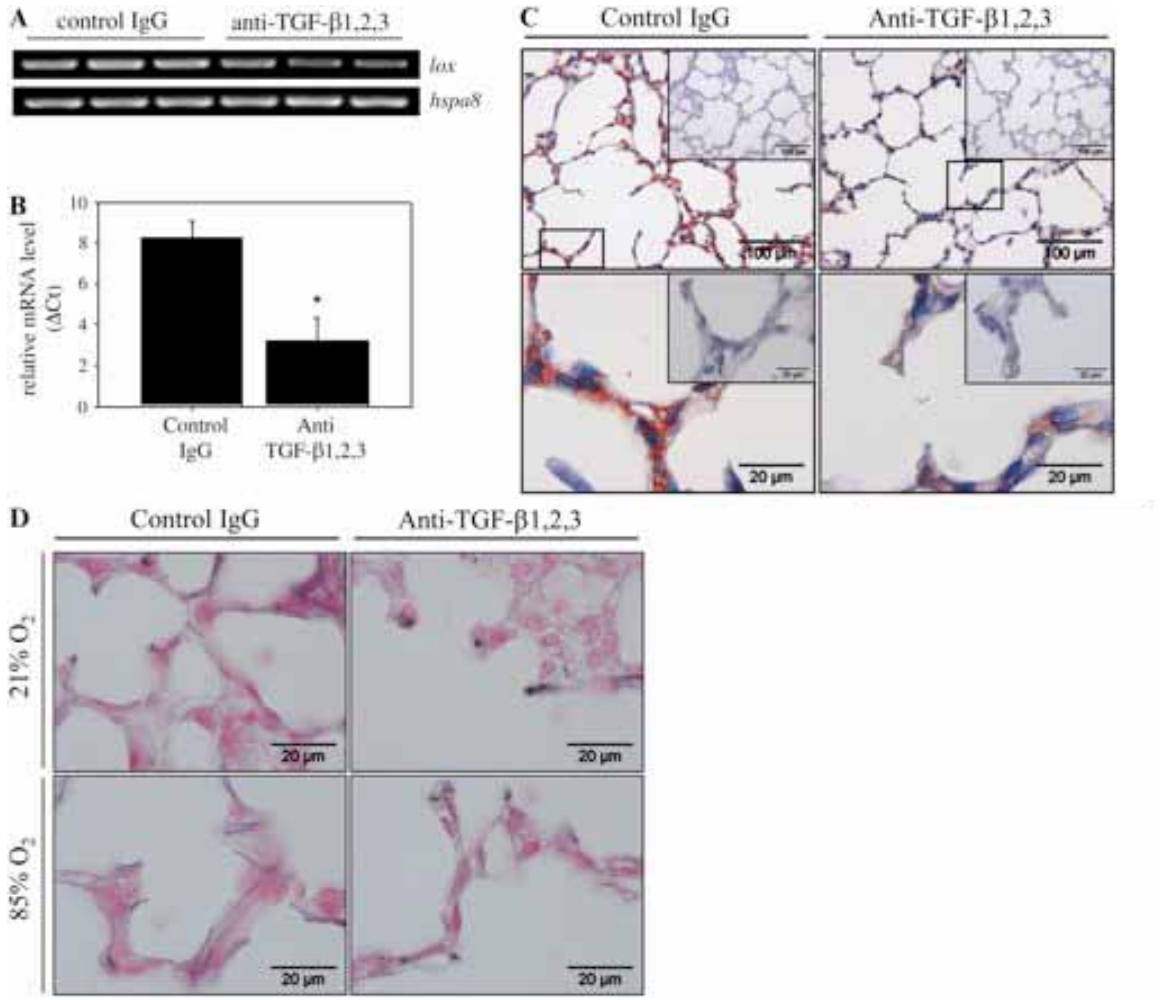

Figure 8.6 (See page 122) Treatment of neonatal mice with transforming growth factor (TGF)-β1,2,3–neutralizing antibodies suppressed the induction of *lox* gene expression by hyperoxia. Neonatal mice were treated either with control IgG or anti–TGF-β1,2,3–neutralizing antibodies before exposure to hyperoxic (85% O_2) or normoxic (21% O_2) conditions for 10 days, and then killed. The expression of lysyl oxidases was assessed in mRNA from mouse lungs by (*A*) semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR), where expression of the *hspa8* gene was used as a loading control; or (*B*) real-time quantitative RT-PCR (n = 5); or (*C*) by immunohistochemistry. Antibody specificity was confirmed by preadsorption of antibodies with a competing peptide, which had been used as the immunogen for antibody generation (*insets*). Additionally, (*D*) lung sections from mice were screened for assessment of elastin deposition in the developing septa by Hart's elastin stain. **P* < 0.01.

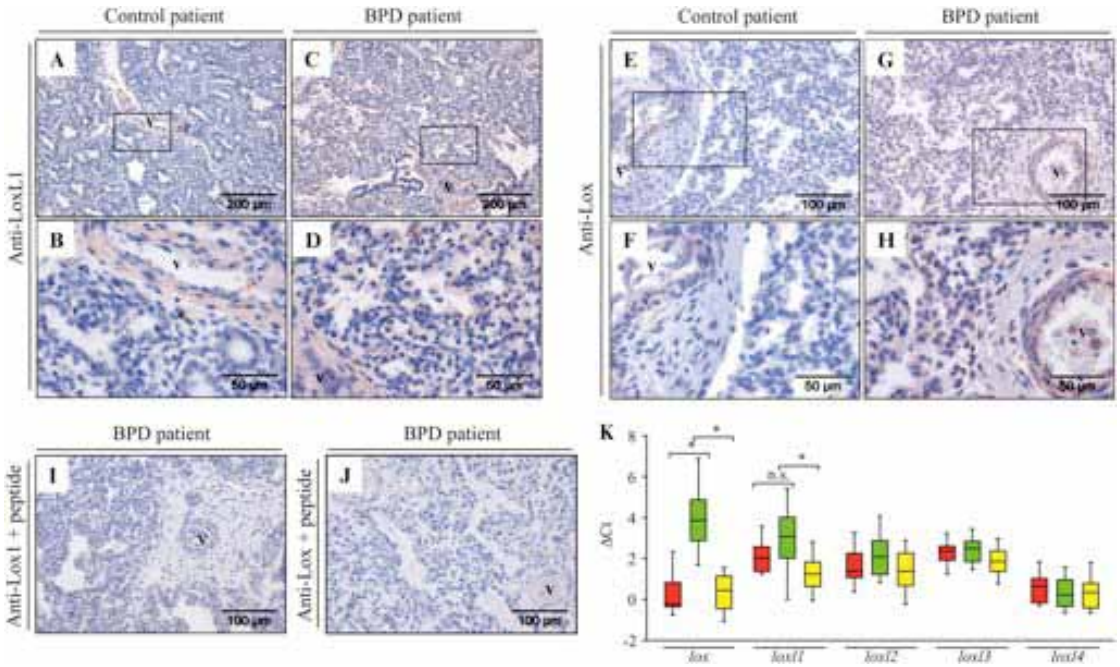

Figure 8.7 (See page 123) The expression of Lox and LoxL1 was elevated in the lungs of neonatal patients who died with BPD or were at risk for BPD. The expression of lysyl oxidases was assessed in the lungs of patients with BPD or at risk for BPD, as well as in control lungs. A low-power (200-µm scale) view of representative sections from (*A*) patients in control group 2 (the histopathology of six patients [Table 1] was examined; in this case, sections from patient 11 are illustrated) and from (*C*) patients in the BPD group (the histopathology of seven patients [Table 2] was examined; in this case, sections from patient 24 are illustrated), stained for LoxL1. (*B*, *D*) High-power views (50-µm scale) are also illustrated for LoxL1 staining in the same sections (the magnified area is demarcated in the low-power view by a *black box*). Representative medium-power views (100-µm scale) are illustrated for the same two patients, stained for Lox (*E*, *F*), with the corresponding high-power views (50-µm scale) illustrated in *F* and *H* (the magnified area is demarcated in the low-power view by a *black box*). (*I*, *J*) Antibody specificity was confirmed by preadsorption of antibodies with a competing peptide, which had been used as the immunogen for antibody generation, before staining a section of lung tissue from the same patient with BPD. Staining was consistently more intense in patients with BPD, and the sections illustrated are representative of the trends observed in a total of five patients assessed per group (as indicated in Tables 1 and 2). The expression of lysyl oxidase mRNA was also assessed in mRNA isolated from the lungs of seven patients in control group 1 (*red*), seven patients with BPD or at risk for BPD (*green*) and five patients in control group 2 (*yellow*) by quantitative real-time reverse transcriptase–polymerase chain reaction (*K*). The *bars* represent the data range and the *boxes* represent lower and upper quartiles. The *line* within the quartile box indicates the median; **P* < 0.01.

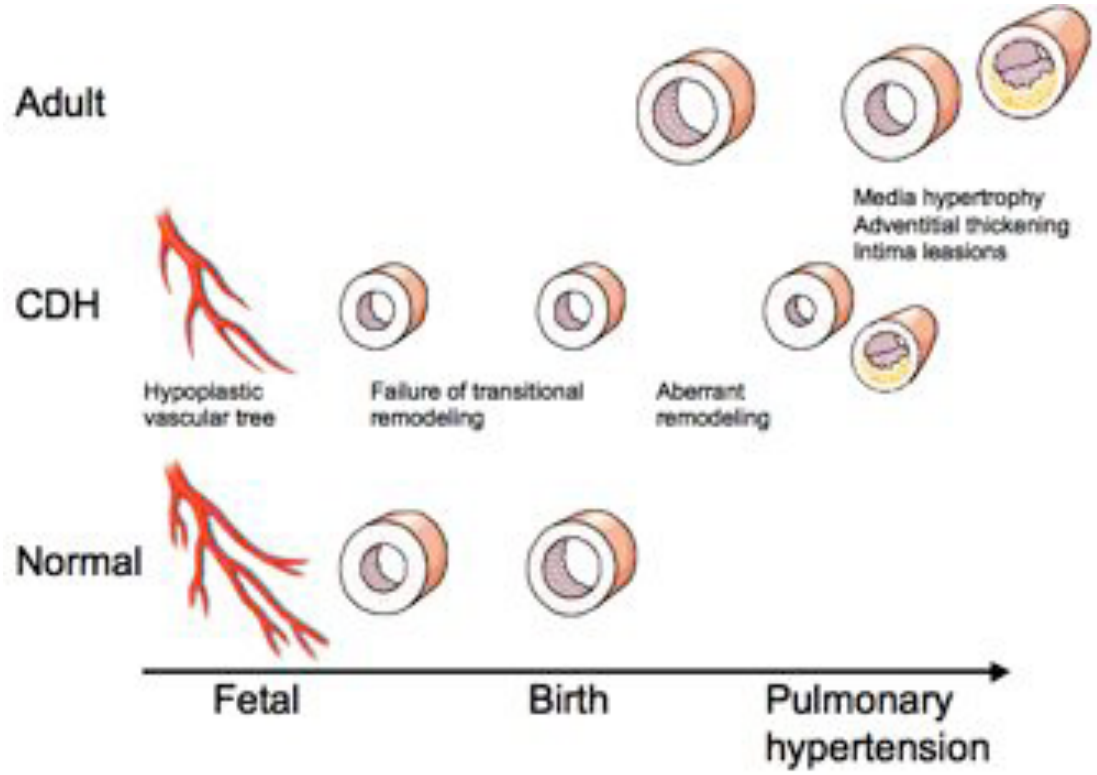

Figure 9.1 (See page 130)
Aberrant development of the pulmonary vasculature in CDH.
(Figure was produced using Servier Medical Art)

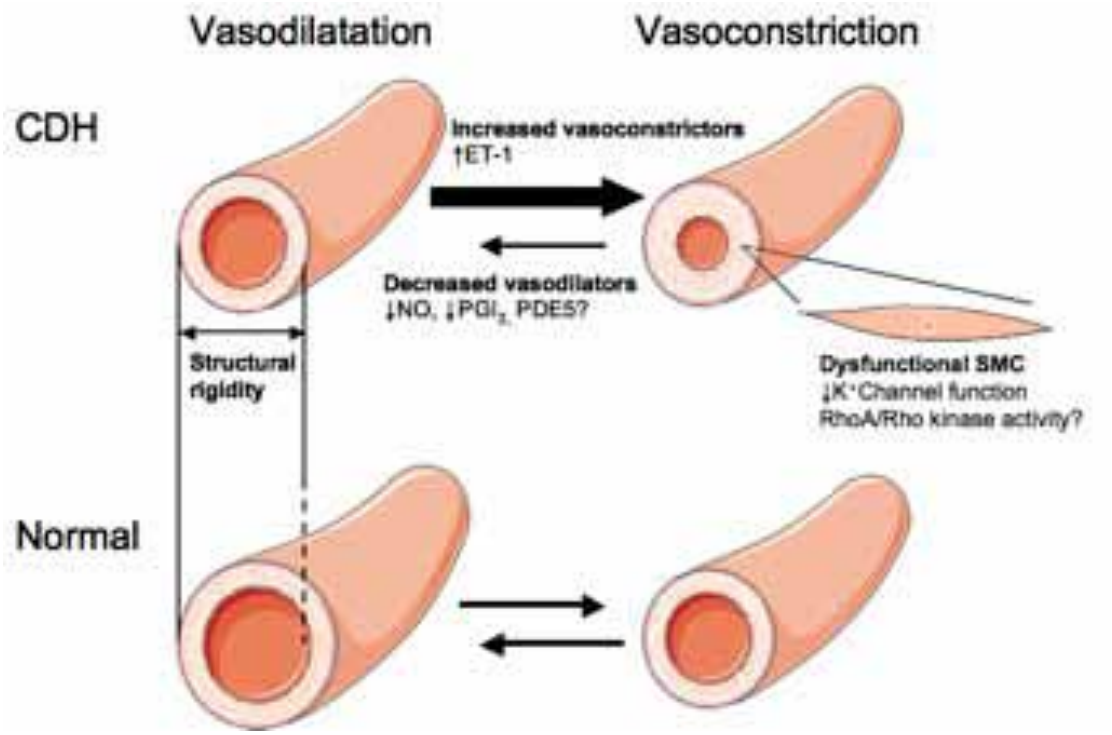

Figure 9.2 (See page 137)
Pulmonary vascular tone in CDH is characterized by increased vasoconstrictors, decreased vasodilators, and dysfunctional SMC. The aberrant vascular development appears to fix the vessels in an incomplete dilated state. ET-1; endothelin, NO; nitric oxide, PGI_2; prostacyclin, PDE5; phosphodiesterase 5.
(Figure was produced using Servier Medical Art)