DYING FOR OXYGEN

Roles of Hypoxia Inducible Factor 2α and 3α during lung development

Yadi Huang

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TABLE OF CONTENTS

CHAPTER 1.1

GENERAL INTRODUCTION: CONGENITAL LUNG LESIONS-UNDERLYING MOLECULAR MECHANISM

Seminars in Pediatric Surgery (2010) 19, 171-179

Seminars in Pediatric Surgery (2010) 19, 171-179

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Congenital lung lesions—underlying molecular mechanisms

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Congenital lobar emphysema;
Pulmonary sequestration

Congenital lung lesions comprise a broad spectrum of rare but clinically significant developmental abnormalities, including congenital cystic adenomatoid malformation, bronchopulmonary sequestrations, congenital lobar emphysema, and bronchogenic cysts, which are commonly surgically treated. Although the terms congenital cystic adenomatoid malformation, bronchopulmonary sequestrations, congenital lobar emphysema, and bronchogenic cysts are entrenched in clinical usage and comfortably correspond to rigid pathologic definitions, there is a considerable overlap in the findings. Disregarding the controversy about lesion nomenclature and classification, it is widely accepted that congenital lung lesions result from perturbations in lung and airway embryogenesis. It is generally accepted that both place (level in the tracheobronchial tree) and timing (gestational age) of the embryologic insult correlates with the type of lesion and histopathology that is manifested. The objective of this review is to briefly review normal lung development and to analyze the known molecular mechanisms underlying those diseases.

Congenital lung diseases represent a wide variety of developmental abnormalities of the respiratory tract, which are relatively rare and commonly have a good prognosis. Some of these congenital lung diseases, such as abnormalities of surfactant production, alveolar-capillary dysplasia, and some pulmonary vascular malformations affect all the lung parenchyma and are not commonly approached by the surgeon, and therefore will be outside of the scope of this review, as will be lung hypoplasia, which has a fully-dedicated review inside this issue. Thus, we will focus on those congenital lung lesions that are understood as resulting from a focal (both in space and in time) development malformation, and present as cystic lesions, as are congenital cystic adenomatoid malformation (CCAM), bronchopulmonary sequestration (BPS), congenital lobar emphysema (CLE), and bronchogenic cysts (BC). Classically, most of these lesions were noticed within the first weeks to months of life, but currently these lesions are diagnosed in the prenatal period, providing an opportunity for a more accurate and planned treatment.[1,2]

Moreover, the opportunity to follow these lesions antenatally is providing new information to better understand their pathophysiological mechanisms, namely fetal hydrops

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that is the harbinger of fetal and neonatal death. Pathologists using surgical removed specimens have done a terrific job classifying these lesions, but little has been discovered about the molecular mechanisms. However, during the last 2 decades important contributions have been made by developmental biologists searching for a better understanding of the molecular regulation of normal lung development, and by using different methodologies, such as knocking-down or up-regulating gene expression, are making relevant discoveries about the molecular mechanisms underlying congenital lung lesions. Therefore, knowledge of the development of normal lung seems essential for those that want to better understand the underlying molecular mechanisms of congenital cystic lung malformations. Thus, a short overview of normal lung development will be presented herein as an introduction for the current knowledge about the molecular mechanisms underlying these lesions.

Normal lung development

The bronchial tree of the human lung has more than 10^5 conducting and 10^7 respiratory airways arrayed in an intricate pattern crucial for oxygen flow. Lung development is a highly orchestrated process directed by mesenchymal(vessels)-epithelial interactions, which control and coordinate the temporal and spatial expression of multiple regulatory factors required for proper lung formation. Many endogenous and exogenous biophysical and biochemical factors may disturb this delicate process leading to disorders of lung growth, maturation, and function. In humans, lung development begins at 3 to 4 weeks of gestation and comprises 6 different stages: the embryonic stage (4-7 weeks of gestation), the pseudoglandular stage (5-17 weeks of gestation), the canalicular stage (16-26-week gestation), the saccular stage (24-38-week gestation), the alveolar stage (36-week gestation to 2 years of age), and the microvascular maturation (birth to 2-3 years of age). To direct this highly predetermined program, a multitude of controlling factors have been identified, namely transcription factors, growth factors and their receptors, extracellular matrix proteins, and intercellular adhesion molecules. We will give a brief overview of some of the key-genes that have been described to play an important role at different developmental phases of lung development. Recent reviews have described the molecular pathways involved in normal lung formation in more detail.[3-6]

Embryonic stage

The definitive endoderm, which forms after completion of gastrulation, undergoes complex cellular rearrangements to establish the primitive gut. The foregut, the anterior part of the primitive gut, generates the lungs and its neighboring foregut derivatives, like thyroid, esophagus, pancreas, liver, and stomach. The formation of these organs requires inductive, paracrine signals from the surrounding mesenchyme, mostly the notochord, heart, and septum transversum, and the competence of the foregut endoderm to respond to them. The prospective lung field is established by growth factors secreted by the heart, for instance, fibroblast growth factor-1 (FGF1) and FGF2, which form an instructive gradient specifying the endoderm. The endodermal cells express FGF receptors (FGFR) to become liver or lung, and it was shown that intact FGFR4-mediated signaling is required to induce the expression of one of the earliest markers of lung epithelium, thyroid transcription factor (TTF1; Nkx2.1). Retinoic acid (RA), the active form of vitamin A, is synthesized in the foregut by retinaldehyde dehydrogenase 2 (RALDH2), and mice deficient for this enzyme fail to develop lungs, if they are rescued from early lethality by maternal RA.

Furthermore, RA controls the activity of transforming growth factor-beta (TGFβ) in the prospective lung field through its receptors RARα and RARβ, which in turn regulate the local mesenchyme expression of FGF10.[7] Note that deletion of both RARα and RARβ results in pulmonary agenesis, tracheoesophageal fistula, and lobar agenesis.[8] Another factor influencing FGF10 expression, at least in chicken, is the transcription factor T-box 4 (TBX4). Aberrant expression of TBX4 leads to the activation of FGF10 expression and abnormal formation of lung-buds. However, TBX4 knockout mice do not display lung abnormalities, and this emphasizes the redundancy effect of the 6 TBX factors, a phenomenon that likely occurs with other signaling pathways during lung development.[9] In this way, the ventral foregut cells in the prospective lung field express FGFR2 and respond to FGF10 by cellular movements into the mesenchyme towards the FGF10 source, forming the primary lung-buds.

Among the subset of HOX family genes, which encodes transcription factors involved in patterning of the early embryo, it was described that HOXb5 is important in patterning of airway branches during mouse lung morphogenesis.[10] Interestingly, the addition of RA to lung explants induces the expression of HOXb5, which suggests a possible role for endogenously produced RA.[11] Moreover, HOXb5 is strongly expressed in thoracic mesenchyme just before the budding of the lung primordial diverticulum from the embryonic thoracic foregut.

These early phases in lung development require the interplay of many other factors, both in the endoderm as well as in the mesenchyme. Moreover, several transcription factors, like SOX2 (a member of SOX family of HMG containing transcription factors), forkhead box 2 (FOXA2; also known as HNF-3β), GATA4, and GATA6 (members of the GATA family of zinc finger transcription factors), have been described as regulating target genes involved in the early patterning of the lung.[12-14] There are also secreted morphogens, and the downstream pathways thereof that play an important role in the inductive moments of lung development, like the sonic hedgehog pathway (Shh).[15]

Inactivation of Shh, or members of its signaling cascade (Gli, Ptc, Smo, hip), has a deleterious effect on the formation of the lung, with varying severity. Interestingly, several of these factors that play an important role in the formation of the primary structures of the future lung are also involved in later phases of differentiation and specification of cell types (like Sox2, Gata6).

Pseudoglandular stage

During this stage branching morphogenesis occurs and the preacinar airways and blood vessels develop. Additional steps in lung development are differentiation of epithelial cells in a centrifugal manner with the appearance of ciliated cells, goblet and basal cells, and production of cartilage, which can already be found around the main bronchi before 10 weeks after conception. A highly ordered sequence of patterning events collectively referred to as branching morphogenesis generates the bronchial tree and defines the proximal-distal axis of the lung by the end of the pseudoglandular stage. This process results from reiterated combination of 3 main processes: domain branching, planar bifurcation, and orthogonal bifurcation.[16] Domain branching is responsible for the formation of the main secondary branches, whereas planar bifurcation subsequently generates the tertiary and later-generation branches. Orthogonal bifurcation results in the formation of 3-dimensional branching of the airways. The repetitive use of these branching modes could explain the complex but highly ordered appearance of the lung. At the molecular level, it is suggested that a delicate interplay between FGF10, FGFR2b, and sprouty 2 (Spry2) are central in the activation of the 3 central branching modes. Lung bud position, outgrowth, and subsequent arrest result from the dynamic change in relative activity of Shh, FGF10, bone morphogenetic protein 4 (BMP4), and Spry2 signaling.

Local FGF10 expression in the distal mesenchyme, the part of the lung that actively grows and branches, binds to the FGFR2b receptor on the epithelial cells. This triggers a signaling cascade that leads to the expression of Spry2, Shh, and Bmp4. These morphogens subsequently fine-tune the response and outgrowth of the lung bud (for detailed review on molecular processes, see Cardoso and Lü[4]). Wingless (Wnt) signaling is also important for the correct formation of the lung.[17] However, the different results obtained with interference of the Wnt signaling cascade (Wnt, β-catenin, Dkk1) makes it difficult to pinpoint the nature of Wnt signaling in lung development. Targeted deletion of β-catenin or the expression of the Wnt antagonist Dickkopf-1 (Dkk1) hampers normal branching partly through the failure to induce the correct level of FGFR2b in the distal lung epithelium. Extracellular matrix components, especially fibronectin and laminin, seem to play a role by accumulating at clefts, specific points that do not branch. This cycle of interaction is then repeated during subsequent branching events. It should be emphasized that molecules involved in mechanical smooth muscle cell contraction or neuroendocrine products, like bombesin and ghrelin, and even interleukins, such as IL-6, might have an intrinsic regulatory capability over global pulmonary branching processes.[18-22] Note that BPS and CCAM (bronchiolar-types I, II and III) are assumed to appear during this stage.

Canalicular stage

This period is characterized by capillaries leaning against the epithelium, marking the beginning of the future blood-air interface. The second landmark of this stage is the differentiation of the pulmonary epithelium into type II cells, the producers of surfactant, and subsequently type I cells, which contribute to the formation of a thinned prospective air-blood barrier. Several transcription factors play a specific part in these differentiation processes. SOX2 and Mash-1 are important for the pulmonary neuroendocrine cells, whereas SOX2, FOXA2, GATA6, and TTF1 for the epithelial cells (basal cells, ciliated cell, Clara cell, goblet cell, type II and type I cells).

Saccular stage

The widening of the peripheral air spaces distal to the terminal bronchioles at the expense of intervening mesenchyme will allow for sufficient gas exchange. The prospective lung parenchyma is gaining in size by dichotomously branching of the terminal generations of the airway tree. Furthermore, during this period, preparation for the real alveolarization starts by deposition of elastic fibers at the localizations where future secondary septa will form. It has been proposed that the formation of acinar-alveolar subtype of cystic adenomatoid malformation (type IV) occurs during this period.[23] As in the canalicular period, lack of amniotic fluid, whether related to renal anomalies (Potter's syndrome) or rupture of amniotic membranes, may cause lung hypoplasia during this period through mechanisms that are still unknown. Krüppel-like factor 5 (KLF5) is required for maturation of the lung and influences the expression of genes regulating surfactant lipid and protein homeostasis, vasculogenesis and smooth muscle cell differentiation.[24]

Alveolar stage and microvascular maturation

The establishment of secondary septa that subdivide the saccules incompletely into smaller units, the alveoli, characterizes this stage. This immature structure does not yet correspond to the adult morphology and will undergo more restructuring, called microvascular maturation.[25] Among the variety of factors that participate in the control of budding of secondary septa, elastin appears to play an essential role.[26] Other factors involved are FGF family, platelet-derived growth factor-A (PDGF-A), and its receptor (PDGFR-α), colagenases, and proteoglycans.[27] VEGF has also been demonstrated to play a role in maintaining alveolar structure, playing an important role in matching the epithelial-capillary interface during lung morphogenesis. The insult that originates CLE is assumed to occur either during late saccular or alveolar stages.

Congenital cystic adenomatoid malformation

CCAMs are relatively rare developmental abnormalities of the lung that can cause significant morbidity and mortality in infants as the result of associated fetal hydrops, lung hypoplasia, and respiratory distress. The lesions are described as hamartomatous lesions, ie, normal lung tissues in a disorganized spatial arrangement. The result of this process is a multicystic mass that replaces the normal lung structure. Human CCAMs are unilateral and usually confined to a single lobe. The exact incidence of CCAM is unknown, although it has been estimated at 1:25,000 to 1:35,000 pregnancies in 1 study.[28]

Nomenclature and classification

A new nomenclature, congenital pulmonary airway malformation, has been proposed for this entity, mainly because neither cystic or adenomatoid features are always present,[29] however we will continue to adopt the term CCAM. Three different histologic types were initially described[30]: type I (50% of all CCAMs), which consist of large cysts (2-10 cm in diameter) lined by a pseudostratified columnar epithelium, with smooth muscle and fibrous tissue around it, and in some cases, cartilaginous plates throughout the lesion as well; type II (35% of all CCAMs), which consist of smaller cysts (0.5-2 cm in diameter) that give the lesion a sponge-like appearance, with a cuboid or columnar epithelium and a fibromuscular layer underneath; and type III (10% of all CCAMs), which are characterized by multiple microscopic cysts (<0.5 cm in diameter) that resemble bronchioles.

To this original classification, 2 other types where added afterwards[31]: type 0, consisting of a solid lesion formed by bronchiole like structures with cartilage and smooth muscle, also referred to as acinar dysplasia, and type IV, consisting of a single large cyst with a flattened nonciliated epithelium, smooth muscle, and cartilage in some cases. Depending on differences in cytodifferentiation, these 5 categories could be assembled into 2 major subtypes: CCAM types I, II, and III, with a bronchiolar-type epithelial differentiation; and a second subtype consisting of CCAM type IV, which has an acinar-alveolar epithelial differentiation.

In an effort to provide clinicians with a practical and useful guideline for evaluation and treatment of cases detected in utero, Adzick et al[32] proposed a classification system on the basis of gross anatomy and sonographic appearance of the fetal lesion. Although this system correlates poorly with histology of the resected specimen, it has a relevant degree of prognostic value, and has thus gained widespread acceptance among surgeons. These authors distinguished between macrocystic and microcystic CCAMs. Macrocystic lesions consist of multiple large cysts (5 mm or larger in diameter) with thin intervening echogenic areas. They appear sonographically as fluid-filled cysts. Microcystic lesions are smaller than 5 mm in diameter. They have a homogeneous echogenic appearance in the fetal lung, with no visible cystic spaces. When microcystic pulmonary malformations are large, they are frequently associated with mediastinal shift, pulmonary hypoplasia, polyhydramnios, and nonimmune hydrops.[33] In fetuses in which a congenital pulmonary airway malformation grows rapidly and develops hydrops, intervention is often required for survival. Macrocystic lesions have been thought to have a more favorable prognosis because they tend to grow less rapidly; however, some of these fetuses do develop hydrops and require antenatal intervention also.

Etiology

It is generally accepted that CCAM is characterized by abnormal airway patterning during lung branching morphogenesis and is formed by abnormal branching of the immature bronchioles. Cystic structures arise from an overgrowth of the terminal bronchioles with a reduction in the number of alveoli. Although CCAM pathogenesis remains unknown, several authors have hypothesized that different types of CCAM originate at distinct stages of lung development. The bronchiolar subtype (CCAM types I, II, and III) may develop at the pseudoglandular stage, and the acinar-alveolar subtype (CCAM type IV) may be caused by a late event in the saccular period (between 22 and 36 weeks) that disrupts branching of the distal acinar structures.[34] Increased proliferation and decreased apoptosis were verified in CCAM specimens.[35] All these data, strongly suggest a focal arrest in lung maturation during the fetal period.

Several studies in which the authors used human fetal resected CCAM tissue, tried to discern factors that could be responsible for this pathology. Volpe et al[36] demonstrated that HOXb5, a transcription factor important for the proliferation and patterning of the last 7 subdivisions of the smaller airways, in human fetal resected CCAM tissue, was maintained at a higher level of expression, characteristic of early stages of lung development. More recently, Jancelewicz et al[37] analyzed gene expression from laser dissected epithelium and mesenchyme of human fetal and postnatal CCAM. They demonstrated that markers of early lung development, such as HOXb5 and TTF1, are overexpressed in fetal CCAM. They also demonstrated that FGF9 was overexpressed; however, they reported a decrease in FGF7 and no altered expression of FGF10 and FGFR2.[37] Fetal CCAMs that grew rapidly progressed to hydrops and required in utero resection showed increased PDGF-B gene expression and PDGF-B protein production compared with normal fetal lung or term CCAM specimens,[38] but to date there is no evidence that this factor is the causative factor for CCAM. PDGF-B is a mesenchymal growth factor, which increases cell proliferation potentially through downstream effectors, such as FGF7 or TGFβ. However, FGF7 gene expression and protein production were evaluated and no differences were found when compared with normal lungs.[35]

Specific cell adhesion molecules, such as α-2 integrin and E-cadherin, important to lung development and airway morphogenesis were analyzed both in BPS and CCAM tissues, and results show that their expression patterns are altered. They also suggested that CCAM pathogenesis might be associated with potentially altered integrin cytoplasmic signaling. Interestingly, both integrin and E-cadherin are in part regulated by the master regulatory HOX genes.[39] Fromont-Hankard et al[40] observed abnormal and strong glial cell–derived neurotrophic factor expression in epithelial cells lining CCAM cysts that contrasted with the absence of glial cell–derived neurotrophic factor staining in adjacent normal lung and in postnatal lung from individuals without CCAM, suggesting a focal arrest in lung maturation during the fetal period.

Wagner et al[41] performed a gene expression profiling study by using microarray analysis of fetal-resected CCAM tissues compared with age-matched controls. They found 2 candidate genes that met the most stringent criteria for differential expression: Clara cell marker 10 (CC10) and fatty acid binding protein 7 (FABP-7). CC10 expression had already been found to be increased in CCAM,[34] whereas FABP-7 has not been previously implicated in pulmonary development or in the pathogenesis of CCAM. This protein belongs to a family of intracellular lipid-binding proteins with functions related to the transport of long-chain fatty acids, targeting of fatty acids to specific metabolic pathways, and the subsequent regulation of gene expression and cell growth.[42] These authors found that fetal CCAM expresses significantly less FABP-7 than control fetal lung. They speculate that less FABP-7 may result in more long-chain unsaturated fatty acids free in the cytoplasm causing inhibition of the glucocorticoid receptor. Therefore, CCAMs, with low FABP-7, would have less glucocorticoid response than normal lung, resulting in an arrest in development. However, these studies have a common major limitation, which is that the analysis was performed in CCAM specimens that were surgically resected, analysis of which is unlikely to reveal the initial inciting events.

Relevant insights regarding the molecular mechanisms underlying CCAM formation may arise from prospective in vivo studies in animal models. Previous studies in transgenic murine models, where heterotopic overexpression of FGF7[43] and FGF10,[44] and orthotopic overexpression of FGF9[45] resulted in marked perturbations of lung morphogenesis, suggesting that these factors, or others directly dependent, might be implicated in the development of adenomatoid malformations. Gonzaga et al[23] focally and transiently induced FGF10 overexpression in the mesenchymal compartment of the fetal rat lung at different locations and developmental stages using an intraparenchymal gene transfer method. As a result, pulmonary morphogenesis was markedly perturbed with the very rapid appearance of localized cystic lung malformations. The type of malformation observed was developmental stage and location dependent, with the spectrum ranging from macrocystic malformations lined by predominantly bronchial epithelium, to focal microcystic malformations lined by predominantly alveolar epithelium. The striking similarity of these lesions to those seen in human CCAM strongly implicates mesenchymal overexpression of FGF10 in the initial events invoking CCAM formation.

In the light of the current models for branching morphogenesis, researchers have proposed that transient high levels of FGF10 at the pseudoglandular stage of branching morphogenesis would induce a burst of proliferation and outward migration of the epithelium responsive to FGF10, with less proliferation at branch points where antagonists as Bmp4 and Spry2 are expressed. Thus, at the early time point, when FGF10 levels are high-unopposed action of FGF10 allows the rapid formation of large cysts. In contrast, if FGF10 high levels occurred later in development, expression of BMP4 and Spry 2 increase in the epithelium surrounding cystic areas, opposing epithelium proliferation and outgrowth, leading to the formation of smaller cysts. In addition, during the pseudoglandular stage, if the altered levels of FGF10 occur in proximal airways, where fewer branch points exist, and high columnar epithelium predominates, unopposed FGF10 signaling would be predicted to result in large cysts lined by predominantly bronchial epithelium. Although in distal airways, where multiple branch points are already present and cuboidal epithelium predominates, smaller and more numerous cysts would form lined by predominantly alveolar epithelium. During the canalicular stage of lung development only terminal branching occurs, so FGF10 overexpression might stimulate short segments of responsive epithelium into small cyst and differentiated adenomas formation. Studies showed that the pattern difference between the branched airway (dorsal) and the air sac (ventral cystic structure) in chick lung is due to the difference in the diffusion coefficient of FGF10 between these 2 regions,[46] and that the cyst-branch difference in this system is caused by region-specific mesenchymal properties related to Hoxb cluster nested gene expression.[47] In addition, induced expression of Sox2 also induces the appearance of cystlike structures, suggesting that the forced differentiation of epithelium into proximal cells leads to CCAM.[12]

Biological markers involved in cellular proliferation and differentiation as well as blood vessel growth have been studied, with researchers attempting to elucidate the origin of CCAM and its possible linkage to malignant transformation.[34,48,49] Predisposition to malignancy, in particular of type 1 CCAM is still poorly understood. It has been speculated that this change might reflect an inherent instability in the alveolar tissue adjacent to congenital cysts.[50] Specific tumor-associated markers, such as epidermal growth factor receptor (EGFR) and K-RAS oncogene, have been investigated in cases of CCAM, however no firm conclusions can be drawn on this issue.[51] The initial events that disrupt the strict balance of the mesenchymal-epithelium interactions in the developing lung are yet to be determined. It is unknown whether anomalous expression of genes represents causation or a global delay in differentiation.

Bronchopulmonary sequestration

BPS are microscopic cystic masses of nonfunctioning pulmonary tissue that lack an obvious communication with the tracheobronchial tree. Typically, the lung tissue in BPS receives all or most of its blood supply from an anomalous systemic artery, with the origin of this artery being variable. Two forms of sequestration are recognized: intralobar and extralobar. Although rare, both forms can occur simultaneously. Intralobar sequestrations (ILS) are incorporated into the normal surrounding lung, whereas extralobar sequestrations (ELS) are completely discrete from the normal lung and are enveloped by separate pleura. Reported incidences of the 2 forms of sequestration vary substantially.

Extra lobar sequestrations

Historically, most ELS have been diagnosed in infancy; however, these lesions are now increasingly being diagnosed prenatally. Microscopic examination of these lesions frequently demonstrates dilated subpleural lymphatics, dilated bronchioles, alveolar ducts, and alveoli.[52] They are generally located in the left lower chest, with up to 15% found either within or below the diaphragm. Because of their embryologic origin, ELS have a close association with the gastrointestinal tract, and up to 10% are located within the abdomen. Newborns have a broad spectrum of presentations. They may be asymptomatic or their lesions may be incidentally identified. Infants with symptomatic lesions may present with respiratory distress, pneumonia, feeding difficulties, hemorrhage, or even congestive heart failure. The sequestered lobe may cause substantial arteriovenous shunting, leading to high-output cardiac failure. Classical studies report that 40% of infants have other associated anomalies, including chest wall and vertebral deformities, hindgut duplications, and congenital heart disease among others.[53] A total of 5% to 15% of infants with congenital diaphragmatic hernias have one or more ELS.[54] All cases of ELS have a systemic arterial blood supply. In 20% of patients, the feeding artery originates from the infradiaphragmatic aorta. In most instances of ELS, there are systemic venous connections, which include the superior vena cava and the azygous and hemiazygous veins.

Intralobar sequestrations

ILS is most commonly seen on the left side, especially in the medial basal or posterior basal segments of the lower lung lobes. In contrast to infants with ELS, children with ILS are otherwise generally normal. Also, their lesions typically present later in childhood or adulthood. However, with the routine use of antenatal US screening, this classic pattern of presentation may change.[55] Older children may present with signs and symptoms related to inadequate tracheobronchial drainage caused by the lesion or adjacent atelectatic lung. This results in recurrent pneumonia, lung abscess, and hemoptysis. Communication between the esophagus and stomach occurs in approximately 10% of patients. Optimally, any communication should be identified preoperatively by a contrast study of the gastrointestinal tract or intraoperatively by endoscopy and recognition of anatomic communication between the airway and gastrointestinal tract. As with ELSs, ILSs have a systemic artery blood supply, although venous drainage may occur through the pulmonary veins.

Etiology

The etiology of sequestrations has been the subject of great debate. Five major causes were identified: vascular traction, vascular insufficiency, coincidental occurrence, acquired pathology after infection, and developmental insult.[56] The most widely accepted embryologic theory for the development of BPS, and which provides a single mechanism for the spectrum of pathology described in the literature, is that a supernumerary lung bud arises caudal to the normal lung bud and continues to migrate caudally with the esophagus.[57] As mentioned previously, the primitive bronchial tree begins as a ventral diverticulum of the foregut at 3 weeks, which bifurcates into right and left lung-buds by 26 days. Definitive lobes of the lung form between weeks 5 and 8 of gestation. Therefore, BPS is likely to arise between weeks 4 and 8 of gestation.[57] If this lung bud arises prior to the development of the pleura, it is invested with adjacent lung and becomes an ILS. If supernumerary lung development occurs after pleura formation, the bud will grow separately and acquire its own pleural covering, forming an ELS. Some authors claimed that the high incidence of congenital diaphragmatic hernia in patients with ELS points to a defect occurring prior to week 6 of gestation.[58] Volpe and coworkers[36] have demonstrated that HOXb5 is necessary for normal airway branching and development and have suggested that the developmental abnormalities seen with BPS is related to abnormal expression of the homeobox genes. Vascular traction of the developing lung such that a portion separates from the main lung mass was initially pointed out as major cause for ELS.[59] Although, there is no known genetic predisposition to these sequestrations, there are many associated anomalies, particularly with ELS.

Congenital lobar emphysema

CLE is a term used to describe a distended, hyperlucent lobe on plain radiographs, usually the left upper or the right middle lobe.[60] Pathologically, a distinction is made between a polyalveolar lobe, in which the number of alveoli is greatly increased, and congenital lobar overinflation, in which the alveoli are markedly distended. CLE is rarely diagnosed prenatally, possibly because of its low prevalence in utero or the increased echogenicity of the lungs, which could be too subtle to be evaluated in utero.[61] However, the differential diagnosis of an echogenic lung should include CLE apart from CCAM and pulmonary sequestration. The prevalence of this rare condition is 1 per 20,000 to 30,000 deliveries, whereas the prevalence during pregnancy is unknown.[62] CLE may be associated with cardiovascular anomalies in 14% of cases. Although uncommon, renal, gastrointestinal, musculoskeletal, and cutaneous malformations may also occur.[63]

Etiology

The pathophysiologic mechanism of CLE consists of disruptions of bronchopulmonary development due to abnormal interactions between embryonic endodermal and mesodermal components of the lung, resulting in progressive lobar hyperinflation. Several mechanisms have been postulated for the formation of CLE: air trapping in the emphysematous lobes, which is thought to be caused by dysplastic bronchial cartilage, creating a ball valve–type effect; endobronchial obstruction from extensive mucosal proliferation and infolding; extrinsic compression of the bronchi from aberrant cardiopulmonary vasculature; and diffuse bronchial abnormalities that may be related to infection.[64] However, in many instances an exact cause cannot be determined.[65]

Bronchogenic cysts

BC result from abnormal budding of the foregut. As foregut duplication cysts, they share common features with esophageal duplication cysts but are characterized by the presence of cartilage, smooth muscle and glands in their wall. The majority is located in the mediastinum, usually adjacent to the distal trachea or proximal main stem bronchi, but they can also be found within the parenchyma of the lung.[66] They are usually unilocular, filled with fluid or mucus and generally do not communicate with the airway. They may become symptomatic by a mass effect on the trachea or bronchus and are one of the causes of congenital lobar overinflation (CLO). They can also cause dysphagia, become infected or bleed, leading to hemoptysis or hemothorax. Rare cases of malignancy have also been described. De Perrot demonstrated increased proliferative activity consistent with atypical adenomatous hyperplasia in some areas of a bronchogenic cyst, although in some reports the differentiation between intrapulmonary bronchogenic cyst and type 1 CCAM could be questioned.[67]

Concluding remark

The spectrum of congenital lung lesions likely results from disordered embryologic interactions, which occur during the course of fetal lung development. Although some molecular links are being suggested, it is still too early to establish any molecular pathway as the cause of these congenital lesions.

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CHAPTER 1.2

GENERAL INTRODUCTION: HYPOXIA INDUCIBLE FACTORS IN EMBRYONIC DEVELOPMENT AND DISEASES

REGULATION OF HYPOXIA INDUCIBLE FACTORS

The founding member of the Hypoxia Inducible Factors (HIFs), HIF1, was originally identified as the factor inducing the expression of the *EPO* gene, which encodes for erythropoietin. [1-2] HIFs are important transcription factors in the cellular adaptation to hypoxia by regulating different sets of genes involved in angiogenesis, metabolism and cell homeostasis. They are heterodimeric transcription factors consisingt of two structurally related subunits, one is an oxygen sensitive HIFα subunit (HIF1α, HIF2α or EPAS1 and HIF3α) and the other is the constitutively expressed subunit, HIF1β/ARNT-subunit (Aryl hydrocarbon Receptor Nuclear Translocator). The Human HIF1α protein has a C-terminal region spanning residues 526–652 which is the oxygen-dependent degradation domain (ODD) and two minimal transactivation domains localized at amino acid residues 531–575 and 786–826[3-4]. Inter-species protein homology is well conserved for the HIF-1α and HIF-1β subunits, with over a 90% similarity between human, rat and mouse.[5] The protein structure of HIF1α, HIF2α, HIF3α and HIF1β is shown in Figure 1.[6] HIF1α, HIF2α and HIF3α are structurally highly related proteins, which are post-translationally regulated by one of three prolyl hydroxylase domain-containing enzymes (Egln1, Egln2 and Egln3).[7] Under normoxic conditions, HIFα proteins are hydroxylated on one or both of the prolyl residues

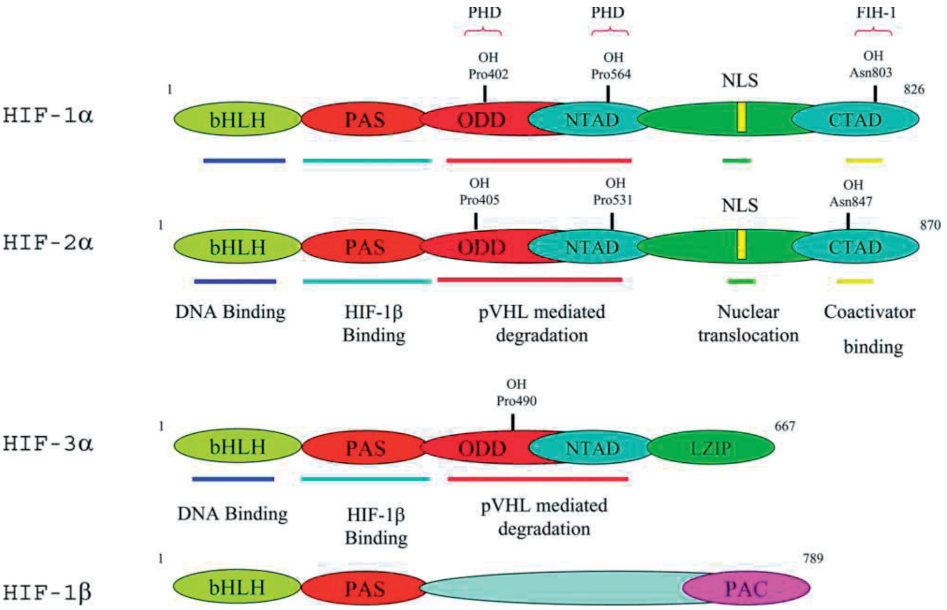

Figure 1: Schematic diagram showing structural domains of HIF family members HIF-1α, HIF-2α, HIF-3α and HIF-1β. The modifications of specific residues are highlighted above each protein, and the proteins that perform those modifications are shown. Coloured bars below each protein delineate particular interaction regions within HIF proteins. CTAD, C-terminal transactivation domain; LZIP, leucine zipper; NLS, nuclear localization signal; NTAD, N-terminal transactivation domain; PAS, Per/ARNT/Sim domain; PAC, PAS-associated C-terminal domain.[6]

located in the ODD, which generates a binding site for the von Hippel-Lindau (pVHL) tumor suppressor protein. pVHL is a component of an ubiquitin ligase complex, which polyubiquitinates the hydroxylated Hifα proteins leading to their proteasomal degradation. [8]

In contrast, under low oxygen conditions, the HIFα proteins are stable and can dimerize with HIF1β, which can bind to hypoxia responsive elements (HRE, consensus RCGTG) in the promoter regions of specific genes and transcriptionally activates downstream target genes which contain hypoxia response elements, such as EPO and VEGF.[9,10] (Figure 2)

Hif1α

Hypoxia inducible factor 1α is a highly conserved transcription factor and present in almost all cells types. Hif1α mRNA is detected in almost all human organs, including heart, lung, liver, placenta, brain, skeletal muscle, kidney and pancreas.[11] Immnohistochemical examination revealed that the Hif1α protein is present in brain, kidney, liver, heart and skeletal muscle in healthy mice under normoxic conditions, the

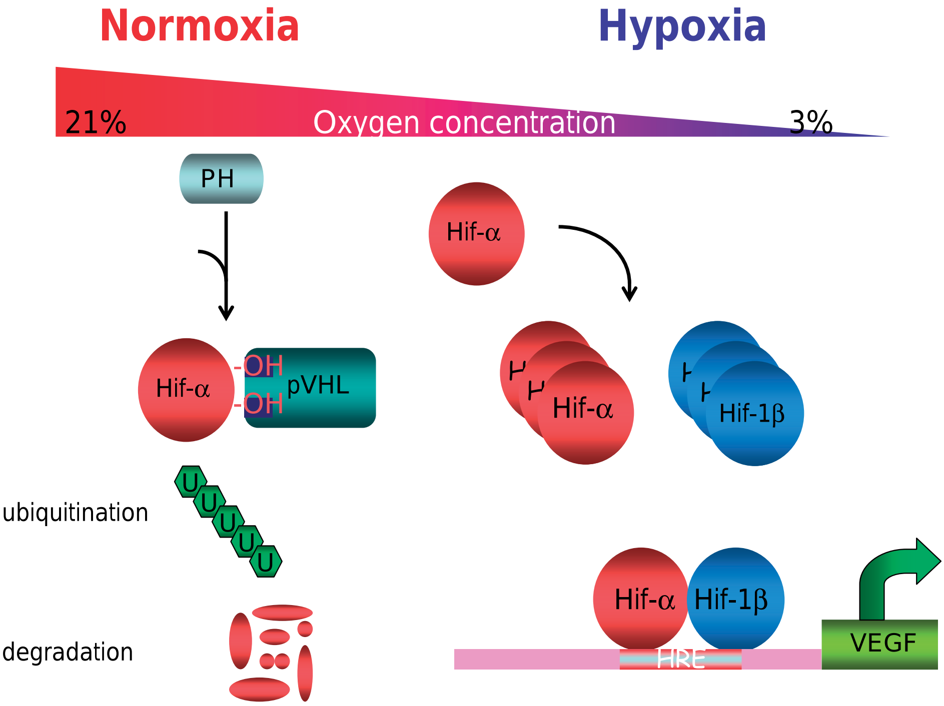

Figure 2: The activity of hypoxia inducible factors is regulated by oxygen levels. HIFα proteins are hydroxylated on one or both of the proly residues under normoxic conditions, pVHL can recognize the hydroxylated proteins and then target them for proteasomal degradation; HIFα proteins are stabilized and can dimerize with HIF1β under hypoxia, which will bind to HRE (hypoxia response elements) in the promoter region of specific genes l activating transcription of the target genes.

expression increased after exposure to hypoxia for 30 to 60 minutes.[12] Hypoxia induces Hif1α in all cultured pulmonary cells, while pulmonary arterial smooth muscle cells also express Hif1α at normoxic conditions.[13]

Hif1α in embryonic development

Hif1α is essential for embryonic development and is important in cardiac and vascular development and embryonic survival. In wild type mice, the expression of Hif1α increases between embryonic day 8.5 and 9.5. Complete deficiency of Hif1α leads to embryonic lethality around day 11. Hif1α knockout mice have multiple developmental defects in neural tube development, vascularization, heart development, neural crest migration, and display massive cell death in the cephalic mesenchyme. The vascular defects were spatially and temporally correlated with cell death.[14,15-16] Hif1α knockout mice demonstrated decreased VEGF mRNA expression compared to wild type animals, since VEGF is a direct target gene of Hif1α. [9,15] Specific ablation of Hif1α in avascular tissue showed gross skeletal malformations and prenatal death, probably due to tracheal abnormalities.[17] In addition, the Hif1β/ARNT knockout mice revealed important functions of hypoxia inducible factors, including Hif1α, in placentation during embryonic development. [18] Both a conditional knockout and over-expression of Hif1α in lung epithelial cells in mice showed respiratory distress syndrome and abnormal lung development.[19,20] Collectively, these findings indicate that Hif1α plays important roles during embryonic development.

Hif1α in diseases

Since oxygen homeostasis is very important for human development and physiology, and Hif1α is required for a variety of physiological responses to chronic hypoxia, it is not surprising that Hif1α is involved in many different types of human diseases including cardiovascular disorders, pulmonary hypertension, pregnancy disorders, and tumorigenesis.[21] One study found increased levels of Hif1α to be an early response to myocardial ischemia or infarction, and may be considered as a useful marker of acutely jeopardized myocardium.[22] PR39, a macrophage-derived peptide that can inhibit degradation of Hif1α, was shown to induce myocardial angiogenesis and may be used as a potential therapeutic strategy to promote angiogenesis within ischemic myocardium.[23] It was also shown that Hif1α is involved in the induction of cardioprotective molecules, such as inducible nitric oxide synthase (iNOS), hemeoxygenase 1 (HO-1), and erythropoietin (EPO), which in turn alleviate myocardial damage caused by harmful events such as ischemia-reperfusion injury.[24]

Several studies have shown that Hif1α is required for a variety of physiological responses in the lung to chronic hypoxia, which leads to the development of hypoxia-induced pulmonary hypertension.[2] Mice containing one functional copy of Hif1α showed a significant delay in the development of pulmonary hypertension.[25] The Fawn-hooded rat, a genetic model for pulmonary arterial hypertension, displays similar characteristics as the human disease and has an increased level of Hif1α.[26] Patients with non-hypoxia associated pulmonary arterial hypertension showed elevated expression of HIF1α by immunohistochemistry.[27] Bronchopulmonary dysplasia (BPD) which is a developmental lung defect requiring prolonged life support for newborns, is associated with decreased levels of Hif1α. [28-29]

In Sheep, HIF1α is expressed normally in healthy fetal skin. Its expression is markedly upregulated in scarring fetal wounds, and correlated with the expression pattern of transforming growth factor β3 which is a potent anti-scarring cytokine. Thus, HIF1α also plays a role in wound healing in embryos.[30]

Rapidly growing tumors are usually exposed to hypoxia or even anoxia due to inadequate blood supply. Since Hif1α is induced by hypoxia, it might be involved in tumorigenesis.[31] A direct link between Hif1α and tumorigenesis is the observation that v-SRC, the oncogene of the Rous Sarcoma Virus, can induce the activity of the Hif1α protein even in normoxic conditions.[32] The expression of HIF1α in different types of human cancers, such as brain, colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinomas, indicate its role in tumor progression. Hif1α expression has also been correlated with the aberrant accumulation of the tumor suppressor protein p53.[33-34] HIF1α is also over-expressed in both small cell and non-small cell lung cancers.[35-36] It is also present in human prostate cancer cell lines under normoxic conditions and even expressed at higher levels in response to hypoxia.[37] Thus, HIF1α is a potential therapeutic target for cancer treatment. For example, PX-478, one of the HIF1α inhibitors demonstrated effectiveness against tumor growth in the mouse model of human lung cancer.[38] Another HIF1α inhibitor, RX-0047, can also inhibit the formation of human lung metastasis in a xenograft mouse model.[39]

Hif2α

Hif2α (also called EPAS1/HRF/HLF/MOP2), which shares high similarity with Hif1α also binds to hypoxia response elements (HRE) in the promoter region of different genes.[9,40-42] Hif2α is expressed in almost all organs upon hypoxic induction, such as brain, heart, lung, kidney, liver, pancreas and intestine; Hif2α is also present in vascular endothelial cells, kidney fibroblasts, and liver cells, epithelial cells of the intestine and heart, and bronchial cells and type II pneuomocytes of the lung.[43-47] Hif2α mRNA expression is increased in the human lung during different gestational stages.[48]

Hif2α in embryonic development

Gene ablation of Hif2α leads to different developmental defects, depending on the genetic background of the mouse. The first Hif2α knockout mice die at mid gestation, and showed substantially reduced catecholamine levels.[49].In an ICR/129 Sv outbred background, Hif2α knockout mice died in utero between embryonic day (E)9.5 and E13.5 and developed severe vascular defects both in the yolk sac and embryo.[50] While in 129/Sv x Swiss genetic background, Hif2α knockout mice die immediately after birth, and showed pulmonary surfactant deficiency which resulted in neonatal respiratory distress.[47] There were no surviving Hif2α knockout mice from isogenic 129S6/SvEvTac or congenic C576BL/6J matings, whereas F_1 hybrid matings yielded Hif2α knockout mice at one-fourth of the expected number. These mice showed abnormalities in multiple organs i.e. retinopathy, hepatic steatosis, cardiac hypertrophy, skeletal myopathy, hypocellular bone marrow, azoospermia and also impaired homeostasis of reactive oxygen species.[51] However, conditional over-expression of Hif2α in airway epithelial cells also leads to pulmonary surfactant deficiency resulting in neonatal respiratory distress and lack of maturation of type II cells.[46] (Chapter 2) These two studies indicate a critical role of Hif2α in lung development and surfactant production, especially inhibiting the differentiation and maturation of alveolar epithelial cells. Hif2α knock-down mice showed 80-20% expression of Hif2α depending on different tissues. This study showed that Hif2α is a key factor in premature retinopathy by affecting the expression of erythropoietin which is an important angiogenic factor.[52] A genetic knock-in of Hif1α into the Hif2α locus showed an expansion of Oct-4 expression, which is a transcription factor essential for maintaining stem cell pluripotency. Such mice showed defective hematopoietic stem cell differentiation in embryoid bodies, and large embryonic stem cell (ES)-derived tumors.[53] In adult mice, lack of Hif2α leads to an apparent mitochondrial disease state,[54] and it is required for normal hematopoiesis.[55]

Hif2α in diseases

HIF2α has also been associated with a number of diseases. Heterozygous deficiency of Hif2α protects mice from developing hypoxia induced pulmonary hypertension.[56] We found that HIF2α is highly associated with human pulmonary hypertension and Congenital Diaphragmatic Hernia (CDH), which generally develop neonatal pulmonary hypertension (Chapter 3).[57] Indigenous highlanders of the Tibetan plateau live at high altitude (3,200-4,300 m) and thus under hypobaric hypoxia, but they are resistant to developing chronic mountain sickness, have thin-walled pulmonary vessels and a high blood flow. Three independent studies using genome-wide scans revealed a natural, positive selection on *EPAS1* (*HIF2a*) and *EGLN1*, indicative for adaptation of Tibetans at high altitude.[58-60] The Gly537Trp mutation in HIF2α leads to stabilization of this protein, which resulted in increased synthesis of EPO, which is associated with familial erythrocytosis, probably through the HIF2α-mediated regulation of EPO expression.[61-62] Another study indentified two new heterozygous HIF2A missense mutations, M535T, and F540L, are also both associated with erythrocytosis.[63] HIF2α was also found to be upregulated in human and rat polycystic kidney diseases, and appears to be correlated with increased EPO production.[64] Conditional ablation of Hif2α in the intestinal epithelium showed that Hif2α plays a crucial role in maintaining iron balance in mice. Hif2α can directly regulate the transcription of the principal intestinal iron transporter, the divalent metal transporter 1 (DMT1). This finding provides a basis for the development of new strategies, specifically in targeting HIF2α, to improve iron homeostasis in patients with iron disorders.[65]

Like HIF1α, HIF2α is also associated with different cancers. HIF2α is over-expressed in non small cell lung cancer in human.[35] Hif2α leads to increased tumor size, invasion, and angiogenesis in a mouse model of non small cell lung cancer.[66]

However, gene expression profile analysis of lung tissues from both human and mouse lung adenocarcinoma patients showed downregulation of human *Hif2*α mRNA.[67] Hif1α is expressed at various levels in different human breast cancer cell lines, while the expression of HIF2α was lower or even absent from the more invasive cell lines.[68] It was also shown that deletion of HIF2α in a mouse model of non small cell lung cancer resulted in increased tumorigenesis.[69] Thus, these studies suggest a complex regulation of tumor growth and invasion that is not only controlled by the absence or presence of HIF2α.

In summary, Hif2α is required for embryonic development and is association with different diseases.

HIF3α/IPAS/NEPAS

There are at least three splice variants of the *HIF3*α locus: IPAS, HIF3α and NEPAS. The splicing patterns and three protein structures are shown in Figure 3.[70] One of the three is IPAS (inhibitory PAS protein)[71], which is hypoxia inducible. IPAS lacks both the N-terminal transactivation domain (NTAD) and the C-terminal transactivation domain (CTAD) and it acts as a dominant negative regulator of Hif1α and Hif2α (Figure 3). Ectopic expression of IPAS impairs the induction of genes involved in adaption to a hypoxic environment, such as vascular endothelial growth factor (Vegf).[72]

The N-terminal domain of the HIF3α protein, another splicing variant, shares 57% and 53% amino acid sequence identity with Hif1α and Hif2α respectively, and the C-terminus of Hif3α protein shares 61% identity with Hif1α.[73] However, the transcriptional activity of the Hif3α protein is much weaker than that of Hif1α and Hif2α, because the C-terminal transactivation domain (CTAD) is absent. Reporter gene analysis shows that Hif3α suppresses the expression of hypoxia inducible genes. Thus, Hif3α acts as a competitor of Hif1α and Hif2α by recruiting the common partner Hif1β and by binding to the same HRE sequences.[74] Hif3α is expressed abundantly in lung epithelial cells (A549 cells) and the expression is induced under hypoxia in several organs including cortex, hippocampus, lung, heart, kidney, cerebral cortex.[75] [76] [77]

The other splicing variant of the Hif3α locus is NEPAS (neonatal and embryonic PAS) protein). The first eight amino acids of NEPAS are encoded by the IPAS-specific

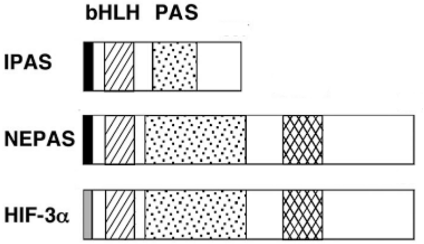

Figure 3: Comparison of NEPAS, IPAS, and HIF3α showing each splicing pattern (NEPAS, thick line; IPAS, dashed line; HIF3α, thin line) and the primary structure. bHLH, basic-loop-helix; PAS, Per-ARNT-Sim; NTAD, N-terminal transactivation domain.[70]

first exon, followed by exons 2 to 15 of the Hif3α gene. (Figure 3) It is expressed almost exclusively in the late embryonic and neonatal stages, and the expression is predominant in the lung and heart, while Hif3α mRNA is rarely detectable during embryonic and neonatal stages. Homozygous mutant mice are alive after birth, but they display enlarged right ventricle and impaired lung remodelling, which suggests that NEPAS/Hif3α is important in lung and heart development during the embryonic and neonatal stages.[70] In our studies, we found that Hif3α plays important roles in pulmonary development, especially in the regulation of epithelial cell differentiation. (Chapter 4)

Role of HIFs in lung development

Since embryonic development occurs in a hypoxic environment, it is not surprising that HIFs play important roles during embryogenesis, especially pulmonary development[2].[78] In human, *HIF1*α and *HIF2*α mRNA and proteins levels are quite high in fetal lung, and are already expressed as early as 8 weeks of gestation, which suggests that HIF1α and HIF2α proteins play critical roles during lung development. [78]

Lung bronchiolar and epithelium specific Hif1α knockout mice showed respiratory distress syndrome and die within hours after birth. Alveolar epithelial cell differentiation is impaired and a complete loss of surfactant proteins including high levels of glycogen was observed in the lungs of these mice.[19] Conditional expression of a transgenic Hif1α in pulmonary epithelium results in lung hypoplasia characterized by defective branching morphogenesis, impaired epithelial maturation, leading to severe neonatal respiratory distress. The lungs also show increased levels of glycogen in pulmonary epithelial cells, concomitant with decreased surfactant.[20] Moreover, in an experimental model of severe respiratory distress syndrome in preterm lambs a decrease of Hif1α expression is observed.[79] These findings show that Hif1α is important during lung development, and the level of protein expression must be controlled tightly.

As mentioned before, the role of Hif2α in lung development has been studied by genetic mouse models to reduce or induce the expression of Hif2α. Both Hif2α knock-out and Hif2α over-expressing mice suffered from neonatal respiratory distress. These studies showed that Hif2α plays a critical role in surfactant production and the differentiation and maturation of alveolar epithelial cells.[46-47] Another study using an experimental model of severe respiratory distress syndrome in preterm lambs also showed decreased protein expression of both Hif1α and Hif2α.[79]

In our studies, we investigated the roles of Hif2α and Hif3α during lung development by generating conditional Hif2α and Hif3α over-expressing mice in airway epithelial cells (Chapter 2 and Chpater 4). We found that Hif2α plays a critical role in surfactant production and alveolar type II cell differentiation. Hif3α impaires branching morphogenesis and inhibits the differentiation of distal epithelial cells. Hif3α appears to induce one of the proximal cell markers, Sox2, which leads to the induction of proximal cells type at the expense of the distal cells (Chapter 4).

Genes regulated by Hypoxia inducible factors

Hypoxia inducible factors (HIFs) transcriptionally regulate the expression of several target genes, and many processes involved in oxygen homeostasis are mediated by HIFs. [80]

Genes regulated by Hif1α

Hypoxia inducible factor 1α is the most extensively studied hypoxia inducible factors. It acts as a master regulator of oxygen-regulated gene expression, and genes regulated by Hif1α have shown to play important roles in various physiological pathways.[5,80-81] Hif1α target genes are involved in oxygen transport including erythropoiesis and iron transport.[80-81] Hif1α can activate erythropoietin Hep3B cells subjected to hypoxia.[82] *In vivo*, Hif1α deficiency mice show dysregulation of erythropoiesis signaling and iron homeostasis during mouse development with a decreased expression of Epo, the Epo receptor (EpoR), Hemoglobin and transferrin receptor (TfR) mRNA.[83] Other studies also showed Hif1α can transactivate transferrin (Tf) and its receptor protein.[84-86]

Hif1α also transactivates genes involved in angiogenesis and vascular regulation.. Vascular endothelial growth factor (Vegf) is one of the angiogenic factors and is found to be induced by Hif1α.[87,88] Other examples of genes directly regulated by Hif1α are Endothelin-1 (ET-1) [89], the inhibitor of plasminogen activator Pai-1, involved in several physiological and pathophysiological processes such as atherosclerosis or thrombosis.[90-91] Hif1a also regulates Adrenomedullin (ADM) which is involved in the regulation of cardiovascular tone.[92]

As shown by different studies, genes involved in glucose uptake and glycolytic pathways are ubiquitously expressed and stimulated by hypoxia.[80] So, it is not surprising that Hif1α regulates a number of genes involved in glucose metabolism, and transcriptional activates genes encoding glycolytic enzymes in hypoxic cells. Some genes encoding enzymes essential in the glycolytic pathway, such as ALDA, PGK1, enolase 1, lactate dehydrogenase A, and phosphofructokinase L (PFKL), contain Hif1α binding sites in their promoter sequences.[82,93] Hif1α deficient embryonic stem cells also showed a decreased expression of genes involved in glucose uptake such as glucose transporter 1 and 3 (Glut1 and Glut3).[94] Genes involved in glycolysis, such as Gapdh and Aldoc, also showed decreased expression in Hif1α deficient embryonic stem cells.[94] Hif1α can also transactivate fructose-2,6-bisphosphate (Pfkfb3) which is a key regulator of glycolytic flux .[95-96]

Genes regulated by Hif2α

Since Hif1α and Hif2α are structurally related, Hif2α also associates with Hif1β and transactivates target genes in common with Hif1α, but also some Hif2α-specific targets.[97-98] Like Hif1α, Hif2α can also transactivate genes involved in angiogenesis such as Vegf , Vegf receptor Flt-1 and Adrenomedullin.[97,99-100,101] Inactivation of Hif2α in cells results in a decreased expression of Vegf and its receptor.[98,102-103] These findings show that Hif2α, like Hif1α, plays an important role in the regulation of Vegf expression.

However, specific over-expressing of Hif2α in airway epithelial cells in mouse lung did not induce Vegf expression(Chapter 2),[46] suggesting that Vegf expression induced by Hif2α is cell type specific. Hif2α may also induce the expression of another angiogenic factor, Pai-1.[46,104] In human kidney cells, the expression of EPO was largely dependent on the activation of the HIF2α,[105] which was confirmed by an *in vivo* study showing that Epo is regulated by Hif2α in the mouse liver.[106]

Hif1α is associated with upregulation of glycolytic genes in multiple cell types, whereas Hif2α is not involved in regulating the glycolytic pathway.[97] However, Hif2α may still stimulate expression of the Glucose transporter 1 (Glut1) in airway epithelial cells,(Chapter 2)[46] as well as Glut3.[100] Recently, it was shown that Hif2α can induce the expression of Sox2, which suggest a putative role for Hif2α in regulation of stem cell differentiation. [107]

Genes regulated by Hif3α

As mentioned in the above section, Hif3α lacks the C terminal transactivation domain, and so the transcriptional activity is much weaker than that of Hif1α and Hif2α.[74] In cells from the mouse cornea, IPAS inhibits the expression of Vegf.[72] It has been shown that Hif3α and NEPAS repress HRE-containing promoters in transient luciferase assays. Moreover, in both NEPAS/Hif3α null mice and endothelial cells an enhanced expression of ET-1(Endotheline-1) was observed, suggesting that Hif3α may suppress the expression of ET-1.[70] Collectively, these findings resulted in the hypothesis that Hif3α acts as a competitor for Hif1α and Hif2α, since all three isoforms compete for the binding partner Hif1β and the heterodimers bind to the same promoter region. Since the Hif3α containing dimer has a much weaker transcriptional activity, it serves as a dominant negative factor of Hif1α and Hif2α.. However, since we observed that Hif3α can activate Sox2 gene, which is also one of the target genes of Hif2α, Hif3α may activate specific targets in some cases.(Chapter 4)

Hypoxia inducible factors (Hif1α, Hif2α and Hif3α) play important roles in different physiological processes in response to hypoxia. Both Hif1α and Hif2α are well studied proteins and have major impact in cancer and vascular diseases.[5] As mentioned above, all of the three proteins may have important functions during lung development and disease. Hif2α contributes to alveolar type II cells maturation and differentiation during lung development (Chapter 2), and Hif3α inhibits alveolar epithelial cells differentiation. (Chapter 4)

SCOPE OF THIS THESIS

It was shown previously that HIFs are expressed in lung development.[2] Hif2α displays a very specific expression pattern, being increasingly expressed towards the end of gestation. Our preliminary data showed that the expression of Hif2α resided in the endothelial cells, as well as the type II pneumocytes. Therefore, we wanted to analyse the function of Hif2α in type II cells using an inducible transgenic approach. We found that Hif2α is required for the terminal differentiation of type II cells as well as the production of certain phospholipids of the pulmonary surfactant (chapter 2).

Next, we were interested to analyze the expression of HIF2α protein in samples from patients who suffered from persistent pulmonary hypertension of the newborn (PPHN), since it was published that Hif2α may contribute to the development of pulmonary hypertension. We indeed found a number of cases of PPHN with elevated levels of HIF2α. (Chapter 3)

In addition to studying the role of Hif2α, we also analyzed the potential role of Hif3α as a modulator of the hypoxia response in type II pneumocytes. We applied the same inducible transgenic approach as for our Hif2α studies, and found that the forced expression of Hif3α in the embryonic pulmonary epithelium resulted in a significant inhibition of the formation of distal epithelium at the expense of proximal epithelium (chapter 4).

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CHAPTER 2

HYPOXIA-INDUCIBLE FACTOR 2α PLAYS A CRITICAL ROLE IN THE FORMATION OF ALVEOLI AND SURFACTANT

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Hypoxia-Inducible Factor 2α Plays a Critical Role in the Formation of Alveoli and Surfactant

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Alveolarization of the developing lung is an important step toward the switch from intrauterine life to breathing oxygen-rich air after birth. The distal airways structurally change to minimize the gas exchange path, and Type II pneumocytes increase the production of surfactants, which are required to reduce surface tension at the air–liquid interface in the alveolus. Hypoxia-inducible factor 2α (Hif2α) is an oxygen-regulated transcription factor expressed in endothelial and Type II cells, and its expression increases toward the end of gestation. We investigated the role of Hif2α in Type II cells by conditionally expressing an oxygen-insensitive mutant of Hif2α in airway epithelial cells during development. Newborn mice expressing the mutant Hif2α were born alive but quickly succumbed to respiratory distress. Subsequent analysis of the lungs revealed dilated alveoli covered with enlarged, aberrant Type II cells and a diminished number of Type I cells. The Type II cells accumulated glycogen in part by increased glucose uptake via the up-regulation of the glucose transporter 1. Furthermore, the cells lacked two crucial enzymes involved in the metabolism of glycogen into surfactant lipids, lysophosphatidylcholine acyltransferase and ATP-binding cassette sub-family A member 3. We conclude that Hif2α is a key regulator in alveolar maturation and the production of phospholipids by Type II cells.

Keywords: Hif2α; lung development; surfactant

CLINICAL RELEVANCE

Our research contributes to the understanding of Type II cell biology. Moreover, it may contribute to an understanding of the origin of certain surfactant deficient diseases.

Lung development begins as an endodermal bud invades the surrounding mesenchyme as early as 4 weeks of gestation in humans and at 9.5 days of gestation in mice. This primitive bud then branches and grows in a repetitive manner to form the basic architecture of the bronchial tree, as reviewed by Morrissey and Hogan (1). During development, a vascular network is present from the earliest onset of primitive lung-bud formation, and it gradually expands with the branching airways (2). The transition at birth from prenatal, fluid-filled lungs to postnatal, air-filled lungs requires a thinning of peripheral lung tissue by dilatation of the alveoli and a closer association of the pulmonary capillaries with epithelial cells, to facilitate optimal gas exchange. Aside from these structural adaptations to life, the epithelial cells of the lung start producing surfactant to reduce surface tension at the air–liquid interface in the lungs. Surfactant is also required to protect the lung from exposure to the environment, and thus serves as a defense mechanism. Surfactant consists of phospholipids and four apolipoproteins, surfactant-associated proteins A, B, C, and D (SP-A to SP-D, respectively), which are primarily produced by Type II pneumocytes. The importance of surfactant is clear in newborns with untreatable respiratory distress syndrome, which led to the identification of several mutations in different genes (3).

Hypoxia-inducible factors (HIFs) are critical transcription factors in the cellular response to hypoxia. They regulate the expression of genes involved in angiogenesis, metabolism, and cell homeostasis. HIFs are heterodimers composed of two structurally related subunits, an oxygen-sensitive Hifα subunit and a constitutively expressed Hif1β/aryl hydrocarbon receptor nuclear translocator subunit (4). Three structurally related Hifα proteins have been characterized (Hif1α, Hif2α or Epas1, and Hif3α), which have two critical proline residues that are hydroxylated by one of three prolyl hydroxylases (Egln1–3) under normoxic conditions, leading to the poly-ubiquitinylation and proteasomal degradation of the Hifα subunit through the von Hippel-Lindau tumor suppressor pathway (5). However, under hypoxic conditions, the Hifα subunits are stable and dimerize with Hif1β to regulate downstream target genes by binding to hypoxia-responsive elements (consensus RCGTG) (6). Although Hif1 (i.e., the Hif1α/Hif1β dimer) and Hif2 (i.e., the Hif2α/Hif1β dimer) activate common target genes, they also activate specific sets of genes (7–9). The transcriptional activity of the Hif3α protein is weak, because the C-terminal transactivation domain is absent (10).

The importance of different Hifα factors was established by gene ablation studies. Hif2α null mice display a pleiotropic phenotype ranging from premature death to postnatal abnormalities, depending on the background of the murine strain (11–14). Interestingly, the inactivation of Hif2α resulted in respiratory distress in newborns, and heterozygous Hif2α mice are protected from developing pulmonary hypertension upon exposure to mild hypoxia (13, 15). An endothelial-specific Hif2α knockout mouse showed increased vessel permeability, indicating that Hif2α is required for the stabilization of vascular integrity. Hif2α is expressed in distinct cell types, such as vascular endothelial cells, kidney fibroblasts, liver cells, and epithelial cells of the intestine and heart (16, 17). In the lung, Hif2α is primarily expressed in endothelial cells, bronchial cells, and Type II pneumocytes (18). We recently described the increase of Hif2α

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mRNA expression in the human lung during different gestational stages (19).

Because Hif2α is expressed not only in vascular endothelial cells, but also in the developing airways and Type II pneumocytes, we investigated the precise role of Hif2α in Type II cells by generating an inducible Hif2α transgene. To study the effects of Hif2α independent of oxygen level, we used a previously described stable human HIF2α containing two amino-acid substitutions (P531A and N847A) (20). Mice expressing this mutant HIF2α (mutHIF2α) transgene in developing airways showed dilated alveolar structures, and newborns suffered from respiratory distress. The mutHIF2α-expressing Type II pneumocytes appeared immature, and maintained vast amounts of glycogen, a precursor of phospholipids. Moreover, the distal airways contained a significantly reduced number of Type I cells. Hif2α induced genes involved in the glucose synthetic pathway, such as glucose transporter 1 (*Glut1*) and UTP-glucose-1-phosphate uridylyltransferase(*Ugp2*), and mutHIF2α-expressing Type II pneumocytes lacked lysophosphatidylcholine acyltransferase and ATP-binding cassette sub-family A member 3 (Abca3), two important enzymes in the production of phospholipids. We hypothesized that Hif2α prepares Type II cells toward the end of gestation to produce surfactant lipids by stimulating the accumulation of glycogen and gradually increasing the proteins involved in producing phospholipids.

MATERIALS AND METHODS

Generation of Transgenic Animals

The myc epitope sequence was cloned after the endogenous ATG codon of the full-length cDNA encoding a constitutively active, oxygen-insensitive mutant human HIF2α (mutHIF2α; P531A and N847A; a generous gift of Daniel J Peet) (20), and subcloned into a modified pTRE-Tight vector (Clontech, Leusden, The Netherlands). Transgenic lines were produced by a pronuclear injection of FVB/N fertilized eggs, and initially three independent lines were analyzed, which all produced identical phenotypes. Mice were kept under standard conditions, and all experiments were performed according to the guidelines of the local ethics committee. Lung-specific expression of the myc-mutHIF2α transgene was obtained by crossing the myc-mutHIF2α lines with SPC-rtTA transgenic mice (a gift of Jeffrey Whitsett) (21). The administration of doxycycline to pregnant mothers from gestational age 6.5 days onward in the drinking water (2 mg/ml 5% sucrose) resulted in lung epithelium–specific expression. Each experiment was performed with at least three independent litters containing double-transgenic, single-transgenic, and wild-type pups that all shared the FVB/N genetic background.

Histochemistry

Fetal lungs were dissected and fixed in formal saline overnight at 4°C by direct immersion, whereas the lungs of newborn mice were gently fixed under pressure. Next, the lungs were processed and paraffin-embedded, according to routine protocols. For periodic acid–Schiff (PAS) staining, sections were rehydrated and incubated with 1% periodic acid (Klinipath, Duiven, The Netherlands), followed by incubation with Schiff's reagents (Klinipath). Sequential sections were incubated with 0.1% amylase before proceeding with the PAS staining. Immunohistochemistry was essentially performed as previously described, with the antibodies Myc (Roche, Mannheim, Germany), β-tubulin IV (bioGenex, San Ramon, CA), pro-SPC (Chemicon, Amsterdam, The Netherlands), T1α (University of Iowa Hybridoma Bank, Iowa City, IA), TTF (Thermo, Amsterdam, The Netherlands), CCSP (rabbit polyclonal, kindly provided by B. Stripp), Glut1 (Abcam, Cambridge, UK), LPCAT1 (Seven Hills Bioreagents, Cincinnati, OH), ABCA3 (Seven Hills Bioreagents), and SMA (Thermo) (22).

Lungs were imaged using an Olympus BX41 microscope and DP71 camera (Olympus, Zoeterwoude, The Netherlands). Subsequent measurements of alveolar size and cell counting were performed with SIS Software Cell D (Olympus) Three independent samples of control and double-transgenic lungs were used to measure 10 randomly selected airspaces, and the average area of the airspaces was calculated.

Microarray Analysis

Lungs of three control and three double-transgenic male embryos were dissected on Embryonic Day 18.5, and the middle and caudal lobes were used for the isolation of total RNA with Trizol reagent (Invitrogen, Breda, The Netherlands). cDNA was synthesized using the GeneChip Expression 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix, Santa Clara, CA). Biotinylated complementary RNA probes were generated and hybridized onto Affymetrix Mouse Genome 430 2.0 microarray chips, according to standard conditions. After normalization, the data were analyzed with OmniViz software, version 3.6.0 (Omniviz, Inc., Maynard, MA).

Quantitative PCR

The isolation of RNA and subsequent quantitative PCR analysis was essentially performed as previously described (19). The gene-specific primers used in this study are shown in Table E1 in the online supplement.

RESULTS

Conditional Expression of mutHIF2α in Lungs Leads to Respiratory Distress in Neonatal Pups

The importance of Hif2α during vascular development was shown previously by gene ablation studies in mice (12, 23, 24). In addition to vascular defects, other organs such as the lungs are affected by the loss of Hif2α, depending on the genetic background (13, 14). However, the precise role of Hif2α during the formation of the lung is not fully understood. The Hif2α protein becomes significantly expressed during late phases of development in alveolar Type II pneumocytes (Figure E1A), confirming previous findings in the Hif2α knockout model (13) and our own findings in human tissue samples (19).

To determine the precise role of Hif2α in the epithelium during lung development, and more specifically in Type II pneumocytes, we generated transgenic mice expressing a myc-epitope tagged HIF2α under the control of a doxycycline-inducible tet-on promoter (Figure E1B). We used an oxygen-independent, constitutively active mutant HIF2α containing two amino-acid substitutions at critical regulatory residues (mutHIF2α; P531A and N847A) (20), to create an artificial hypoxic environment in cells that express this protein. The expression of mutHIF2α in embryonic lung epithelia was established by crossing the mutHIF2α transgenic line with the SPC-rtTA line, which drives the expression of the rtTA gene in epithelial cells of the embryonic lung (21). Lungs isolated from noninduced or doxycycline-induced single mutHIF2α or SPC-rtTA transgenic mice, or lungs from noninduced double-transgenic mutHIF2α/SPC-rtTA double-transgenic animals, appeared indistinguishable from normal control lungs (Figure E2). Pregnant females from timed matings between SPC-rtTA and mutHIF2α mice received doxycycline to induce the expression of the mutHIF2α transgene in double-transgenic fetuses. Double-transgenic pups were born at a Mendelian ratio, but mutHIF2α-expressing mice readily suffered from respiratory distress, as demonstrated by their cyanotic appearance, and these pups succumbed within 6 hours after birth. This indicated that the gas exchange surface area was sufficiently developed, but the pups most likely suffered from surfactant deficiency.

Expression of mutHIF2α Causes Alveolar Dilatation

To determine how the perinatal lethality developed, we analyzed lungs of double-transgenic animals and control lungs at different gestational ages. Macroscopic analyses of isolated lungs did not show clear abnormalities in double-transgenic animals at gestational ages 14 and 16 days (Figure E1). Starting after gestational age 16 days, significantly dilated alveolar structures were evident macroscopically in the lungs of double-transgenic animals (Figures 1A and 1C versus Figures 1E and 1G). Histological examinations of these developmental stages showed enlarged alveolar spaces in mutHIF2α-expressing lungs (Figures 1B and 1D versus Figures 1F and 1H). Quantification of the alveolar spaces revealed a significant increase of distal surface area in double-transgenic animals both on Embryonic Day 18.5 and postnatally (Figures 1I and 1J). Staining with a myc-epitope–specific antibody confirmed the expression of transgenic mutHIF2α protein in the epithelium of double-transgenic lungs (Figures 1F and 1H and their *insets*). Although mutHIF2α-expressing cells were present in double-transgenic lungs at earlier gestational ages, they did not cause macroscopic alterations at these stages (Figure E1).

Because mutHIF2α-expressing lungs showed abnormal dilated alveoli, and newborns readily succumbed to respiratory distress, transgenic lungs were analyzed with cell-specific markers to investigate the integrity and differentiation of fetal lungs. Thyroid transcription factor (Ttf1) was expressed in nearly all epithelial cells in both control and double-transgenic lungs (Figures E3A and E3B). Also, the vasculature (CD31) and smooth muscle cell component of the mesenchyme (Sma) did not reveal striking differences (Figures E3C–E3H). The ciliated (β-tubulin) and Clara cells (CCSP) of the proximal conducting airways were normally present (Figure E4), as were distal airway Type I (T1α) and Type II pneumocytes (Sftpc; Figures 2A–2D). However, we observed clear, aberrant Type II epithelial cells in the dilated alveolar structures that were positive for Ttf1 and Sftpc (*arrows* in Figures E3B and E2D). Detailed analysis of these cells revealed an enlarged cytoplasm, as indicated by toluidine blue staining (Figures 3B and 3D). These cells appeared to express the transgenic mutHIF2α protein, as shown by positive staining for the myc epitope (Figure 3C). The mutHIF2α transgene was expressed in distal epithelial cells, and although the differentiation potential of the epithelial cells was not affected, we quantified the number of differentiated Type I and Type II cells. Real-time PCR analysis showed a clear decrease in expression of the Type I cell–specific *Aqp5* gene, whereas the expression of the Type II cell–specific *Sftpb* gene was comparable to the control sample in reference to the general epithelial marker gene *Ttf1* (Figure 2E). The latter was confirmed by counting the number of Sftpc-positive cells (Figure 2F). In addition, the expression of two other surfactant-associated proteins, *Sftpa* and *Sftpc,* was also unaffected (Figure E5). Collectively, these findings indicate that the expression of the mutHIF2α transgene leads to a reduction in number of Type I cells, suggesting that Type II cells are blocked in their differentiation to Type I cells.

The respiratory distress of the newborns, combined with the abnormal appearance of Type II cells, warranted ultrastructural analysis. Electron microscopy showed Type II pneumocytes in control lungs with lamellar bodies, which are indicative of secretory organelles rich in phospholipids (Figure 3G). However, in double-transgenic Type II cells, lamellar bodies were less frequent and varied in shape (Figures 3H–3J). This suggested that the production or secretion of phospholipids was hampered by the expression of mutHIF2α in Type II cells. The neonatal death and appearance of enlarged alveolar epithelial cells suggested that the production of surfactant was affected. Therefore, we performed PAS staining on both control and double-transgenic lungs of neonatal pups. In control lungs, only the cells of the upper, conducting airways contained PAS-positive material (Figure 4A), whereas in double-transgenic lungs, alveolar epithelial cells also contained PAS-positive material (Figure 4B). When sequential sections were treated with amylase, the pink PAS-

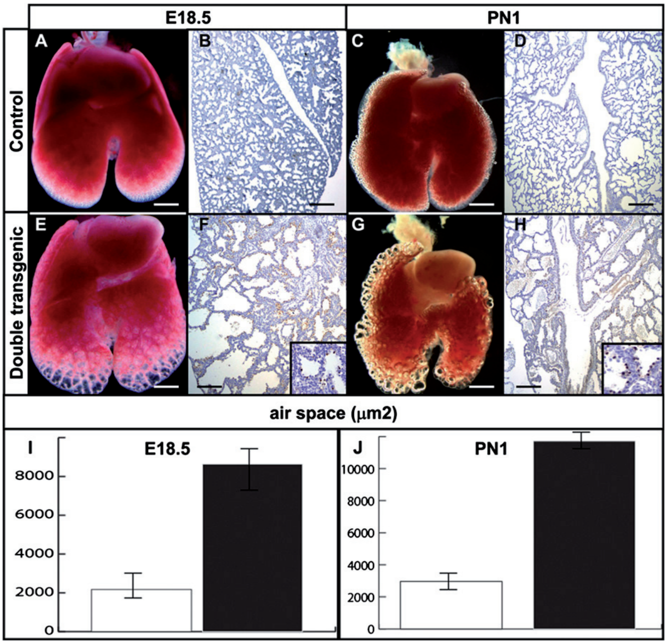

Figure 1. The expression of mutant hypoxia-inducible factor–2α (mutHIF2α) leads to abnormal alveolar spaces. External appearances of control (*A* and *C*) and mutHIF2α-expressing lungs (*E* and *G*) at two different gestational ages display enlarged airspaces late in gestation. Analysis of transgenic Hif2α expression (Myc epitope) clearly showed expression in double-transgenic lungs (*F* and *H*), which is absent in control lungs (*B* and *D*). *Insets*: Higher magnification, to clarify myc-positive cells. *Scale bars*, 2 mm (*A*, *C*, *E*, and *G*) or 500 μm (*B*, *D*, *F*, and *H*). The alveolar spaces in mutHIF2α-expressing lungs were significantly increased compared with control lungs (*I* and *J*). The alveolar space area of mutHIF2α lungs on Embryonic Day (E) 18.5 is 8,633 ± 1,000 μm^2, versus 2,171 ± 600 μm^2 for control lungs ($n = 3$ each, $P = 0.002$). On Postnatal Day (PN) 1, the area of alveolar space in mutHIF2α lungs is 11,709 ± 300 μm^2, versus 2,961 ± 400 μm^2 for control lungs ($n = 3$ each, $P = 0.018$).

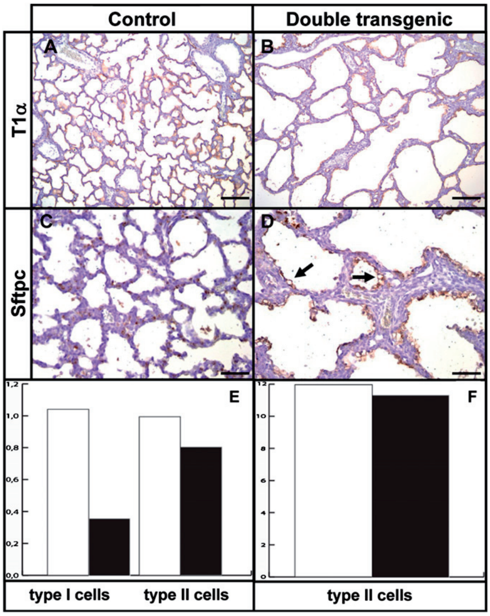

Figure 2. Expression of lung-specific cell markers in mutHIF2α-expressing lungs. The expression of T1α (*A* and *B*) and Pro-SPC (Sftpc; *C* and *D*) was determined in control and mutHIF2α double-transgenic lungs isolated 4 hours after birth. The sites and extent of expression are comparable between control lungs (*A* and *C*) and mutHIF2α double-transgenic lungs (*B* and *D*). Note the dilated alveolar spaces and enlarged cells in mutHIF2α double-transgenic lungs, which are also positive for Sftpc (*arrows* in *D*). *Scale bars*, 100 μm (*A–D*). (*E*) The number of Type I cells was significantly decreased, as monitored by the decreased expression of Type I cell–specific marker aquaporin 5 (Aqp5), compared with the general epithelial marker Ttf1 (0.36 ± 0.1 versus control 1.04 ± 0.2, $n = 3$ each, $P = 0.017$), whereas the expression level of Type II cell marker Sftpb is similar between control and mutHIF2α double-transgenic lungs when compared with the expression of Thyroid transcription factor (Ttf1) (0.8 ± 0.3 versus control, 1 ± 0.06; $n = 3$ each). (*F*) Counting the number of positive Sftpc cells by immunohistochemistry confirmed the result obtained by quantitative PCR, showing comparable numbers of Type II cells between control and double-transgenic animals.

positive staining disappeared (*arrows* in Figures 4E–4G), indicating that the majority of PAS-positive material in these cells was glycogen. In addition, staining the sequential section with an antibody against the myc-epitope confirmed that the PAS-positive, glycogen-rich cells are indeed mutHIF2α-expressing cells (Figures 4E–4G). Thus, the expression of mutHIF2α in Type II pneumocytes leads to the accumulation of glycogen.

Expression of mutHIF2a in Type II Cells Leads to Aberrant Surfactant Production

Three mutHIF2α-expressing lungs and three control lungs were processed at gestational age 18.5 days for microarray analysis, to elucidate the origin of the aberrant alveolar structures and Type II cell abnormalities caused by mutHIF2α. We first analyzed the expression of the best established downstream target gene of the Hif transcription factor family, vascular endothelial growth factor (VEGF). However, both the microarray and quantitative PCR did not show significant up-regulation of VEGF in mutHIF2α-expressing lung tissue (Figure E5). Because we used total lung tissue for the microarray and quantitative PCR analysis, we investigated whether the expression of VEGF was locally up-regulated in the lung. Therefore, we performed immunohistochemistry to examine the expression of VEGF protein in neonatal and newborn lungs, but no significant changes were observed (Figure E6).

Next, a select number of genes that were specifically changed in mutHIF2α-expressing lungs are listed in Tables E2A and E2B.

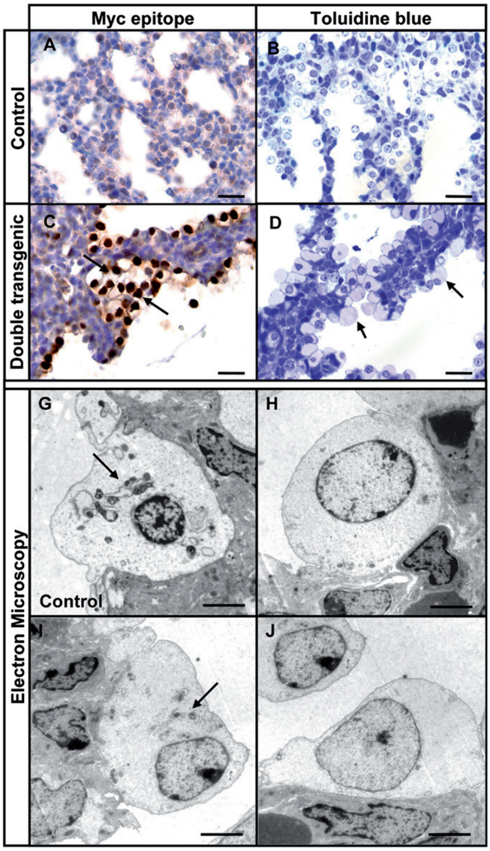

Figure 3. mutHIF2α induces abnormal Type II pneumocytes with variable lamellar bodies. Transgenic mutHIF2α induces enlarged Type II pneumocytes on Embryonic Day 18.5, which are positively stained with the myc-epitope antibody (*arrows* in *A* and *C*), and are readily apparent upon toluidine blue staining of ultrathin sections (1 μm) when control lungs (*B*) are compared with double-transgenic (*D*) lungs. Electron microscopic images of control alveolar epithelial cells on Embryonic Day 18.5 shows normal presence of lamellar bodies (*arrow* in *G*), whereas the lamellar bodies in Hif2α-overexpressing cells appear less frequent and variable in shape (*arrows* in *H–J*). *Scale bars*, 50 μm (*A–D*), 1.4 μm (*G*), or 1.9 μm (*H–J*).

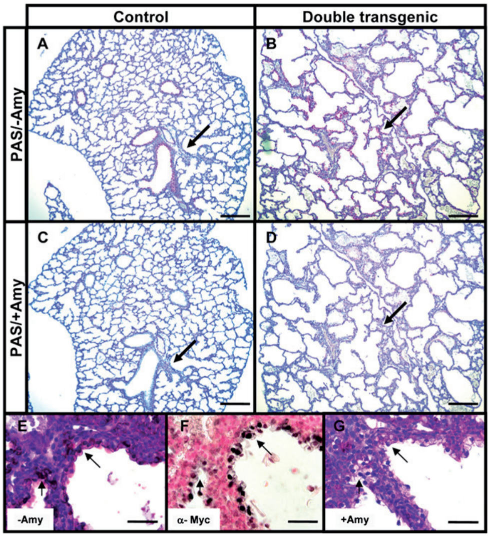

Figure 4. Hif2α induces the accumulation of glycogen in Type II pneumocytes. Periodic acid–Schiff (PAS) staining of mutHIF2α double-transgenic and control lungs of newborns. In control lungs, pink PAS-positive material is present in cells of the proximal upper airways (*arrow* in *A*), whereas in Hif2α double-transgenic lungs, pink PAS-positive material is also evident in distal alveolar epithelial cells (*arrow* in *B*). Sequential sections treated with amylase (PAS/+Amy) loosed the PAS-positive material in both control lungs (*arrow* in *C*) and mutHIF2α double-transgenic lungs (*arrow* in *D*), indicating that the pink material is glycogen. High-power images of sequential sections show that the PAS-positive, glycogen-rich cells in double-transgenic lungs (*E* and *F*) express transgenic mutHif2α (α-Myc; *G*). *Scale bars*, 500 μm (*A*–*D*) or 100 μm (*E*–*G*).

As expected, we found a number of genes previously reported to be induced directly by either hypoxia or Hif2α, such as *Pai1*, *Egln3*, *Sc2A1*, *Adm*, *Gbe1*, and *Plod2* (7, 25). In support of these microarray results, we validated some of the genes by quantitative PCR (Figure E5A). We also analyzed the expression of *Hif1α* and some of its direct targets, *Gys1*, *Pdk1*, *Pfkfb4*, and *Aldoc*, but found no changes in level of expression compared with control samples (Figure E5B). These findings affirm that the observed phenotype is caused by genes (directly) downstream of Hif2α, and is not the result of compensation by Hif1α. Aside from being direct targets of Hif1α, Pdk1, Pfkfb4, and Aldoc are also important enzymes involved in glycolysis. Therefore, the expression of mutHif2α did not influence the glycolytic pathway, supporting the increased storage of glycogen.

Because we showed that mutHIF2α-expressing cells stored large quantities of glycogen (Figure 4), we analyzed the expression of Glut1 (Slc2a1), a glucose transporter directly induced by Hif2α (8, 26). Both the microarray and quantitative PCR analysis of control and double-transgenic lungs showed a clear up-regulation of *Glut1* by the Hif2α transgene (Figure E5A). Moreover, the number of Glut1-expressing epithelial cells was increased in lungs of double-transgenic animals relative to control lungs (Figures 5A and 5B; for overview, see Figure E7). Staining sequential sections with the myc-epitope antibody indicated that many mutHIF2α-expressing cells were also Glut1-positive (Figures 5C and 5D). Furthermore, we found that *Ugp2*, a gene involved in the biosynthesis of glycogen, was up-regulated both in the microarray analysis and by quantitative PCR (Figure E5A). We concluded that the expression of mutHIF2α in Type II cells shifts glycogen metabolism toward an increased generation and storage of glycogen.

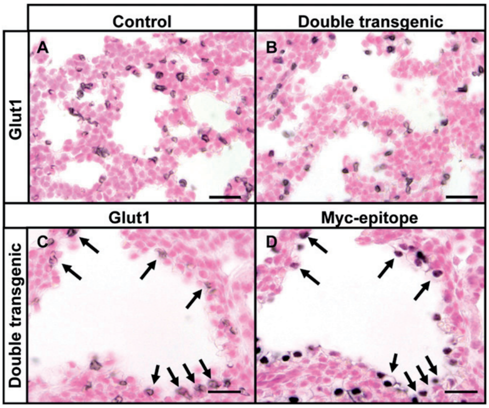

Figure 5. Hif2α induces the expression of Glut1 in Type II pneumocytes. The expression of glucose transporter 1 (Glut1) was determined in mutHIF2α double-transgenic newborn lungs (*B*) and control lungs (*A*). The colocalization of Glut1 and transgenic mutHIF2α was shown by staining sequential sections with specific antibodies for Glut1 (*arrows* in *C*) and the Myc-epitope (*arrows* in *D*). Mark the positive erythrocytes in *A* and *B* which express Glut1 as well. *Scale bars*, 100 μm (*A* and *B*) or 50 μm (*C* and *D*).

Next, we analyzed the expression of genes and proteins known to be involved in the production of lipid components of the surfactant. Two genes involved in surfactant synthesis, *Lpcat1* and *Abca3*, were down-regulated in lungs of double-transgenic animals according to microarray and quantitative PCR (Figure E5C). Abca3 is an ATP-binding cassette transporter expressed in lamellar bodies of Type II pneumocytes (27), and Lpcat1 converts unsaturated phosphatidylcholine into dipalmitoylphosphatidylcholine (DPPC), the main phospholipid responsible for reducing surface tension in the lung (28). Lpcat1 and Abca3 are mainly expressed in alveolar epithelial Type II cells of control lungs (Figures 6A and 6B, *arrows*; for overview, see Figure E7), whereas almost no expression of Lpcat1 and Abca3 can be detected in the alveolar epithelial cells of mutHIF2α-expressing lungs (Figures 6C and 6D). Sequential sections showed that myc-positive, mutHIF2α-expressing cells are devoid of Lpcat1 and Abca3 (Figures 6C–6F, *arrows*), whereas cells that did not express mutHif2α expressed both proteins (Figures 6C–6F, *asterisks*). Other genes, such as stearoyl-CoA desaturase (*Scd1*), *Fasn*, and choline–phosphate cytidylyltransferase 1α (*Pcyt1A*), are implicated in the phospholipid metabolic pathway and were also down-regulated by microarray analysis, and confirmed by quantitaive PCR (Figure E5C). *Scd1* catalyzes the biosynthesis of monounsaturated fatty acids required for the synthesis of triglycerides, cholesteryl esters, and phospholipids (29, 30). *Fasn* encodes a fatty-acid synthase, which catalyzes in the presence of nicotinamide adenine dinucleotide phosphate–reduced the synthesis of palmitate from acetyl–CoA and malonyl–CoA (31). *Pcyt1A* is a key enzyme involved in the *de novo* synthesis of phosphatidylcholine (32). Collectively, these data show that expression of

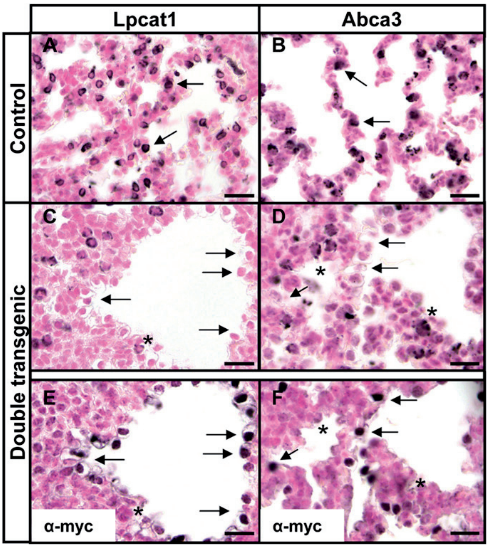

Figure 6. Hif2α hampers the expression of crucial phospholipid metabolic enzymes. The expression of lysophosphatidylcholine acyltransferase (Lpcat1) (*A* and *C*) and ATP-binding cassette sub-family A member 3 (Abca3) (*B* and *D*) was reduced in double-transgenic neonates (*C* and *D*), compared with control mice (*A* and *B*). Staining sequential sections with the Myc-epitope antibody (*E* and *F*) revealed that transgenic mutHIF2α-expressing Type II cells lost expression of Lpcat1 (*arrows* in *C* and *E*) and Abca3 (*arrows* in *D* and *F*), whereas cells that lacked mutHIF2α expressed Lpcat1 and Abca3 (*asterisks*). *Scale bars,* 100 μm (*A* and *B*) or 50 μm (*C–H*).

mutHIF2α in Type II pneumocytes leads to a down-regulation of important genes involved in the metabolism of surfactant lipids, which explains the observed neonatal death of double-transgenic animals. The phospholipid composition of total newborn lungs was measured, and although no difference was evident in the DPPC (16:0) content, we did notice significant changes in unsaturated lipids that serve as substrates for Lpcat1 (Figure E8).

Hif2α knockout animals on a specific C57BL6 background survived, but showed multiple organ pathology as a result of mitochondrial dysfunction, primarily at sites with high energy demand, such as skeletal muscle and liver (14). The Juvenile mice demonstrated a high mortality rate of unknown cause, but unrelated to pulmonary dysfunction. To investigate a possible dysfunction of bioenergetics in mutHIF2α-expressing lungs, we analyzed the expression of genes encoding key proteins in energy metabolism, such as *Sod2*, *Fxn*, *Cycs*, *Aco1*, and *Ogdh* (Figure E5D). Both the microarray and quantitative PCR analysis did not show significant changes in expression levels between control and double-transgenic animals of these genes, suggesting that mitochondrial function in mutHIF2α-expressing mice is normal.

DISCUSSION

The transition from a fluid-filled lung to a functional gas-exchanging organ at birth requires the expansion of the alveolar space and a reduction of the blood-gas barrier. Type II pneumocytes increase the production and secretion of surfactants, to reduce surface tension in the alveoli. Type II cells also play an important role in the maintenance of distal airways, and serve as precursor cells for Type I cells, which are closely associated with vascular endothelial cells for optimal gas exchange. The failure to maintain surfactant homeostasis in the alveoli results in acute as well as chronic respiratory problems. Mutations in a number of surfactant-related genes, such as *ABCA3*, *SFTPA*, *SFTPB*, and *SFTPC* were found in human newborns suffering from surfactant deficiency (3). Here we describe the involvement of Hif2α in the production of phospholipids, the major constituent of surfactant, as well as a mediator in the differentiation of Type II pneumocytes of the distal airway epithelium.

Mice expressing an oxygen-insensitive, mutant Hif2α in epithelial cells were born at a normal Mendelian ratio, but succumbed within hours of respiratory distress. Macroscopic analyses of the lungs showed an apparently normal bronchial tree, but with dilatated alveolar spaces. Recently, a murine model of bronchopulmonary dysplasia has been described which had enlarged alveoli combined with a thickening of the septal walls (33). Although we also observed significantly larger airspaces, we could not determine significant differences in the thickness of septa between control and double-transgenic lungs. Subsequent microscopic analysis revealed numerous alveolar Type II cells with an enlarged cytoplasmic appearance in the epithelia of mutHIF2α-expressing lungs. Furthermore, the total number of Type II cells was not affected, but the number of Type I cells was significantly reduced. Because Type II cells serve as precursors for Type I pneumocytes, this finding supported our observation that Type II cells are blocked in their terminal differentiation. The expression of the mutHIF2α transgene already started early during gestation, which may have induced immature epithelial cells to differentiate prematurely into the Type II pneumocyte pathway, leading to the untimely formation of alveolar structures. This would explain why mutHIF2α-expressing transgenic mice have a reduced number of enlarged alveolar spaces.

Because VEGF is one of the primary targets of HIF factors and was previously implicated in the Hif2α knockout lung phenotype (13), we first analyzed the expression of VEGF. Surprisingly, we did not observe alterations in the expression level of VEGF or in the cellular distribution. In contrast with the knockout, which inactivates the *Hif2α* gene in all cells, the mutHIF2α is expressed specifically in early embryonic epithelium, and is later restricted to Type II cells. The *Vegf* gene could be subject to additional regulatory factors in Type II cells that prevent its overexpression. Additionally, the block in Type II cell terminal differentiation may also affect the expression of *Vegf*. Moreover, the expression of mutHIF2α is induced much earlier than is the normal expression of Hif2α, causing an artificial prolonged hypoxia, possibly resulting in the loss of *Vegf* induction, as shown for rats (34). Although we did not find changes in the expression level of *Vegf*, we observed significant differences in other targets of Hif2α, such as *Pai1*, *Egln3*, *Sc2A1*, *Adm*, *Gbe1*, and *Plod2*.

Close examination of mutHIF2α-expressing lungs showed that enlarged Type II pneumocytes maintained vast amounts of glycogen after birth. Normally, Type II pneumocytes before birth contain glycogen, which is the major substrate for pulmonary surfactant synthesis. It is rapidly converted postnatally into phospholipids, mainly phosphatidylcholine (35, 36), which are primarily required to reduce surface tension at the air–liquid interface of alveoli. After birth, alveolar cells are exposed to air, which causes a degradation of the α subunit of Hif2. However, mutHIF2α is stable, even under normoxic conditions, and thus activates the transcription of hypoxic-responsive genes postnatally. Indeed, the glucose transporter *Glut1* is up-regulated in newborn mice, and is localized to the membrane of mutHIF2α-positive Type II pneumocytes, in agreement with previous findings that hypoxia-inducible factors can induce the accumulation of glycogen (37). Interestingly, we also detected a significant increase in the level of *Ugp2* mRNA, which encodes for an enzyme involved in the biosynthesis of the glycogen precursor UDP–glucose (38). Thus, both the intake of glycogen and the production of intracellular glycogen are increased in Type II cells, causing an overload of the glucose metabolic system. Glycogen is processed to produce the glycerol backbone for phospholipids. Previously, links between glucose metabolism and respiratory distress were indicated by an increased incidence of respiratory distress syndrome in infants of diabetic mothers (39, 40). Furthermore, glucose infusion experiments with fetal lamb lungs showed a decrease of pulmonary disaturated phosphatidylcholine. This suggested that effects on surfactant regulation may be associated with glycogen regulation due to increased substrate influx into the glycogen synthetic pathway (41, 42). Because glycogen is the major source for pulmonary surfactant synthesis in Type II pneumocytes, these findings are in concordance with our results that Hif2α up-regulates glycogen metabolic enzymes such as *Glut1* and *Ugp2* (36).

In addition to the accumulation of glycogen in Type II cells, genes involved in the production of phospholipids, such as *Lpcat1* and *Abca3*, were down-regulated in mutHIF2α-expressing mice. Lpcat1 is involved in the phospholipid remodeling pathway to generate saturated phosphatidylcholine, and is up-regulated toward the end of gestation in Type II cells (28, 43, 44). Abca3, an ATP-binding Cassette family member present in the membrane of lamellar bodies, is required for the vesicular uptake of surfactant lipids, such as phosphatidylcholine, sphingomyelin, and cholesterol. It is also up-regulated in Type II cells upon the end of gestation (45, 46). Both Lpcat1 and Abca3 play critical roles in the adaptation of the lung to gas exchange, since gene ablation studies for both genes produced lethargic mice that failed to thrive (27, 28). The phenotypes associated with the ablation of either Lpcat1 or Abca3 resemble the phenotype of mutHIF2α-expressing mice. Previous reports showed that this finding correlated with a severe reduction or depletion of saturated phosphatidylcholine and phosphatidylglycerol (27, 28). As shown by staining for the myc-epitope, not all Type II cells express the transgene. This incomplete penetrance of transgene expression was observed previously, and most likely reflects the variegated expression of the rtTA protein under the influence of the SPC promoter (22). This may explain why mutHIF2α-expressing mice do not deteriorate immediately after birth, but gradually succumb within 4 to 6 hours. It could also explain the variation in lamellar bodies observed by electron microscopy which resembles the pleiotropic phenotype of incomplete Lpcat1 gene trap inactivation (28). We also found that the gene encoding for the rate-limiting step in *de novo* phosphatidylcholine synthesis, *Pcyt1a*, was significantly down-regulated in mutHIF2α-expressing lungs. Like the Lpcat1 and Abca3 mice, gene ablation of *Pcyt1a* resulted in neonatal respiratory distress (47). Because important enzymes involved in the production of surfactant phospholipids are influenced by Hif2α, we suggest that during the last phases of embryonic development, Hif2α is required in Type II cells to prepare the cellular metabolism for the sudden adaptation to oxygen breathing. Lipids and their precursors accumulate gradually during development to prepare for the first breath, and prenatally mutHIF2α-expressing Type II cells normally accumulate lipids. However, the maintenance of hypoxic conditions by the continuous expression of mutHIF2α after birth causes disturbances in lipid metabolism, leading to death within hours. Measurement of the phospholipid composition of total newborn lungs did not show significant differences in the DPPC (16:0) content immediately after birth. Most likely this occurred because newborn lungs were isolated for lipid analysis directly after birth, to prevent the introduction of artifacts. Newborns still need to adapt to extrauterine life, and therefore significant changes in lipids may only become apparent after a few hours. In addition, we found a significant decrease in unsaturated lipids, which serve as substrates for Lpcat1, indicating that the effects of mutHIF2α could start to become significant only after birth. Interestingly, the turnover time for the major phospholipid, DPPC, is 5 hours, which corresponds very well with the time that mutHIF2α mice start to die (48).

The inactivation of Hif2α in mice resulted in various phenotypes, ranging from death on Embryonic Day 10.5 to respiratory distress in premature mice, depending on the genetic background (13). The neonates that survived suffered from breathing problems and did not produce sufficient surfactant phospholipids and surfactant-associated proteins. Interestingly, the inactivation and ectopic activation of Hif2α showed comparable phenotypes, suggesting that Type II cells require different levels of Hif2α at distinct phases of Type II cell maturation. However, these gene ablation studies lacked Hif2α in all expressing cells, making it difficult to compare them directly with our cell-specific expressing mice. Although Hif2α mRNA increases until birth, it is not known whether the protein is present and, if so, whether it is transcriptionally active. Moreover, in adult rat lungs, no baseline expression of Hif2α was evident, but the protein was rapidly induced by mild hypoxia (17). Thus, directly after birth, cells are exposed to normoxic conditions, which degrade the Hif2α protein, and thereby triggering the cells to produce and secrete phospholipids. During the fetal stages, Type II pneumocytes prepare for their important function directly after birth by progressively increasing the expression of Hif2α until birth, leading to the accumulation of substrates required for the lipid components of surfactant, and the prevention of the production of enzymes that process these substrates. The required enzymes are synthesized toward the end of gestation, to prevent the premature secretion of surfactants. At birth, these enzymes, such as Lpcat1 and Abca3, immediately process the lipid precursors to the end products, possibly initiated by the rapid degradation of Hif2α protein or by functional changes in Hif2α.

In conclusion, Hif2α contributes to the maturation of Type II cells and to the regulation of genes encoding for important proteins involved in the production of surfactant. Moreover, the level of *Hif2α* expression is tightly regulated to ensure proper phospholipid homeostasis. We conclude that Hif2α is a key regulator in the formation of mature alveoli and in the differentiation of Type II cells.

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CHAPTER 3

HYPXIA INDUCIBLE FACTOR 2α IS ASSOCIATED WITH DEVELOPMENT OF PULMONARY HYPERTENSION IN HUMAN NEWBORNS

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ABSTRACT

Persistent pulmonary hypertension in newborns (PPHN) is a life threatening condition and affects about 2 in 1000 live births. It is known that chronic hypoxia causes pulmonary hypertension and is related to high-altitude sickness. Recently, Hypoxia Inducible Factor (HIF) 1α and 2α were associated with pulmonary hypertension, both in human and in experimental animal studies. HIFs are crucial factors in the cellular response to hypoxia by transcriptionally activating genes encoding proteins involved in angiogenesis and metabolism.

Here we evaluated the protein expression pattern of HIF2α in the lungs of human neonatal pulmonary hypertension patients in comparison to age-matched controls, and we extended the analysis to lungs from congenital diaphragmatic hernia (CDH) patients at different developmental ages who also develop pulmonary hypertension.

We found that HIF2α is expressed highly in some patients with pulmonary hypertension, while HIF2α is absent in age-matched controls. HIF2α is expressed in every patient with CDH, while only half of the age-matched controls express HIF2α.

Our data suggest a clear association between HIF2α and the development of pulmonary hypertension.

INTRODUCTION

Pulmonary hypertension of the newborn (PPHN) is a life threatening condition of newborns and requires significant clinical support. During development, the lung is poorly supplied with blood, because of the high vascular resistance before birth. However, in order to facilitate the transition to gas exchange by the lung, a dramatic cardiopulmonary transition occurs at birth, which is characterized by an increase in pulmonary blood flow and a rapid drop in pulmonary artery pressure through the relaxation of the contracted vascular smooth muscle cells.[1] During late gestation, the fetus prepares for this transition by increasing pulmonary expression of different signaling molecules.[2,3-4] A failure in this normal cardiopulmonary transition leads to the persistence of the pulmonary hypertension in newborns. The incidence of pulmonary hypertension in newborns (PPHN) is about 2 per 1000 live birth. [1,5-6] PPHN maybe be an isolated disease, but is sometimes associated with congenital disorders, such as congenital heart diseases, lung diseases and congenital diaphragmatic hernia (CDH).[7] CDH is an anatomical defect of the diaphragm with pulmonary hypoplasia as a result of compression of the developing lung by intra-abdominal organs.[8] The underlying causes of persistent neonatal pulmonary hypertension are still largely unknown, although several genetic and epigenetic mechanisms have been described and hypothesized.[9-10] For example, the BMP/TGF-β signaling pathway is associated with pulmonary hypertension,[8] and mutations in the *BMPR2* gene (bone morphogenetic protein type II receptor), a transmembrane receptor for TGF-β family members, have been found in 70% of families with heritable pulmonary arterial hypertension. In addition, up to 25% of patients with apparently sporadic IPAH (idiopathic pulmonary arterial hypertension) harbour mutations. [9-10] Moreover, it was show that there is a failure of BMPR trafficking in pulmonary artery hypertension, and reduced expression level of BMPR2 protein. [11-12]

It is already known that prolonged exposure to hypoxia is a significant cause of pulmonary hypertension, and the key component of the cellular response to hypoxia is the Hypoxia Inducible Factor (HIF). Hypoxia inducible factors (HIFs) are heterodimeric transcription factors composed of an oxygen-sensitive Hifα subunit and a constitutively expressed Hif1β/aryl hydrocarbon receptor nuclear translocator (ARNT) subunit. Currently, three structurally related α-isoforms are identified, Hif1α, Hif2α or Epas1 and Hif3α. Under normoxic conditions, Hifαs are hydroxylated by one of the three prolyl hydroxylases (Egln1-3) and targeted for poly-ubiquitinylation and proteasomal degradation through the von Hippel-Lindau tumor suppressor protein.[13] However, under hypoxia, the Egln proteins are inactive, resulting in stalbe Hifα subunits that dimerize with Hif1β to form HIF1 (HIF1α/HIF1ß), HIF2 (HIF2α/HIF1β) and HIF3 (HIF3β/HIF1β) complexes. HIF1 and HIF2 activate the transcription of downstream target genes by binding to hypoxia-responsive elements (consensus sequence RCGTG).[13-15] The HIF3α subunit lacks the C-terminal transactivation domain, and as a result only minimally activates the transcription of target genes. Moreover, the *HIF3*α locus is alternatively spliced, leading to yet another subunit, the

Inhibitory PAS Domain Protein (IPAS), which also impairs the hypoxia response by competing with the HIF1α and HIF2α isoforms for the HIF1β subunit.[16]

Several lines of evidence suggest that hypoxia inducible factors are involved in the development of pulmonary hypertension.[17] Indigenous highlanders of the Tibetan plateau live at high altitude (3,200-4,300 m) and thus under hypobaric hypoxia, but they are resistant to developing chronic mountain sickness, have thin-walled pulmonary vessels and a high blood flow. Three independent studies using genome-wide scans revealed a natural, positive selection of *EPAS1* (*HIF2α*) and *EGLN1*, indicative for adaptation of Tibetans to high-altitude.[18-19,20] Hypoxia also induces signs of pulmonary hypertension in rodents, but mice containing one functional copy of either Hif1α or Hif2α show a significant delay in the development of pulmonary hypertension.[21-22] In Fawn-hooded rats, a genetic model for pulmonary arterial hypertension, show similar characteristics of the human disease with activated Hif1α as indicated by its translocation to the nucleus. [23]

Based on these studies, we examined the ontogeny of HIF2α protein expression in a developmental series of normal lung tissue. Furthermore, we analyzed HIF2α in lungs of a cohort of CDH patients, including appropriate controls, since CDH patients suffer not only from a structural defect of diaphragm, but also from associated pulmonary hypoplasia, lung immunity and persistent pulmonary hypertension of newborns. [7,24-26]

We found that 3 out of 11 newborns that have clinical signs of pulmonary hypertension show high expression level of Hif2α in alveolar epithelial cells and endothelial cells around the pulmonary vessels, whereas Hif2α expression is hardly detectable in other patients and age-matched controls. In CDH patients, Hif2α is expressed in all cases at different gestational ages and after birth, while only half of the age matched control ones show expression of Hif2α. From these results, we conclude that Hif2α is associated with the development of PPHN.

MATERIALS AND METHODS

Human lung tissue collection:

With the approval of the Erasmus MC Medical Ethical Committee and the informed consent of the parents, lung tissue was obtained from the archives of the Department of Pathology, Erasmus MC (Rotterdam). The selected lung tissues were obtained after elective termination of pregnancy (TOP) or at autopsy. The collection of lung specimens was within one hour post mortem. After harvesting the tissues, the samples were directly snap-frozen and stored in liquid nitrogen. The duration from harvesting to freezing did not exceed one hour. The characteristics of the 17 CDH patients are reported in Table 1. Lung tissues from 13 age-matched fetuses without pulmonary abnormalities served as controls and were handled and sampled as the CDH ones. Characteristic of PH patients and controls are shown in Table 2.

Table 1. Characteristics of CDH patients and controls selected for tissue microarray.

	Developmental stage	n	Gestational age in weeks	Postnatal age	Birth weight in grams
Control	Immature/ Premature	9	31,5 (15 – 36)	1 hr (0 – 24 hrs)	2000 (57 – 2700)
	Term	8	39,5 (38 – 41)	36 hrs (1 hr – 1 wk)	3220 (2490 – 3950)
CDH	Immature/ Premature	7	31,5 (15 – 36)	1 hr (0 – 48 hrs)	1032 (30 – 2515)
	Term	6	39 (37 – 40)	7 hrs (1 hr – 3 days)	2835 (2000 – 3800)

Median and ranges (in brackets) of gestational age, postnatal age and birth weight of CDH patients and controls divided in immature / premature and term subgroups

Tissue microarray array (TMA)

Tissue micro arrays were constructed as described by Kononen et al. [27] For each sample, three tissue core biopsies of 1.5 mm in diameter and 3.2 mm in depth were taken from preselected regions to ensure adequate representation of all lung structures. These biopsies were placed in linear arrays into empty recipient paraffin blocks, two for the normal developmental stages, one for CDH, and one for CDH control. Tissue cores of adult multi-slides were used as controls.

Immunohistochemistry

Paraffin embedded lungs were sectioned in 5µm. Antigen retrieval was performed with microwave treatment in Tris-EDTA buffer (1.2 gram Tris with 0.37 gram EDTA in demi-water, adjusted to PH=9 with HCl). Sections were blocked with 5% BSA in PBS for 30min and incubated with primary antibody diluted in antibody dilutant (Invitrogen) overnight at 4C. Hif2α (1 in 500 dilution) antibody from Gentex was used. Images were taken by Olympus BX41 microscope and the cell D programme provided by Olympus.

RESULTS

HIF2α is highly expressed in lungs of newborns with pulmonary hypertension

Previously, we showed that the expression of *HIF2*α mRNA, as analyzed by quantitative PCR, gradually increased during development.[28] Since the HIF2α protein is post-translationally regulated by prolyl hydroxylases, we analyzed normal lungs from a series of different gestational ages. HIF2α was expressed in half of the number of fetal cases that were analyzed starting from 15 until 36 weeks of gestation. In addition, neonatal lungs born after week 37 were also positive for HIF2α (Figure 1,A-F). HIF2α was expressed both proximally and distally in the airway epithelial cells and alveolar epithelial cells, and no obvious differences were observed in the intensity of the

Table 2: Characteristic of pulmonary hypertension patients and controls
A: Pulmonary hypertension patients

Patient no.	Gestational stage (in weeks)	Postnatal age	Ventilation	Diagnose/Co-morbidity
1	40	9 weeks	yes	Transposition Aorta/pulmonary, Thrombus vena cava, Persistent chylusproduction
2	37	5 days	yes	Trachea-Oesophagal fistle, Tracheomalacie, Respiratory acidosis
3	40	unknown	yes	Meconium aspiration syndrome, asphyxia,
4	42	852 weeks	unknown	Chromosomal disorder, Multiple VSD's, ASD's
5	40	10 days	Yes	alveolar capillar dysplasia
6	37	1 day	Yes	trisomy 13
7	34	2 day	Yes	longhypoplasie, anhydramnion, cystenieren
8	35	4 days	Yes	Trisomy 21, AVSD, Open ductus Botall
9	42	5 days	Yes	Trisomy 21, severe cerebral damage
10	38	43 weeks	Yes	Congenital Heart Disease: abnormal pulmonary vene position
11	35	566 weeks	Yes	Large VSD, Persistent Ductus Botall

Patient no1 are abducted after two days, the rest are all abducted the next day.
B: Controls

Patient no.	Gestational stage (in weeks)	Postnatal age	Ventilation	Diagnose/Co-morbidity
1	36	2 weeks	Yes	Alveolar Proteonosis with surfactant protein B-deficiency. Possible liver congestion
2	40	18 hours	Yes	Extracerebral bleeding, Livercongestion, Multiple asphyctic bleeding
3	40	12 hours	Yes	Hypovolemic shock with postischaemic encephalopathy, Subarachnoidal Bleeding
4	37	1 week	Yes	Pneumothorax, Liverrupture, Anemiae, Severe asphyxia (asphyctic bleeding in lung), Chronic hypotension, Adrenal necrosis and calcification, Haemorhaegic kidneys
5	40	3 weeks	Yes	Possible Aicardi syndrome, Suspicion metabolic disorder, Dermoidtumor in pharynx
6	40	6 weeks	Yes	Shunt: Norwood I reconstruction
7	42	2 days	Yes	Trisomy 21
8	40	3 days	Yes	Possible congenital pneumonia with hypertension, Potter I malformation in kidney
9	37	2 days	Yes	Congestion of lungs and liver. Large thrombus in left myocardial ventricle causing decompensatio cordis

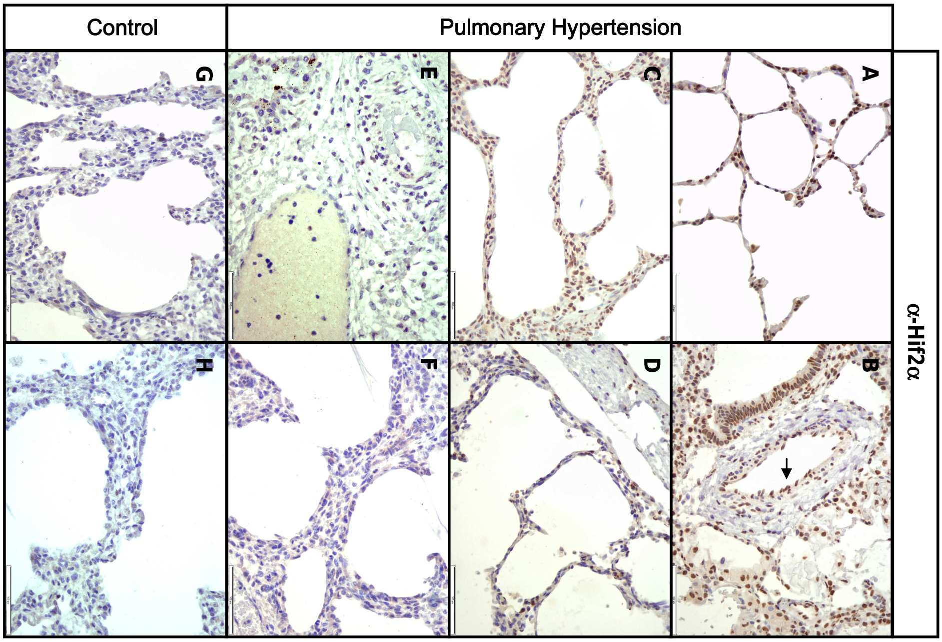


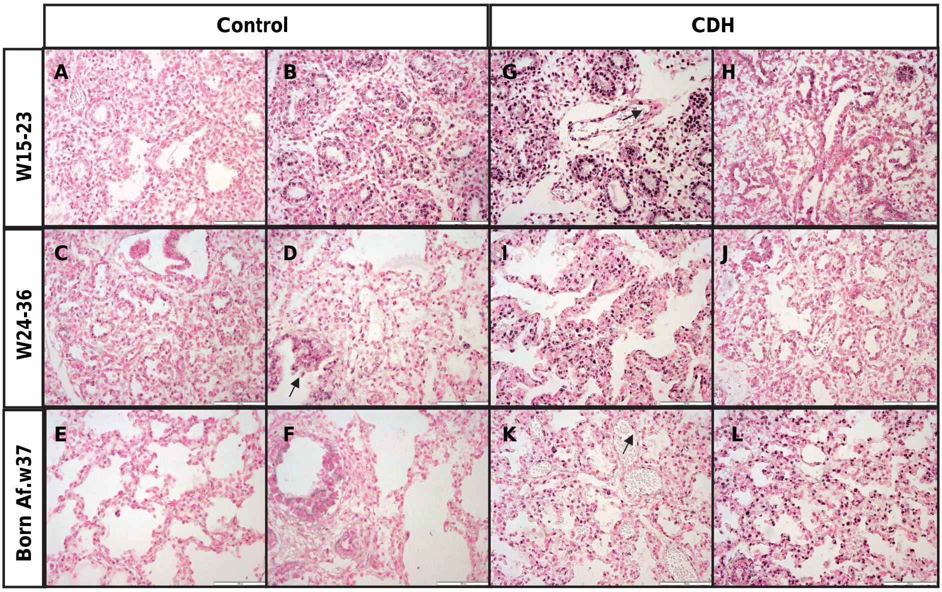
Figure 1: Upper panel: HIF2α is expressed in some lungs of different gestational ages: Week15-Week23 (Figure 1B); Week24-W36 (Figure 1D); born after week 37 (Figure 1F); while HIF2α is absent in some lungs of different gestational ages: Week15-Week23 (Figure 1A); Week24-Week36 (Figure 1C)l; Born after week 37 (Figure 1E). HIF2α is expressed in alveolar type II cells and also epithelial cells in airways (Figure 1D, arrow). HIF2α is expressed in lungs of all the CDH patients (Figure 1 G-L). Scale bar: 100μm. Lower panel: there are half of the control lungs positive for HIF2α from gestational age Week15 until birth; while all the lungs are positive for HIF2α in CDH patients from gestational age Week15 until birth.

staining. (Figure 1 A-F, arrows). Hif2α is also highly expressed in alveolar type II cells in lungs as we and others have shown before for mouse lungs ([29, 30]).

Next, we analyzed a series of patients with neonatal pulmonary hypertension (PPHN), since the hypertension my cause (local) hypoxia and activate HIF2α protein. Clear staining patterns were observed in the endothelial cells of the vessels of patients with PPHN (Figure 2B, arrow) and in alveolar epithelial cells (Figure 2 A-D, arrows). While in some cases of neonatal pulmonary hypertension (PPHN), HIF2α is not expressed at all (Figure 2 E, F). HIF2α is barely expressed in age-matched controls. (Figure 2 G, H)

Hif2α is highly expressed in lungs of Congenital Diaphragmatic Hernia (CDH) patients

The main clinical challenge for the treatment of CDH is the associated pulmonary hypertension. [7,25-26] Therefore, the expression of HIF2α was analyzed in lungs from a cohort of CDH patients of different gestational ages until birth. Contrasting the expression of HIF2α in normal, unaffected lungs (Figure 1 A-F), we found that HIF2α is prominently present in all CDH cases analyzed (Figure 1, lower panel). The sites of



Age	Control: No. of Hif2α positive	Control: No. of total cases	CDH: No. of Hif2α positive	CDH: No. of total cases
W15-23	**3**	**4**	**2**	**2**
W24-36	**2**	**5**	**5**	**5**
Born Af. W37	**4**	**8**	**6**	**6**

Figure 2: HIF2α is expressed in the lungs of some cases of neonatal pulmonary hypertension patients. (Figure 2 A-F); HIF2α is expressed in alveolar type II cells (Figure 2 A-F) and also endothelial cells around vessels (Figure 2 B, arrow). HIF2α is not expressed in the lungs of neonatal age matched control (Figure 2 G, H). Scale bar: 100μm.

HIF2α expression was both in alveolar epithelial cells and all endothelial cells of the blood vessels (Figure 1 G-L, arrows). Moreover, in some CDH lungs the level of HIF2α appeared very high as indicated by the intense staining (Figure 1 G, I and L).

Therefore, we analyzed the expression of HIF2α in lungs from eleven neonatal pulmonary hypertension patients and compared them to age-matched control lungs. Three of the affected lungs were clearly positive for HIF2α (Figure 1C, D and E), mostly in alveolar type II cells, where HIF2α is also expressed in neonatal mice.[29] However, in the other eight cases of neonatal pulmonary hypertension, HIF2α expression is hardly detectable in the lung (Figure 1F). In the neonatal control human lung, there are only few cells positive for HIF2α (Figure 1G and H). Our data suggests that expression of HIF2α is maintained in some of the clinical cases of pulmonary hypertension, supporting the relative hypoxia of these patients.

We conclude that HIF2α may contribute to development of pulmonary hypertension in CDH patients.

DISCUSSION

Previously, HIF2α has been correlated with adaptation to hypobaric hypoxia in Tibetan highlanders,[20] and animal studies also found Hif2α to be involved in hypoxia induced pulmonary hypertension [22]. Moreover, we and others have shown a gradual increase of *HIF2*α mRNA during gestation, [28] but no significant differences were detected in mRNA expression levels between CDH patients and controls at different developmental stages.[31] However, we observed increased expression of HIF2α in CDH patients than controls at protein level, even before birth, we found significant higher HIF2α expression in CHD patients than controls. The neonates CDH patients suffered respiratory distress, suggesting that hypoxic exposure is the cause of high expression of HIF2α.

Since the HIFs are post-translationally regulated, we analyzed the expression of HIF2α protein in CDH and neonatal pulmonary hypertension patients in order to better understand the role of Hif2α in the development of pulmonary hypertension.

Our data suggest that pulmonary hypertension is associated with high levels of HIF2α expression. Since prolonged exposure to alveolar hypoxia is a significant cause of pulmonary hypertension,[17] and the lungs of patients with pulmonary hypertension are under constant hypoxic conditions, it may cause the elevated levels of HIF2α in these patients. Although the HIF2α expression in control lungs was present early in gestation until birth, it appeared variable at best. However, not all neonatal cases of Pulmonary Hypertension express high level of HIF2α, some cases show no expression of HIF2α at all. The etiology of individual pulmonary hypertension cases are therefore not correlated with expression level of HIF2α.

In all fetal CDH cases we studied, HIF2α was expressed in the lung during embryonic development and at a much higher level than controls. Moreover, HIF2α is also highly expressed in lungs of patients suffering from neonatal pulmonary hypertension. This suggests that already early in gestation the lungs of CDH patients are intrinsically different from control lungs, and the high levels of HIF2α may contribute to the postnatal pulmonary hypertension, which patients with CDH develop after birth.

One of the results of prolonged hypoxia, which is also observed in PPHN patients, is the structural changes of the pulmonary vasculature characterized by a thickening of the vascular wall of small pulmonary arteries leading to an increased vascular resistance and a worsening of gas exchange.[32] We recently showed that the perivascular cells in the developing CDH lung had a different expression pattern of smooth muscle marker components compared to control lungs, suggesting that the vascular component prematurely differentiate (Sluiter et al, submitted). Furthermore, a significant decrease in the expression of *VEGF-A* mRNA was observed in the alveolar stage of lung development in CDH patients, while the mRNA level of other angiogenic factors, such as *pVHL*, *HIF1α*, *HIF2α*, *HIF3α*, *eNOS*, *iNOS* and *FLK1*, did not show significant changes.[31] However, the pVHL protein was expressed more frequently in the arterial smooth muscle cells of CDH lungs compared with controls, and HIF1α was expressed less frequently in the endothelium of arteries, veins and capillaries of CDH lungs. [33] The observed increase in HIF2α expression in CDH lungs does not seem to correlate with the reported decrease in *VEGF-A* mRNA expression or the lack of significant changes in VEGF-A protein.[31,33] However, we previously showed that a significant increase in Hif2α in type II cells did not induce an increased expression of Vegf-A (Chapter 2), suggesting that the upregulation of HIF2α in CDH patients may be independent of the VEGF signaling pathway.

Interestingly, we have shown that ectopic expression of Hif2α in type II pneumocytes leads to a severe surfactant deficiency as newborn mice succumb perinatally because of respiratory distress. At the cellular level, we showed that there was a significant lack of lamellar bodies in alveolar type II cells and a lack of the production of the phospholipid component of surfactant, [29] Human CDH patients also have a deficiency in their surfactant system as indicated by fewer lamellar bodies in alveolar type II cells. [34] So, it may be that the elevated levels of HIF2α observed in CDH patients are responsible for the deficient production of surfactant.

Endothelin-1 (ET-1) is a signaling peptide derived from the vascular endothelium, which induces vasoconstriction of the pulmonary vasculature. The expression of *ET-1* increased in mouse lungs after prolonged exposure to hypoxia, resulting in pulmonary hypertension.[26,35-36] An enhanced expression of *ET-1* was also observed in the lungs of NEPAS/Hif3α knockout mice,[37] suggesting that Hif3α knock out mice may suffer from pulmonary hypertension. Since Hif3α may act as a dominant negative competitor of Hif2α by recruiting the common partner Hif1ß/Arnt and occupy the same HRE sequences, [38-39] it may be that elevated levels of HIF2α in patients with pulmonary hypertension may shift the balance between HIF2α and HIF3α iin such a manner that downstream target genes, such as *ET-1*, are induced and contribute to the development of pulmonary hypertension.

Although the mechanisms of HIF2α activity and its role in the development of pulmonary hypertension is still incompletely understood, it may be a putative target for future therapies to reduce the clinical challenges faced by the treatment of patients with pulmonary hypertension. In this respect, it may be interesting to investigate the potential of a recently described Hif2α specific competitor, FM19G11.[40]

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CHAPTER 4

HYPOXIA INDUCIBLE FACTOR 3Α PLAYS A CRITICAL ROLE IN THE DISTAL EPITHELIAL CELL DIFFERENTIATION IN LUNG DEVELOPMENT

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Submitted

ABSTRACT

Hypoxic inducible factors (HIFs) are oxygen-controlled transcription factors that play important functions during embryonic development. HIFs are heterodimers of an oxygen-sensitive subunit, Hif1α, Hif2α or Hif3α, and a constitutively expressed subunit, Hif1β. The Hif1α/Hif1β and Hif2α/Hif1β dimers are gene activators, whereas the Hif3α/Hif1β factor lacks major transcriptional activity. Previously, we and others have shown the importance of the Hif1α and Hif2α factors in lung development, and here we investigated the role of Hif3α during pulmonary development. We conditionally expressed Hif3α in airway epithelial cells during gestation. Hif3α transgenic newborn mice were alive and appeared normal, but their lungs showed clear abnormalities, including aberrant branching morphogenesis and a decreased number of alveoli. Although differentiation into the various epithelial cell lineages seemed not entirely blocked, Hif3α expressing lungs displayed a reduced number of the alveolar epithelial type I and type II cells, as well as the Clara cells. Interestingly, Sox2 was specifically upregulated in the Hif3α expressing lungs, and we show that Hif3α directly activates the Sox2 promoter. Moreover, Foxp2, a transcriptional repressor of the Clara cell specific Ccsp promoter and several other distal cell-specific markers, was also found to be upregulated. We conclude that Hif3α is a key regulator in the differentiation of both type I and type II epithelial cells, which may be caused by the specific activation of proximal makers.

Key words: Hif3α, lung development

INTRODUCTION

Hypoxia inducible factors (HIFs) are critical transcription factors that are stabilized under hypoxic conditions and subsequently regulate cellular adaptation by activating different sets of genes which are involved in angiogenesis, metabolism and cell homeostasis. HIFs were originally identified to bind a specific DNA element, the hypoxia response element (HRE), of the EPO gene which encodes erythropoietin.[1-2] HIFs are heterodimeric transcription factors which have two structurally related subunits, an oxygen sensitive Hifα subunit (Hif1α, Hif2α or Epas1 and Hif3α), and the constitutively expressed subunit, Hif1ß or ARNT-subunit (Aryl hydrocarbon Receptor Nuclear Translocator [ARNT]). The Hifα subunits contain a basic Helix-Loop-Helix (bHLH) and a Per/ARNT/Sim (PAS) domain at the N-terminus, an Oxygen-Dependent Domain (ODD) in the center of the protein, an N-terminal transactivation domain (NTAD) and a C-terminal transactivation domain (CTAD). The latter is absent in the Hif3α subunit, which significantly reduces the transcriptional activity of the protein. The three α subunits are post-transcriptionally regulated by prolyl hydroxylase domain-containing enzymes (Egln1-3), which hydroxylate the Hifα subunits at two critical prolyl residues in the ODD under normoxic conditions. The hydroxylated proteins are poly-ubiquitinylated and targeted for proteosomal degradation through the von Hippel-Lindau (pVHL) tumor suppressor pathway.[1] In contrast, under low oxygen conditions, the Egln proteins are inactive, so the non-hydroxylated HIFα proteins are stable and able to dimerize with HIF1β, leading to the transcription of target genes, such as EPO and VEGF, through the binding to the HREs.[1,3]

The most recent identified α-subunit is Hif3α, which is one of several splice variants of the *Hif3*α gene and is expressed in adult thymus, lung, brain, heart, and kidney. The N-terminal domain of Hif3α protein shares 57% and 53% amino acid sequence identity with Hif1α and Hif2α respectively, and the C-terminus of Hif3α protein shares 61% identity with Hif1α.[4] Hif3α associates with Hif1ß and this Hif3α dimer binds to HRE sites in promoter regions, but the transcriptional activity of is much weaker than that of Hif1α and Hif2α, because it lacks the CTAD. HRE luciferase reporter gene analysis indicated that Hif3α may compete with Hif1α and Hif2α to associate with Hif1ß and as such, Hif3α may modulate the hypoxic response by Hif1α and Hif2α. [5] Hif3α is abundantly expressed in lung epithelial cells (A549 cells) and the expression is induced under hypoxia in several organs including cortex, hippocampus, lung, heart, kidney, cerebral cortex [6] [7] [8] .

There are two major splice variants of the *Hif3*α gene, one is the inhibitory PAS protein (IPAS) and the other is neonatal and embryonic PAS protein (NEPAS/Hif3α). IPAS is hypoxia inducible and lacks both the NTAD and CTAD domains producing a dominant negative regulator of Hifs.[9] NEPAS is another splice variant of Hif3α gene and is almost exclusively expressed during late embryonic and neonatal stages of development, especially in the lung and heart, while Hif3α mRNA is rarely detectable during embryonic and neonatal stages. Homozygous mutant mice (NEPAS/Hif3α$^{-/-}$) were alive at birth, but displayed enlarged right ventricle and impaired lung remodelling, suggesting that NEPAS/Hif3α is important in lung and heart development during embryonic and neonatal stages.[5] Interestingly, the *Hif3*α gene contains hypoxia response elements in its promoter region and has been shown to be a transcriptional target of Hif1α. [10]

In order to understand the precise role of Hif3α during pulmonary development, we generated transgenic mice with an inducible *Hif3*α gene. Mice expressing the *Hif3*α transgene in the developing airways showed aberrant branching morphogenesis, although this did not lead to perinatal lethality. The lungs of the induced Hif3α expressing mice appeared to have more mesenchyme than control lungs, and the number of airspace is decreased in Hif3α transgenic animals compared to control ones. Furthermore, analysis of the distribution of specific markers for different epithelial cells showed a clear reduction in the number of type II cells in the alveolar spaces and an aberrant distribution of Sox2 and p63 positive proximal cells. The lungs of the Hif3α expressing mice showed an upregulation of several genes normally expressed in the proximal parts of the lung, such as *Sox2*, *Rar*β, *p63*, while genes only expressed in distal parts of the lung were downregulated, such as *Sftpd, Scd1, Abca3, Aqp5*. Moreover, Foxp2, a repressor of distal cell markers, such as T1α, Spc, and Ccsp, was found to be induced in the lungs of Hif3α expressing mice, which in part explains the reduction in the number of distal cell types. We also found that Hif3α is capable of inducing the expression of *Sox2* gene, which could explain the appearance of aberrant Sox2- and p63 positive cells in the Hif3α expressing lungs. Our results show that Hif3α is involved in the correct formation of the distal lungs, and that ectopic Hif3α expression impairs branching morphogenesis, leading to the induction of proximal cell types at the expense the distal cells.

MATERIALS AND METHODS

Generation of transgenic animal

The myc epitope encoding sequence was cloned directly after the endogenous ATG start codon of the full length cDNA encoding Hif3α and subcloned into a modified pTRE-Tight vector.[11] Transgenic lines were produced by pronuclear injection of FVB/N fertilized eggs, and tail tip DNA of transgenic lines was initially genotyped by Southern blot analysis, after which positive lines were routinely checked by PCR, using transgene-specific primers (sense: 5’-GTCAAGCTTATGGCGCTGGGGCTGCAGCG; antisense 5’- GCATCTAGATCAGTCAGCCTGGGCTGAGC). Three independent lines were initially analyzed, which all produced the same phenotype as described in this manuscript. Mice were kept under standard conditions and all experiments were performed according to the guidelines of the local ethics committee. Lung-specific expression of the Hif3α transgene was obtained by crossing the myc-Hif3α lines with the SPC-rtTA transgenic mice (A generous gift of Jeffrey Whitsett). Administration of doxycycline to pregnant mothers from gestational age 6.5 onward in the drinking water (2mg/ml, 5% sucrose) resulted in lung epithelium-specific expression. Each experiment was performed with at least three independent litters containing double transgenic, single transgenic and wild type pups. All double transgenic animals receiving doxycycline expressed Hif3α in the pulmonary epithelium and showed the described phenotype.

Immunohistochemistry

Immunohistochemistry was essentially performed as previously described [12]. Briefly, lungs were dissected and fixed in formal saline (BDH) overnight at 4°C before processing for paraffin embedding according to routine protocols Antigen retrieval was performed with microwave treatment in 10mM citric acid buffer pH 6.0 or Tris-EDTA. Sections were blocked with 5% BSA or 5% ELK in PBS for 10min and incubated with primary antibody diluted in 5% BSA or 5% ELK in PBS overnight at 4. The following antibodies were used: Myc (Roche), Hif3α (Abcam), ß-tubulin IV (bioGenex), proSP-C (Chemicon), T1α (University of Iowa Hybridoma bank), Ttf1 (Thermo), Ccsp (seven hills), Sox2 (seven hills), Foxp2 (Abcam), Lpcat1 (Seven hills Bioreagents), α-Sma (Thermo), Ki67, cGRP Secondary antibodies against the correct IgG species were conjugated with peroxidase (Dako).

Lungs were imaged using an Olympus BX41 microscope and DP71 camera (Olympus, Zoeterwoude, The Netherlands). Subsequent airspaces counting were performed with SIS Softward Cell D (Olympus). Three independent samples of control and double-transgenic lungs of gestational age E18.5 were used to count the number of airspaces. The number of airspaces was counted by fix selected surface area (140000μm^2) on those selected lung samples.

Microarray analysis

Lungs of three control and three double transgenic embryos were dissected at E18.5 and the middle and caudal lobes were used for total RNA isolation with Trizol reagent according to the manufacturer's instructions (Invitrogen life technologies, Carlsbad, CA, USA). RNA was purified using the RNeasy MinElute Cleanup kit. (Qiagen, Valencia, CA, USA) and cDNA was synthesized from 3 μg RNA using the GeneChip Expression 3'-Amplification Reagents One-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA, USA). Biotin-labelled cRNA synthesis, purification and fragmentation were performed according to standard conditions. Fragmented biotinylated cRNA was subsequently hybridized onto Affymetrix Mouse Genome 430 2.0 microarray chips. After normalization, the data were analysed with OmniViz software, version 3.6.0 (Omniviz, Inc., Maynard, MA, USA).

RT-PCR

RNA isolation and subsequent quantitative PCR analysis was essentially performed as previously described [13]. Gene-specific primers used in this study are shown in supplemental table S.

Luciferase reporter activity assays

0,5*10^5 HEK293T cells were seeded onto 12 well plates one day before transfection. Duplicate wells were transiently transfected with Lipofectamine 2000 (Invitrogen) with a total concentration of 500ng DNA/well, using 9*HREluc (Gift from Manuel Landazuri), pGL3-mpSox2 and pGL3-mpSox2delta (Gift from Victoria Moreno), Hif2α, (gift from Carole Peyssonnaux), Hif3α or pcDNA3. Cells were lysed with passive lysis buffer (Promega) 24-hours after transfection and processed for lucifease analysis by the addition of the LARII reagent (Promega), which was quantified with the VICTOR luminometer. A construct containing the renilla gene (10 ng/well) was co-transfected in each well to serve as a control for transfection efficiency. The renila luciferase activity was quantified by addition of Stop&Glio reagent and also detected in VICTOR luminometer.

RESULTS

Ectopic expression of Hif3α causes aberrant branching morphogenesis

Previously, it was shown that homozygous NEPAS/Hif3α knockout mice were viable, but displayed an enlarged right ventricle and impaired lung remodelling, suggesting that Hif3α plays an important role during pulmonary development. However, the precise role of Hif3α during the formation of the lung is not fully understood. In order to determine the precise role of Hif3α in the epithelium during lung development, and more specifically in type II pneumocytes, we generated transgenic mice carrying a myc-epitope tagged Hif3α under the control of a doxycycline-inducible tet-on promoter (i-Tg-mycHif3α). Expression of Hif3α in embryonic lung epithelium was established by crossing the i-Tg-mycHif3α transgenic line with the established SPC-rtTA line, which drives the expression of the rtTA gene in epithelial cells of the embryonic lung.[14] Lungs isolated from doxycycline-induced or non-induced single i-Tg-mycHif3α or SPC-rtTA transgenic mice, or lungs from double transgenic i-Tg-mycHif3α/SPC-rtTA animals appeared indistinguishable from wild type lungs.

Pregnant females from timed matings between SPC-rtTA and i-Tg-mycHif3α mice received doxycycline to induce the expression of the Hif3α transgene in double-transgenic fetuses. Double-transgenic pups were born at Mendelian ratio and did not show obvious external abnormalities compared to their control litter mates.

In order to determine whether expression of Hif3α leads to pulmonary development defects, we analyzed lungs of double-transgenic animals and control lungs at different gestational ages. Macroscopic analysis of isolated lungs did not show clear abnormalities in double-transgenic animals at gestational ages E16.5, E17.5, E18.5 days and postnatal day 1 (PN1) (Figures 1A and B, E and F, I and J, M and N, respectively). Histological examinations at E16.5 did not show clear differences between control and Hif3α transgenic lungs (Figures 1C and D). However analysis of a series of developmental ages clearly showed aberrant alveolar airspaces in Hif3α expressing lungs starting at E17.5 compared to controls. Hif3α expressing lungs contained significant fewer alveolar

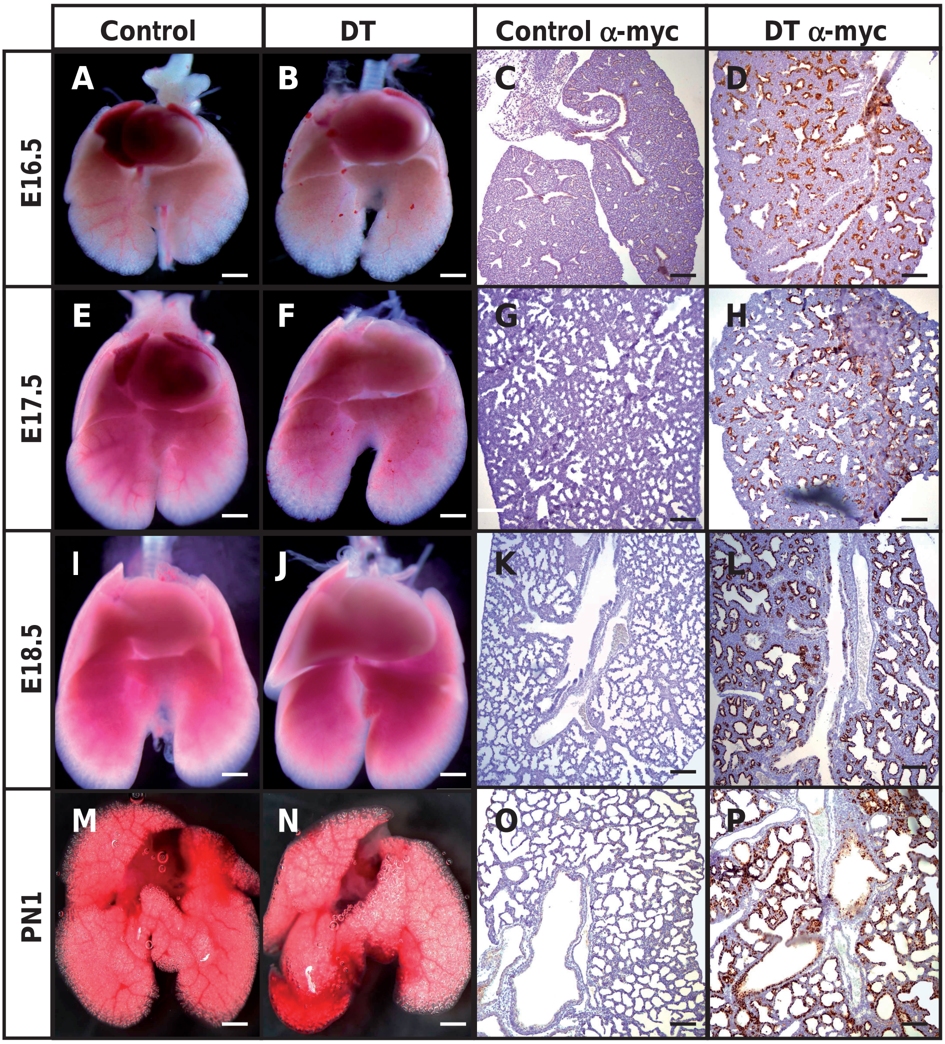

Figure 1: Expression of Hif3α leads to aberrant branching morphogenesis. External appearances of control (A, E, I and M) and Hif3α transgenic lungs (B, F, J and L) at four different gestational ages showed no apparent differences. Histological analysis of control (C, G, K, O) and Hif3α transgenic (D, H, L, P) lungs showed decreased number of air spaces and aberrant branching morphogenesis in Hif3α transgenic lungs. Anti-Myc epitope staining showed expression of the-Hif3α transgene in double transgenic lungs at different gestational ages (D, H, L and P), which is absent in control lungs (C, G, K and O). Scale bars: 2mm (A, B, E, F, I, J, M and L) or 200μm (C, D, G, H, K, L, O and P)

spaces compared to control ones (Figures 1G, K, and O versus Figures 1H, L, and P; Figure S1). Staining with a myc-epitope-specific antibody confirmed the expression of transgenic Hif3α protein in the epithelium of double-transgenic lungs (Figures 1D, H, L and P). The abnormal alveolar spaces remain present in the PN1 stages, but apparently, the mice do not suffer from respiratory distress, indicating that the initial requirements for life are present. So, we conclude that Hif3α expression in epithelial cells leads to aberrant alveolar formation and may affect branching morphogenesis during pulmonary development.

Since we observed significant alveolar changes and aberrant branching morphogenesis, we analyzed the integrity and differentiation potential of fetal transgenic lungs by immunohistochemistry with cell-specific markers. The smooth muscle cell component of the mesenchyme (α-Sma) did not reveal striking differences between control and transgenic lungs (Figures 2A, B). Thyroid transcription factor (Ttf1) was expressed in nearly all epithelial cells in both control and transgenic lungs (Figures 2C, D). Ciliated cells (β-tubulin) and neuroendocrine cells (cGRP) were present in proximal conducting airways of control and transgenic lungs at gestational age E18.5 (Figures 2E, F and 2G, H, arrows). Sox2 is a proximal cell marker, and the expression of Sox2 is expressed in both control and transgenic lungs at gestational age E18.5 (Figures 3A, B). Moreover, both type I (T1α) and type II pneumocytes (Lpcat1; Figures 3C, D and E, F) were present in the alveolar regions. These results indicate that differentiation into the various epithelial cell types is not hampered by Hif3α, although the total number of each cell type may be different. In addition, no differences were observed in the proliferation between control and transgenic lungs as indicated by Ki67 staining (Figure 3G, H).

Hif3α expression inhibits Clara cells differentiation

Three Hif3α-expressing lungs and three control lungs were processed at gestational age 18.5 days for microarray analysis, to elucidate the origin of the aberrant branching morphogenesis (Table I and Table II). Hierarchical clustering of differentially expressed genes revealed large differences between controls and double transgenic lungs (Figure 4, left panel) and the major biological processes (Figure 4, right upper panel) and molecular functions (Figure 4, right lower panel) are indicated. Although Hif3α does not prevent the differentiation of epithelial cells into Clara cells, we noticed that the number of Clara cells was significantly reduced. Both in the microarray analysis as well as the qPCR validation showed downregulation of the Ccsp gene in Hif3α transgenic mice. These gene expression results were confirmed by immunohistochemistry, showing that Ccsp positive cells were less prominent in the proximal airways of the Hif3α expressing lungs compared to control lungs (Figures 5A-D). Quantification of the total number of Clara cells revealed a significant reduction in the double transgenic mice (Figures 5G). Our data suggest that Hif3α expression inhibits Clara cells differentiation during pulmonary development.

Hif3α expression induces airway epithelial cells to differentiate into proximal cell types

Analysis of the microarray data revealed that genes associated with proximal cell types of the lung appeared to be upregulated, whereas genes specifically expressed in distal epithelial cells were downregulated. The induction of proximal markers is reflected by the significant downregulation of genes specific for the distal lung epithelium. The type 1 pneumocyte cell marker Aquaporin 5 (*Aqp5*) was dowregulated in the Hif3α

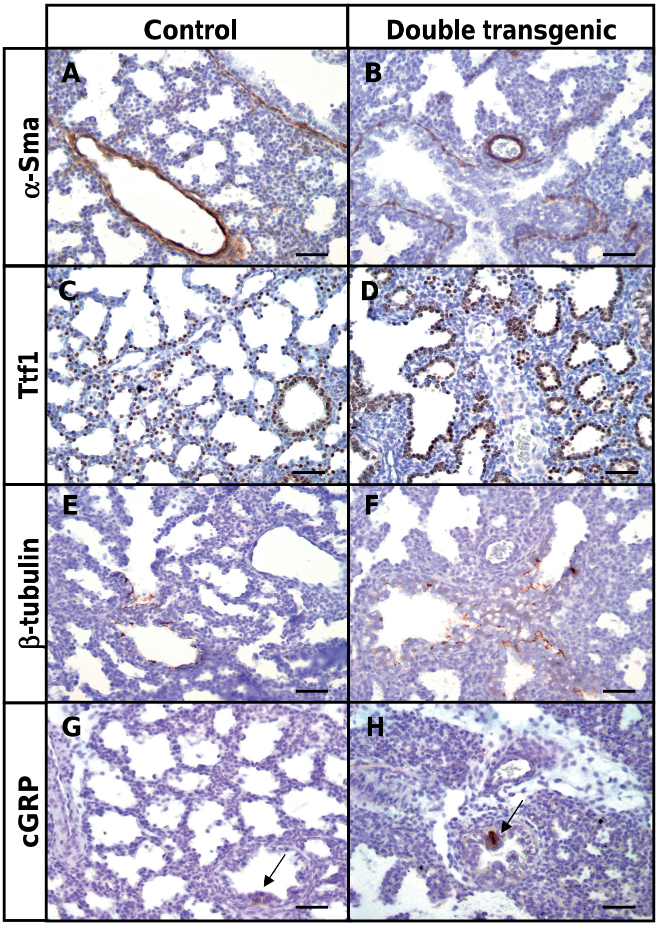

Figure 2: Normal differentiation of proximal epithelial cells in Hif3α transgenic lungs. The site and expression pattern of α-Sma (A and B), β-tubulin (C and D), Sox2 (E and F) and cGRP (arrows in G and H) are comparable between control and Hif3α double transgenic lungs at gestational age E18.5. Scale bars: 100μm.

expressing mice, as were three genes specifically expressed in type II pneumocytes, stearoyl-coenzyme A desaturase (*Scd1*), surfactant associated protein D (*Sftpd*) and ATP-binding cassette (ABC) subfamily A3 (*Abca3*) (Figure 5E),[15-17] Quantitation of the number of type II pneumocytes present in the Hif3α expressing lungs using *Sftpd* in reference to *Ttf1* confirmed a significant reduction in these cells (Figure 5F). Since we are inducing the Hif3α family member of hypoxia inducible genes, we analyzed the expression of Hif1α and Hif2α in the transgenic lungs. Although no apparent difference could be detected for Hif1α (Figure S2), but we did notice a significant downregulation of Hif2α (*Epas1*) (Figure 5E). Previously, we showed that Hif2α is involved in maturation of type II pneumocytes, so the reduction of *Epas1* expression confirmed the loss of type II cells.

Table I. Upregulated genes based on Mircroarray Analysis.

Gene symbol	Gene Title	Entrez ID	Fold Change
Dub2a	deubiquitinating enzyme 2a	384701	6,22
Naaladl2	N-acetylated alpha-linked acidic dipeptidase-like 2	635702	2,16
Cldn6	claudin 6	54419	2,14
Hspa1a	heat shock protein 1A	193740	2,14
Fbn2	fibrillin 2	14119	2
ATP6	ATP synthase F0 subunit 6	17705	1,87
Rimklb	ribosomal modification protein rimK-like family member B	108653	1,83
Sema3e	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3	20349	1,8
Tinag	tubulointerstitial nephritis antigen	26944	1,71
Mia1	melanoma inhibitory activity 1	12587	1,68
Plac1	placental specific protein 1	56096	1,68
Cdh16	cadherin 16	12556	1,64
Cnksr2	connector enhancer of kinase suppressor of Ras 2	245684	1,64
Mthfd2l	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like	665563	1,63
Pcgf1	polycomb group ring finger 1	69837	1,61
Pfn2	profilin 2	18645	1,61
Hspe1	heat shock protein 1 (chaperonin 10)	15528	1,58
Fmod	fibromodulin	14264	1,54
Cdh3	cadherin 3	12560	1,54
Maob	monoamine oxidase B	109731	1,54
Rpl23a	ribosomal protein L23a	268449	1,53
Flrt2	fibronectin leucine rich transmembrane protein 2	399558	1,53
Lgals12	lectin, galactose binding, soluble 12	56072	1,53
Nnat	neuronatin	18111	1,53
Rasef	RAS and EF hand domain containing	242505	1,53
Egfl6	EGF-like-domain, multiple 6	54156	1,53
Ctnnd2	catenin (cadherin associated protein), delta 2	18163	1,52
LOC674930	similar to suppressor of initiator codon mutations, related sequence 1	674930	1,5
Genes Lung development			
Sox2	SRY-box containing gene 2	20674	1,57
Foxp2	forkhead box P2	114142	1,51

Table II. Downregulated genes based on Mircroarray Analysis.

Gene symbol	Gene Title	Entrez ID	Fold Change
Olfr767	olfactory receptor 767	258315	0,45
Ass1	argininosuccinate synthetase 1	11898	0,53
Pgam2	phosphoglycerate mutase 2	56012	0,56
Gipr	gastric inhibitory polypeptide receptor	381853	0,58
Olfr6	olfactory receptor 6	233670	0,6
Igfbp6	insulin-like growth factor binding protein 6	16012	0,61
Nppa	natriuretic peptide precursor type A	230899	0,61
Dio3	deiodinase, iodothyronine type III	107585	0,63
Mphosph6	M phase phosphoprotein 6	68533	0,64
Plscr2	phospholipid scramblase 2	18828	0,64
Ccin	calicin	442829	0,65
Fabp5	fatty acid binding protein 5, epidermal	16592	0,65
Nudcd3	NudC domain containing 3	209586	0,65
Olfr171	olfactory receptor 171	258960	0,65
Rtl1	retrotransposon-like 1	353326	0,66
Rasgrf2	RAS protein-specific guanine nucleotide-releasing factor 2	19418	0,66
Fabp12	fatty acid binding protein 12	75497	0,66
Scnn1a	sodium channel, nonvoltage-gated 1 alpha	20276	0,66
Surfactant related genes			
Scd1	stearoyl-Coenzyme A desaturase 1	20249	0,31
Sftpd	surfactant associated protein D	20390	0,65
Clara cells marker			
Scgb1a1(ccsp)	secretoglobin, family 1A, member 1 (uteroglobin)	22287	0,65
Type I pneumocytes marker			
Aqp5	aquaporin 5	11830	0,65

Among the upregulated genes are *Sox2* and *Foxp2*, two genes known to play important functions during lung development.[12,18] Foxp2 is important during lung development and is expressed in the distal parts of the lung. It represses the transcription of several distal cell markers, such as T1α, Spc, and Ccsp. In our microarray analysis, *Foxp2* was significantly upregulated, which we validated by quantitative PCR (Table I and Figure 6G). Staining with a Foxp2 antibody show that the distribution of Foxp2 positive cells in Hif3α double transgenic lungs was expanded compared to control lungs (Figures 6A, D), suggesting that Hif3α suppressed the transcription of genes expressed specific for alveolar epithelial cells through the induction of Foxp2. In addition, *Rar*β, which is expressed at proximal sites in the lung from embryonic day 11 to 12 and not in the distal epithelium of the lung,[19-20] was significantly induced in Hif3α transgenic mice (Figure 6G), confirming

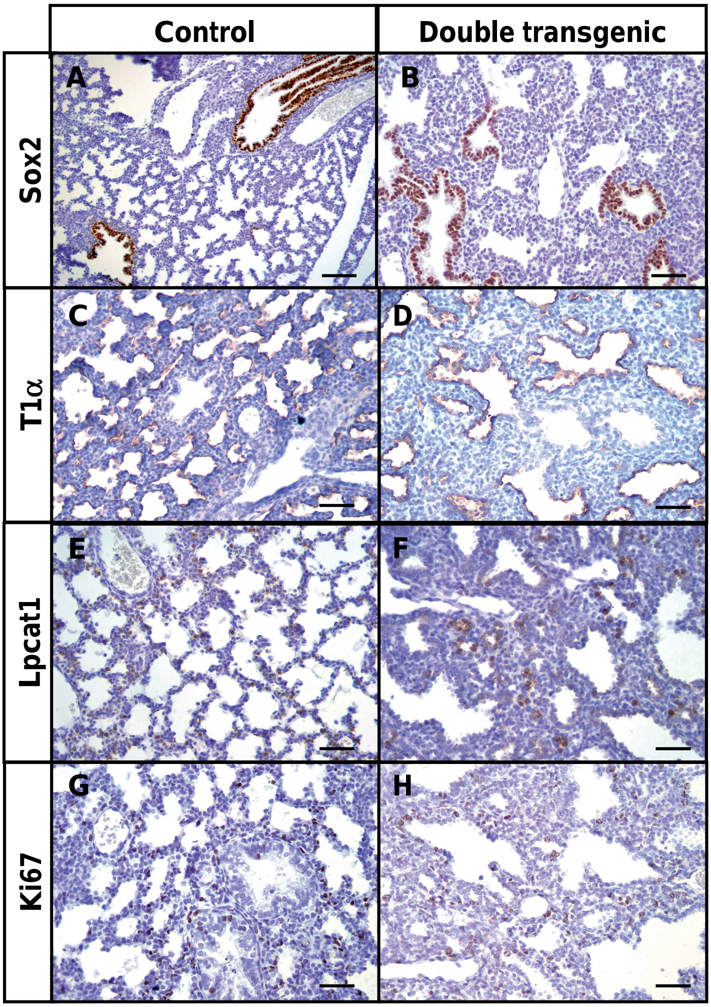

Figure 3: Normal differentiation of distal epithelial cells in Hif3α transgenic lungs. The site and expression pattern of Ttf1 (A and B), T1α (C and D), Lpcat1 (E and F) and Ki67 (G and H) are comparable between control and Hif3α double transgenic lungs at gestational age E18.5. Scale bars: 100µm

the expansion of proximal cell makers in these lungs.[18,21] These results correspond with the previous described findings that Foxp2 is upregulated in Hif3α expressing animals, since Foxp2 is known to transcriptionally repress expression of *Ccsp*. [21]

Sox2 is important for pulmonary branching morphogenesis, epithelial cell differentiation[12] and is exclusively expressed in the proximal parts of the lung. However, in Hif3α expressing lungs, Sox2 is present in epithelial cells of both proximal airways and alveoli at postnatal day 1 suggesting that Hif3α induced proximal cell fate (Figures 6B, E, arrows). The basal cell marker p63 is expressed in the esophagal and tracheal epithelium, but previously we showed that ectopic Sox2 expression induced

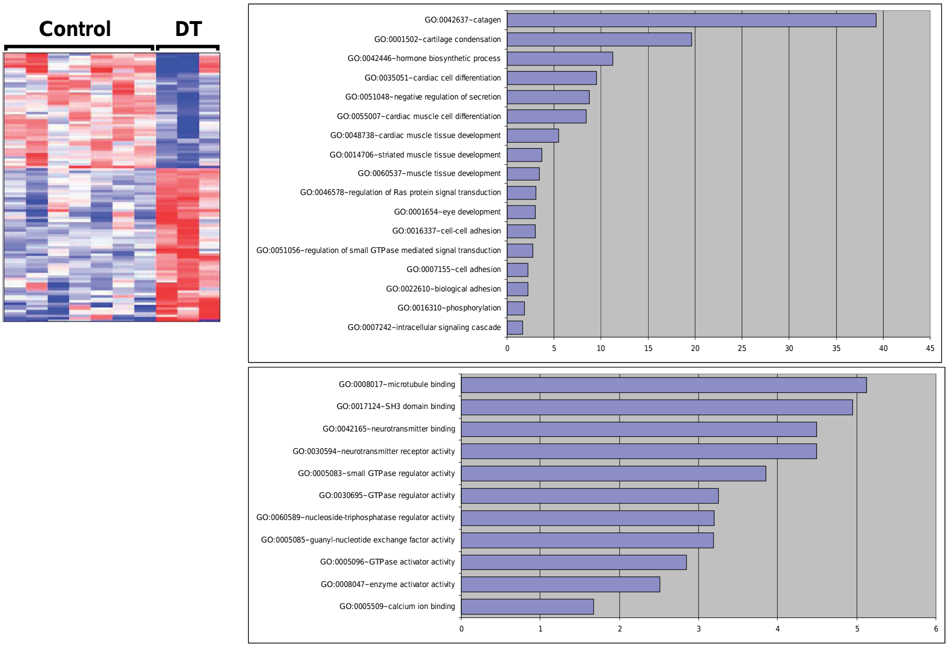

Figure 4: Transcriptome analysis of Hif3α expressing lungs. Treescape showing that the transcriptome of the lungs of the Hif3α expressing animals are significantly different from that of the control lungs (A). The red color indicates the upregulated genes and the blue color indicates downregulated genes. The expression of the genes presented in the treescape is at least 1.5 fold changed with a false discovery rate (FDR) of 10%. The top 10 biological processes (B) and molecular functions (C) of the differentially expressed genes are shown.

the appearance of p63 positive cells in the epithelium of the bronchioles and enlarged distal airspaces.[12] Therefore, we analysed the distribution of basal cells in the Hif3α expressing lungs and found that p63 is abnormally expressed in the alveolar epithelial cells of Hif3α expressing lungs, contrasting the unique expression in the trachea of (Figures 6C insert, *arrows* F). Collectively, our data indicate that Hif3α expression induces airway epithelial cells to differentiate into proximal cell types.

Hif3α induces transcription of Sox2

The promoter region of the *Sox2* gene contains two functional HREs, which are bound by Hif2α.[24] Since Sox2 is upregulated in Hif3α transgenic lungs, we analyzed whether Hif3α can directly induce the transcription of Sox2. Therefore, we performed luciferase reporter assay using the luciferase (Luc) reporter gene under the influence of the Sox2 promoter containing either the two HREs or two mutated HREs.[22] Hif3α induced the expression of the reporter gene with Sox2 promoter about 2 fold, which is significantly more than the induction by Hif2α. (Figure 6H). The Sox2 promoter with the two mutated HRE sites was only slightly induced compared to controls (Figure 6H).As a control, we used the artificial hypoxia responsive construct containing 9 HRE sequences. This construct was considerably induced by Hif2α, but only mildly by

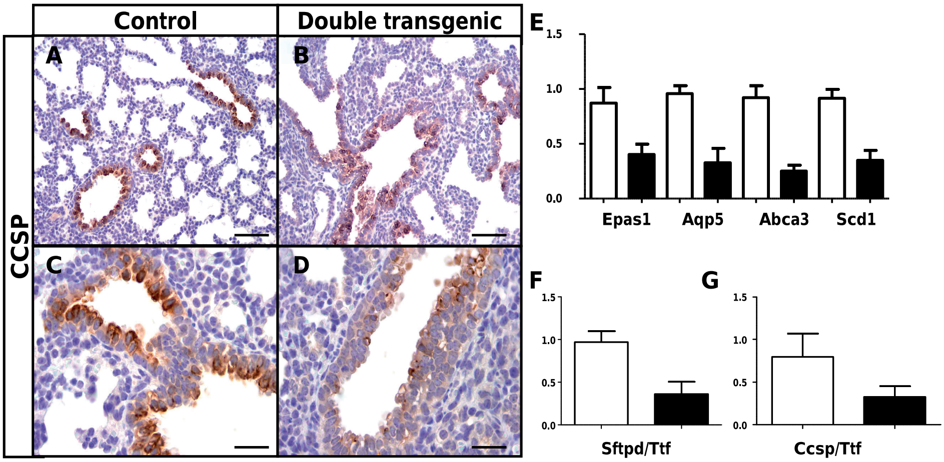

Figure 5: Significant reduction in the number of Clara cells in Hif3α expressing lungs. The expression of the Clara cell marker, Ccsp, was strongly decreased in Hif3α transgenic lungs at gestational age E18.5 compared to controls (A and C versus B and D). Alveolar epithelial cell markers are downregulated in Hif3α transgenic lungs at gestational age E18.5 as shown by quantitative PCR. *Epas1* (0,4 ± 0.1 versus control 0.87 ± 0.1, n=3 each, P=0.012), *Aqp5* (0.33± 0.1 versus control 0.96 ± 0.1, n=3 each, P=0.005), *Abca3* (0.25 ± 0.1 versus control 0.92 ± 0.1 n=3 each, P=0.002), *Scd1*(0.35 ± 0.1 versus control 0.92 ± 0.1, n=3 each, P=0.001). Quantification of the total number of type II pneumocytes (F; *Sftpd* over *Ttf1*, 0.36 ± 0.1 versus control 0.9 ± 0.1; n=3, P=0.01) and Clara cells (G; *Ccsp* over *Ttf1*, 0.3 ± 0.1 versus control 0.82 ± 0.1; n=5, P=0.01) showed a significant reduction of absolute cell number in the Hif3α double transgenic animals. White bars represent control lung samples, black bars represent Hif3α double transgenic lung samples. Scale bars: 100μm (A, B) and 200μm (C,D).

Hif3α, corresponding with the weak transcriptional activity of Hif3α.[5] Identical results were obtained when the assays were performed under hypoxia-mimicking condition, ($CoCl_2$; Figure 6H). So, Hif3α may directly induce Sox2 expression, resulting in an abnormal Sox2 expression in airway epithelial cells in Hif3α transgenic lungs (Figure 6E). Furthermore, it is a strong indication that expression of Hif3α shifts the differentiation of pulmonary cells towards proximal cell types.

DISCUSSION

The hypoxia inducible factors are an important family of proteins involved in the regulation of the cellular response to hypoxia. Gene ablation studies have revealed the specific roles of the different alpha genes during development. Hif1α knockout mice die at early gestation, have multiple developmental defects in neural tube, vascularization, heart development, neural crest migration, [23-25] , whereas depending on the genetic background of the mouse strain, Hif2α knockout out mice ranging from early embryonic lethality to perinatal lethality[26-29]. Previously, it was shown that inactivation of Hif2α resulted in respiratory distress and surfactant deficiency in newborns on a mixed genetic background[28] Recently, we showed the importance of

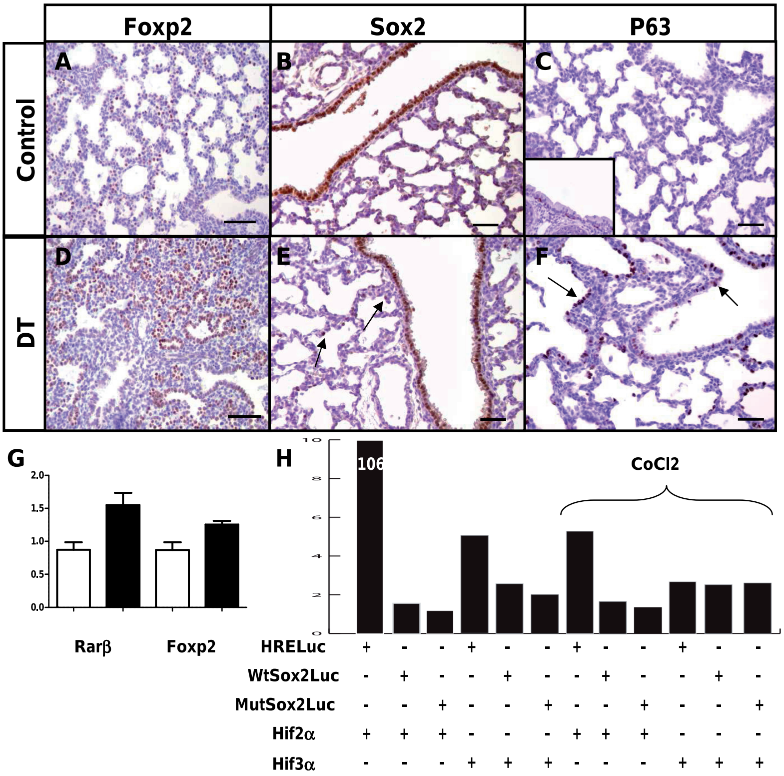

Figure 6: Hif3α induces the expression of proximal differentiation markers. Hif3α induces an expansion of the Foxp2 positive cells in the double transgenic lungs at gestational age E18.5 (A, D), as well as an expansion towards the distal parts of the lungs of Sox2 (B, E) and p63 (C, F). Sox2 was expressed in both proximal airways and alveolar epithelial cells in Hif3α transgenic lungs (*arrows*, E) at PN1. Basal cells, p63 positive, are absent in control lungs (C), but are expressed in basal cells of trachea (C, insert). However, p63 is expressed in the proximal airways and alveolar epithelial cells in Hif3α transgenic lung (*arrows*, F). *Foxp2* and *Rarβ* are significantly upregulated in Hif3α transgenic lungs at gestational age E18.5 as shown by quantitative PCR. (G; Foxp2: 1.25 ± 0.1 versus control 0.87 ± 0.1, n=3, P=0.007; Rarβ: 1.55 ± 0.1 versus control 0.87 ± 0.1, n=3, P=0,009). *Scale bar*: 100μm (A and B). White bars represent control lung samples, black bars represent Hif3α double transgenic lung samples. Both Hif2α and Hif3α induce a 9*HRE artificial promoter (HRELuc) and Sox2 promoter (WtSox2Luc, MutSox2Luc) as measured by the luciferase amount. The fold induction of HRE promoter gene is much higher for Hif2α (106 fold and 5,3 fold under hypoxia) than Hif3α(5 fold and 2.7 fold under hypoxia), while the fold induction of Sox2 promoter is lower for Hif2α (1.6 fold and 1.7 fold under hypoxia) than Hif3α (2.6 fold and 2.5 fold under hypoxic). Data are presented as the induction (n-fold) relative to cells transfected with reporter plasmid and control vector (pcDNA3). The values are the average of two duplicates.

Hif2α in the emergence and maturation of type II pneumocytes. [11] Since Hif3α has been described to function as a dominant negative regulator of the hypoxia response by competing with Hif1α and Hif2α for binding to Hif1ß,[5] we analyzed the role of Hif3α in lung development.

Knowledge of the Hif3α isoform is well described and is complicated by the appearance of different splice variants.[4-5,30-31] Hif3α misses the transactivation domain which is present in the Hif1α and Hif2α isoforms, resulting in a severely reduced transcriptional activity. However, Hif3α is still able to dimerize with the constitutively expressed Hif1ß protein and subsequently this Hif3 complex (Hif3α/Hif1β) binds to HRE sites. This effectively results in the transcriptional competition with Hif1 (Hif1α/Hif1ß) and Hif2 (Hif2α/Hif1ß).[5]

Gene ablation of one of the splicing variants of Hif3α, NEPAS, resulted in mice that were born alive, but displayed enlarged right ventricles and impaired lung remodelling.[5] This hinted at an important role of NEPAS/HIf3α during pulmonary development. Ectopic expression of Hif1α during lung development affected the normal epithelium maturation,[32] and we previously showed that ectopic expression of Hif2α during embryonic pulmonary development leads to surfactant deficiency, impaired alveoli formation, and neonatal respiratory distress.[11] So, the expression level of hypoxia inducible factors may play a critical role during pulmonary development.

Here we conditionally expressed Hif3α in airway epithelial cells during embryonic development in order to elucidate the role of Hif3α in pulmonary development. Hif3α transgenic mice were born at normal Mendelian ratio without obvious respiratory distress. Although macroscopic analyses of the lungs from gestational age day 14.5 until postnatal day 1 did not shown clear differences compared to control animals, histological analyses revealed aberrant branching morphogenesis and a reduced number of alveoli in lungs of Hif3α transgenic mice. Moreover, we found a significant decrease of alveolar epithelial cells and an increase in the number of proximal cells, with the exception of Clara cells.

Detailed analysis of the Hif3α expressing lungs showed an increase in the number of Sox2 positive cells, which were not only present in the proximal airways, but also at in the alveolar epithelium. Moreover, we show that Hif3α is capable of inducing the expression of a reporter construct carrying part of the Sox2 promoter containing two HRE sites. These data correspond well with our previous findings that ectopic expression of Sox2 in airway epithelial cells affect lung branching morphogenesis and epithelial cell differentiation during embryonic development.[12] The phenotype of the Hif3α expressing lungs is not as pronounced, since the Sox2 expression level in Hif3α transgenic lung is not as high. The Sox2 expressing lungs also displayed the emergence of p63 positive basal cells, [12] which we also detected in the Hif3α lungs in the alveolar epithelium.

Besides aberrant expression of Sox2 and p63 in Hif3α transgenic lungs, another gene that is expressed in proximal parts of lung, *Rar*β, was also upregulated. [19-20] Rarβ knockout mice exhibited premature septation, and formed alveoli twice as fast as wild-type mice.[33] So, upregulation of *Rar*β in Hif3α transgenic mice may inhibit pulmonary alveoli formation. Indeed, this phenotype corresponded well with our hypothesis that Hif3α transgenic neonatal pups had a reduced number of alveoli, which appeared to be sufficient for gas-exchange. These results suggest that Hif3α may contribute to alveolar formation during lung development, since ectopic expression of Hif3α leads to upregulation of Rarβ.

Besides the upregulation of proximal cell markers, such as Sox2 and Rarβ in Hif3α transgenic lungs, a number of genes that are expressed distally were downregulated. Among these genes are typical type II pneumocytes markers, such as *Sftpd*, *Scd1* and *Abca3,* indicating that the number of type II pneumocytes are reduced in Hif3α transgenic mice. Previously, we showed a significant downregulation of *Scd1* and *Abca3* in Hif2α expressing transgenic mice, which suffered from respiratory distress and surfactant deficiency[11]. However, the Hif3α transgenic mice appeared to produce sufficient levels of Scd1 and Abca3 to support respiration. The expression of *Sftpd* is low during the entire embryonic period and increases during alveolarisation and lung maturation. [34] So, the low expression of *Sftpd* in the lungs of the Hif3α transgenic mice indicates that Hif3α inhibits alveolarisation and lung maturation, which corresponds well with the observed phenotype of the Hif3α transgenic. Moreover, we observed a block in type II cell differentiation and a reduced number of type I cells in the Hif2α expressing mice, contrasting the Hif3α expressing mice which showed a decrease number of distal epithelial cells in favour of proximal cells.

Aside form the induction of proximal markers, the Hif3α expressing lungs displayed reduced numbers of Clara cells. We also detected an increase of Foxp2, which is able to inhibit the expression of Ccsp.[21] So, the reduced level of Ccsp expression may be directly caused by upregulation of Foxp2. Another recent finding showed that depletion of cells with CCSP promoter activity was associated with alveolar hypoplasia and respiratory failure. [35] Foxp2 is also a factor that inhibits the expression of markers specific for distal epithelial cells, such as Spc and T1α, [18] which corresponds with our results that the number of alveolar Type I and Type II cells are reduced. Thus, the decreased expression of alveolar type I and type II cell markers may be due to upregulation of Foxp2. Sox2 is upregulated in Hif3α transgenic lungs, and we showed that Hif3α induces the Sox2 promoter using an *in vitro* luciferase reporter assay.

Since the transcriptional activity of Hif3α is much weaker than that of Hif1α or Hif2α, because it lacks one of the C-terminal transactivation domains[5]. Therefore, Hif3α may serve as a dominant negative regulator of the hypoxia response induced by either Hif1α or Hif2α. In our Hif2α transgenic mice, we observed that Hif2α target genes, such as *Glut1* and *Pai-1,* were upregulated [11], but their expression does not change in Hif3α transgenic mice. In addition, the Hif3α transgenic mice showed an upregulation of Sox2, a putative target of Hif3α, while Sox2 expression does not change in Hif2α transgenic mice[11]. Although we cannot conclude that the dominant negative role of Hif3α on the hypoxic response is absent, our data do suggest that Hif2α and Hif3α have different target genes, during pulmonary development. This is in line with previous findings describing common targets, as well as specific targets for Hif1α and Hif2α. [36-38] Although Hif3α is considered to be a competitor against Hif1α and Hif2α[5], we did not find genes which are upregulated in Hif2α over-expressing lungs[11] and downregulated in Hif3α transgenic animals.

In conclusion, Hif3α contributes to branching morphogenesis and alveolar formation, possibly by regulating Sox2 expression during pulmonary development. Moreover, the level of *Hif3*α expression is tightly regulated to ensure balance between the total number of proximal cells and distal cells. We conclude that Hif3α plays critical roles during lung development, especially regulating distal epithelial cells differentiation.

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SUPPLEMANTARY MATERIAL

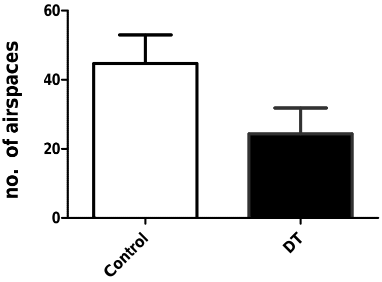

Figure S1: Hif3α reduces the number of alveolar spaces. The number of airspaces was decreased in double-transgenic lungs compared to controls. Three independent samples of control and double-transgenic lungs at gestational age E18,5 were used to count the number of airspaces.

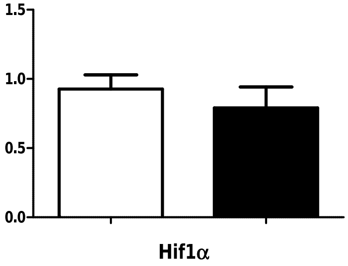

Figure S2: Hif3α does not affect the expression of Hif1α. There is no significant change in the mRNA expression of Hif1α gene 0,8 ± 0.1 versus control 0.7 ± 0.1, n=3 each, P>0.05)

Table S: Primer combinations used in this study.

Gene	NCBI ref.	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
Abca3	NM_013855	TTACGGTCCAAGTTCCTGAG	TAACATCAGCACCTTAGAGCC
Aqp5	NM_009701	GTGGTCATGAATCGGTTCAG	CAAGTAGAAGTAGAGGATTGCAG
Epas1	NM_010137	CTGTGACGACAGAATCTTGG	GGCATGGTAGAACTCATAGG
Foxp2	NM_053242	TGTCATCAGAGATTGCCC	ATAGCCTGCCTTATGAGTG
Rarβ	NM_011243	AACTGCGTCATTAACAAGGTC	TCATTCCTAACAGACTCTTTGG
Scd1	NM_009127	GAGCCACAGAACTTACAAGG	GTACACGTCATTCTGGAACG
Sftpd	NM_009160	GGAAGCAATCTGACATGCTG	GAGGCTCTTCATTTCTGCTC

CHAPTER 5

GENERAL DISCUSSION

GENERAL DISCUSSION

Hypoxia inducible factors are heterodimeric transcription factors which can bind to hypoxia response elements (HREs, core sequence RCGTG) in response to hypoxia. HIF proteins consist of one of the oxygen-sensitive alpha subunits, Hif1α, Hif2α or Hif3α, and the stable Hif1β subunit. HIF proteins play important roles in different physiological and pathological processes including embryonic development, tumor formation, anemia and ischemic vascular diseases.[1] HIF proteins also have important roles in pulmonary development and are associated with certain pulmonary diseases, such as pulmonary hypertension, lung injury and lung cancer.[2] The role of Hif1α in lung development has also been investigated by both conditional lung airway epithelial specific knockout and over-expressing studies. It has been shown that Hif1α has important functions in surfactant production and alveolar epithelial cells differentiation.[3-4] Both Hif2α and Hif3α/NEPAS were also shown to be important in lung development. Loss of Hif2α inhibits Vegf expression during lung development, impairs surfactant production and hampers fetal lung maturation, leading to neonatal respiratory distress in mice. Hif3α/NEPAS knockout mice can survive after birth, but showed impaired lung remodeling, and the expression of endothelin 1 and platelet-derived growth factor b was increased in the lung endothelial cells.[5-6]

In this thesis, we have reported on different aspects of the roles of Hif2α and Hif3α in normal and abnormal lung development. We have shown the role of Hif2α in the formation of type II pneumocytes (chapter 2), the role of HIF2α in pulmonary hypertension (chapter 3), and the role of Hif3α in lung development (chapter 4). Here, we will discuss our results in light of recent developments, a broader perspective.

Hif2α in lung development: alveolar epithelial cells and pulmonary surfactant production

Lung development and maturation has been divided into four stages: pseudoglandular, canalicular, saccular, and alveolar. The tip cells of the airway branches are considered to be precursor cells, and they can differentiate into the different, specialized epithelial cell types of the postnatal lung.[7] Two major types of epithelial cells cover the alveolar space, the flat alveolar type I pneumocytes (type I cells), which are sites for gas exchange and cover about 95% of the alveolar surface, and the alveolar type II pneumocytes (type II cells), which produce surfactant and serve as precursor for the type I cells.[7-8] Since low oxygen levels (3%) ensure normal pulmonary vascular development and epithelial branching morphogenesis[9], and Hif2α is increasingly expressed during gestation in the vascular endothelium and type II pneumocytes.[10-12] We analyzed the role of Hif2α during lung development. Therefore, we generated transgenic mice to conditionally express an oxygen-insensitive Hif2α in airway epithelial cells. As a result, we maintained the expression of Hif2α in the type II cells and when the endogenous Hif2α expression ceased, the mutant Hif2α remained active, even under normoxic conditions. Analysis of fetal lungs at different stages of development revealed that abnormalities of these Hif2α over-expressing lungs start to become visible after gestational day 16, while at earlier embryonic stages almost no obvious morphological differences between Hif2α over-expressing lungs and controls were observed.[12] (Chapter 2) This finding indicates that Hif2α becomes important late in gestation, which corresponds with the previous finding that there is a gradual increase in the expression level of Hif2α during gestation, while after birth the Hif2α expression level goes down. [13]

Analysis of newborn Hif2α over-expressing mice showed that the double transgenic mice suffered from respiratory distress, corresponding to previous phenotypes as a result of a significant surfactant deficiency (Chapter 2). Pulmonary surfactant is essential for the lung as it prevents the lungs from collapsing, it lowers the surface tension and thereby increases lung compliance. Surfactant is a phospholipid-protein complex composed of 90% lipids and 10% proteins, and it is produced by alveolar type II cells. The main lipid component of surfactant is dipalmitoylphosphatidylcholine, which is the primary molecule involved in the reduction of surface tension in the lung. About half of the proteins in surfactant are made up by four apolipoproteins, the surfactant associated proteins Sftpa, Sftpb, Sftpbc and Sftpd, which are also involved in lung immunity against microbes. [14-16]

Since Hif2α is abundantly expressed in alveolar type II cells towards the end of gestation, it is not surprising that Hif2α regulates alveolar epithelial cell differentiation during lung development. Complete loss of Hif2α impairs fetal lung maturation, as indicated by the presence of many immature glycogen positive epithelial cells in the lungs and surfactant deficiency.[5] These lungs lack both alveolar type I and type II cells, which suggest that alveolar epithelial cells are blocked in their differentiation by the loss of Hif2α. Mice expressing the transgenic mutant Hif2α also showed immature, glycogen positive type II cells. Apparently, the maintenance of Hif2α expression inhibited terminal differentiation of type II pneumocytes, as indicated by gene expression profiling and subsequent analysis. One of the known Hif2α target genes, the Glucose transporter 1 (Glut1), was significantly upregulated in the Hif2α transgenic lungs, explaining the appearance of immature, glycogen-loaded type II cells. Glycogen forms the backbone of the most important lipids of pulmonary surfactant, so the accumulation of glycogen may indicate that the terminal synthesis of surfactant is affected. In addition, another Hif2α target, Vegf, did not show significant changes in expression and Vegf is required for surfactant protein synthesis[5]. Moreover, a significant downregulation of Lpcat1 and Abca3 enzymes was observed, both enzymes are involved in the final steps of surfactant secretion. This indicates that ectopic expression of Hif2α hampers the terminal synthesis of surfactant and the secretion in the alveolar space (Chapter 2), leading to respiratory distress in newborn mice.

The phenotype of Hif2α over-expressing mice resembles that of the Hif2α knockout mice, both showing immature alveolar type II pneumocytes and surfactant deficiency which lead to neonatal respiratory distress syndorme.[5,12] This apparent contradiction may be caused by several reason, one of which is the fact that the induced expression of the mutant Hif2α is specific for (immature) epithelial type II pneumocytes, whereas the knockout studies were performed on complete Hif2α gene ablation in all cells. Furthermore, the two studies show that regulation of the level of Hif2α may be important at different stages of type II cell development. Initially, Hif2α may be required to initiate the surfactant production, but later in the maturation of the type II cells, silencing of Hif2α may be required for terminal differentiation of the type II cells. This would fit with previous findings that the expression of HIF2α peaks at the end of gestation, and then returns to baseline levels [13]

Additionally, the Hif2α over-expressing mice did not show changes in the expression of the surfactant associated proteins, contrasting an *in vitro* study showing a repression of the surfactant protein encoding genes in rat alveolar type II cell culture upon induction by Hif1α and Hif2α.[17] On the other hand, Bridges et al used a similar approach to express an oxygen-insensitive Hif1α transgene and found a decreased expression of Sftpb and Sftpc.[4] Collectively, these data suggest that Hif2α plays important roles in regulating enzymes involves in surfactant lipid synthesis (Lpcat1 and Abca3), but does not seem to be the main hypoxic regulator of the surfactant associated proteins. Therefore, it seems that Hif1α may play a more general role during lung development compared to Hif2α.[4] In spite of the differences, both the Hif1α and Hif2α induced expressing studies display defects in the glycogen synthesis pathway as indicated by aberrant glycogen pools in pulmonary epithelial cells and increased expression of glycolytic genes.[4,12] This could be partly due to the impaired glycogen synthesis pathway, since it is known that glycogen is a substrate for surfactant lipid synthesis.

Moreover, the Hif2α over-expressing lungs had a significantly reduced number of type I cells as indicated by reduced expression of *Aqp5*, while the number of type II cells were comparable to controls. However, some of the type II cells were immature as indicated by excessive glycogen, which normally is not present in alveolar epithelial cells of neonatal pups.[12] So, the reduced number of type I cells could be due to reduced number of mature type II cells, suggesting that Hif2α regulates the maturation and differentiation of type II cells. (Chapter 2)

Interestingly, in Hif1α over-expressing lungs, more glycolytic genes changed, including *Aldoc*, *Glut1*, *Glut3*, *Pdk1* and *Pgk1*, while in Hif2α over-expressing lungs, we only found significant increase of *Glut1* and *Ugp2* expression, but other enzymes involved in glycolytic pathways remained the same.[4,12] (Chapter 2) An in *vitro* study also compared different genes regulated by Hif1α and Hif2α in different cell types, and Hif1α was thought to be involved in glycolytic pathways and each would have their own unique target genes.[18-19]

In conclusion, Hif2α directly regulates Glut1 expression, leading to the accumulation of glycogen in alveolar type II cells, and Hif2α over-expression leads to decreased expression of enzymes involved in surfactant synthesis (Lpcat1 and ABCA3). These data suggest that Hif2α may have multiple roles in regulating type II pneumocytes differentiation and surfactant production. The expression level of Hif2α is tightly regulated during pulmonary development, and either reduced or increased expression of Hif2α could contribute to abnormal lung development. Therefore, a balanced regulation of Hif2α is required for maintaining the differentiation of normal alveolar epithelial cells during pulmonary development.

The roles of Hif2α in congenital lung diseases: surfactant deficiency and neonatal respiratory distress

Congenital lung diseases comprise a broad spectrum of rare, but clinically significant developmental disorders, and a lot of signaling molecules play important roles in the development of those congenital lung abnormalities.[20] (Chapter 1) As previously described, Hif2α plays important roles in pulmonary development and therefore, it is not surprising that Hif2α is associated with some types of congenital lung diseases. Hif2α knockout mice showed severe respiratory distress and surfactant deficiency,[5] which is the first indication that Hif2α may be associated with neonatal respiratory distress and surfactant deficiency diseases. In addition, an experimental model of severe respiratory distress syndrome in preterm lambs also showed a decreased expression of Hif2α.[21] Unexpectedly, Hif2α over-expressing lungs also showed neonatal respiratory distress and surfactant deficiency.[12](Chapter 2) In Hif2α knockout mice, the initiation of alveolar type II cells is hampered,[5] while in Hif2α over-expressing lungs, alveolar type II cells do not fully differentiated. The reduced level of Lpcat1 and Abca3 which are crucial enzymes involved in surfactant synthesis is a result of the immature type II cells. (Chapter 2) It has already been shown that either a reduced level of Lpcat1 or the conditional deletion of Abca3 in alveolar type II cells results in surfactant deficiency and severe respiratory distress in new born mice.[22,23] Abca3 is especially critical for the proper formation of lamellar bodies, and mutation of the *ABCA3* gene causes fatal surfactant deficiency and respiratory distress syndrome in human newborns.[24] In addition, mutations in human *SFTPB* and *SFTPC* genes also showed respiratory distress syndrome in infants.[25] So it is obvious that Hif2α directly or indirectly regulates enzymes which are involved in surfactant synthesis, which might explain why mutations in the Hif2α gene leading to Hif2α over-expression could be involved in surfactant deficient diseases and neonatal respiratory distress.

Since Hif2α over-expression leads to surfactant deficiency, our study also suggests a potential clinical therapy for surfactant deficiency treatment, one of the newly identified inhibitor of Hif2α, FM19G11, could be used as a potential drug for treatment of certain surfactant deficient diseases.[26] It would be interesting to test the FM19G11 and other inhibitors in our transgenic mouse model, as well as in the conditional Hif2α knock out mice.

The role of Hif2α in congenital lung diseases: persistent pulmonary hypertension of the newborn (PPHN)

Genome-wide association studies revealed a natural, positive selection on *EPAS1* (*HIF2α*) and *EGLN1*, indicative for adaptation of Tibetans at high-altitude, and those populations are protected from developing high altitude sickness, of which hypoxia induced pulmonary hypertension is one of the symptoms.[27-29] Furthermore, mice with only one functional copy of the Hif2α gene are protected from developing signs of pulmonary hypertension induced by hypoxia.[30] Studies showed that Hif1α is associated with pulmonary hypertension, both in human as in a rat model.[31-32] Our own studies showed elevated levels of HIF2α expression in some cases of neonatal pulmonary hypertension (PPHN). Normally, Hif2α is degraded under normoxia and not expressed in the lung after birth as indicated by controls, The elevated level of HIF2α in PPHN patients showed that Hif2α mis-expression may be associated with pulmonary hypertension in newborns (Chapter 3). However, it is unclear whether Hif2α is the direct cause of pulmonary hypertension in these newborns, or that the expression of Hif2α is caused by lung exposure to hypoxia in these patients. We would like to perform genomic sequencing of the *HIF2*α gene in these patients to find out whether there are any mutations in Hif2α gene causing an aberrant regulation of the expression, splice variants or protein-encoding changes.

Interestingly, the expression level of PAI-1 is elevated in patients with pulmonary hypertension together with a fibrinolytic deficit.[33] So, the impaired fibrinolytic system in pulmonary hypertension patients may be directly due to an elevated concentration of PAI-1(Plasminogen activator inhibitor-1), which is an inhibitor of fibrinolysis.[33] We also found that (Pai-1) to be upregulated in Hif2α over-expressing lungs[12] (chapter 2) and the mouse Hif1α protein induces transcription of a reporter construct containing the Pai-1 promoter region.[34-35] Although binding of Hif2α to the Pai-1 gene was not investigated, these findings suggest that HIF2α expression might induce expression of PAI-1, and the elevated level of PAI-1 could subsequently lead to the development of pulmonary hypertension. However, several experiments need to be done to confirm this, like the analysis of PAI-1 expression in pulmonary hypertension patients, especially the patients with elevated HIF2α expression, as well as chromatin immuno-precipitations using anti-PAI-1 antibodies to directly analyze binding sites of PAI-1 in the genome. This would reveal possible direct targets that may be linked to the development of pulmonary hypertension.

Since many vascular abnormalities are present in human newborns with pulmonary hypertension,[36] it is also worth looking at the expression level of HIF2α downstream targets genes. Especially genes that are linked to angiogensis and vascular development such as VEGF and its receptor, ADM and EPO in the pulmonary hypertension patients with elevated expression of HIF2α in order to better understand the pathology of PPHN.

The aberrant expression of HIF2α in certain cases of PPHN also gives an insight of potential therapy to treat PPHN. One of these therapeutic agents would be the previously mentioned inhibitor of Hif2α FM19G11, which we would loke to test as a potential drug for pulmonary hypertension.[26]

Hif3α in embryonic development

Hif3α shows high similarity to Hif1α and Hif2α. However, Hif3α lacks the C-terminal transactivation domain, and therefore its transcriptional activity is much weaker than that of Hif1α and Hif2α.[37-38] So, Hif3α is considered to work as a dominant-negative competitor of Hif1α and Hif2α for the binding to the general partner Hif1β. Hif3α transcripts are expressed in virtually all embryonic and adult tissues in mice as shown by RT-PCR, [6] while the precise function of Hif3α during embryonic development is still not very well known. The Hif3α locus is subject to alternative splicing, leading to different proteins, like IPAS and NEPAS. Loss of Hif1α in mice leads to early embryonic lethality, whereas loss of Hif2α either leads to early embryonic death or multiple organ defects in mice, depending on different genetic backgrounds.[5,39-44] Although gene ablation of the NEPAS/Hif3α isoform resulted in mice with abnormal heart development and lung remodeling, this phenotype was not lethal.[6] Additionally, airway epithelial cells specific over-expression of Hif3α did not lead to respiratory distress in newborns, but these mice display aberrant lung development. (Chapter4). These data imply that the role of Hif3α in lung development is more subtle than that of Hif1α and Hif2α.

NEPAS/Hif3α knockout mice had enlarged alveolar spaces, which indicated that the process of alveolar formation is impaired.[6] This enlarged airspaces and immature lung phenotype of the NEPAS/Hif3α mice in part resembles the transgenic mice expressing either Hif1α or Hif2α.[4,12] Since Hif3α acts as a competitor for Hif1α and Hif2α, the loss of NEPAS/Hif3α protein may lead to more transactivation of Hif1α and Hif2α downstream target genes. One of the target genes of Hif1α is ET-1,[45] and its expression level is enhanced in NEPAS/Hif3α knockout mice. This finding confirmed the hypothesis that NEPAS/Hif3α indeed acts as a competitor against Hif1α, then there is probably more occupancy of Hif1α to the promoter region of ET-1.

In order to understand more about the function of Hif3α during lung development, we also generated airway epithelial cell specific Hif3α over-expressing mice. Like the NEPAS/Hif3α knockout mice, Hif3α over-expressing mice survived after birth despite an impaired branching morphogenesis and distal epithelial cells differentiation (chapter 4). From these studies, it is obvious that Hif3α is indispensible transcription factor during embryonic development and its effect on embryonic development is more subtle than that of Hif1α and Hif2α. It would be interesting to analyze transgenic mice co-expressing Hif2α and Hif3α together to elucidate the interference between the two proteins.

CONCLUDING REMARKS

In general, hypoxia inducible factors play important roles during pulmonary development. Hif2α is required for alveolar epithelial cell differentiation and surfactant production during lung development. Hif2α is also associated with PPHN and this is probably due to its ability of transactivate different genes involved in vascularization. Hif3α seems to have a more subtle effect on transcription regulation compared to Hif1α and Hif2α, since neither the Hif3α knockout nor over-expression is lethal.

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SUMMARY

SUMMARY

Congenital lung lesions comprise different kinds of rare, but clinically significant developmental abnormalities requiring critical care. As indicated in the first part of chapter 1 of this thesis, lung development commences under the influence of diverse signaling cascades and their downstream target genes. We also discussed that different perturbations in these pathways of lung and airway embryogenesis may result in congenital lung lesions.

Hypoxia inducible factors (HIFs) also contribute to embryogenesis including lung development. In the second part of chapter 1 we reviewed the roles of Hif1α, Hif2α and Hif3α in embryogenesis, especially focused on lung development, as well as their roles in different diseases. Hif2α knockout mice already showed that Hif2α contributes to normal lung development, especially in surfactant production and alveolar type II cells maturation and differentiation. NEPAS/Hif3α knockout mice also showed important roles of Hif3α during lung development. Combined with reports that Hif2α may be involved in pulmonary hypertension, we focused our studies on the roles of Hif2α and Hif3α in lung development.

Initially, we investigated the roles of hypoxia inducible factor 2α during lung development by specifically over-expressing Hif2α in airway epithelial cells during gestation. We found that Hif2α is a key regulator in alveolar maturation and the production of phospholipids by alveolar type II cells. Our results also contribute to an understanding of the origin of certain surfactant deficiency diseases, since Hif2α over-expressing mice showed surfactant deficiency and neonatal respiratory distres. (Chapter 2) Furthermore, our studies contribute to the knowledge of the molecular players involved in the differentiation of distal airway epithelial cells.

Since heterozygous Hif2α mice are protected from developing hypoxia-induced pulmonary hypertension, we hypothesized that Hif2α might be involved in the development of pulmonary hypertension. We evaluated the protein expression pattern of HIF2α in the lungs of human neonatal pulmonary hypertension patients. We found that HIF2α in highly expressed in certain patients with pulmonary hypertension, while HIF2α is absent in age-matched controls. Our data suggest a clear association between HIF2α and neonatal pulmonary hypertension. (Chapter 3)

We also investigated the roles of Hif3α during lung development by specifically over-expressing Hif3α in airway epithelial cells during gestation. In contrast to Hif2α over-expressing mice, Hif3α transgenic newborn mice were alive and appeared normal, but they showed aberrant branching morphogenesis and a decreased number of alveoli in the lung. Lungs of Hif3α over-expressing mice showed a decreased number of the two major distal epithelial cells (Type I, Type II cells) and Clara cells. However, proximal cell marker Sox2 is upregulated in Hif3α transgenic lungs, and we show that Hif3α can directly activate Sox2 promoter. Our data suggest that Hif3α is a key regulator in the differentiation of both alveolar type I and type II cells. (Chapter 4)

Our research revealed that Hif2α is required for normal pulmonary development, especially in alveolar epithelial cell differentiation and surfactant production. Our

data also suggest that Hif2α might play a role in some congenital lung diseases, such as neonatal pulmonary hypertension (PPHN), surfactant deficiency and neonatal respiratory distress. Since neither the Hif3α knockout nor over-expression is lethal, it seems that Hif3α has a more subtle effect compared to Hif1α and Hif2α on transcriptional regulation during lung development. We do find that Hif3α contributes to distal epithelial cell differentiation during lung development.

SAMENVATTING

SAMENVATTING

Congenitale longafwijkingen omvatten een aantal zeldzame, maar klinisch relevante ziektebeelden, welke specifieke en intensieve zorg behoeven. Zoals beschreven in het eerste deel van hoofdstuk 1 van dit proefschrift, vindt longontwikkeling plaats door de integratie van verschillende signaal moleculen die gezamenlijk leiden tot het ontstaan van de ingewikkelde long structuur. Wanneer er verstoringen optreden in één van deze moleculaire cascades tijdens de embryonale ontwikkeling kan dit resulteren in congenitale longafwijkingen. Hypoxia inducible factors (HIF's) zijn belangrijke transcriptie factoren die bijdragen aan de ontwikkeling van diverse embryonale organen, waaronder de longen. In het tweede deel van hoofdstuk 1 wordt de bijdrage van Hif1a, Hif2a en Hif3a in het proces van de embryogenese, met name de longontwikkeling en hun rol in verschillende ziekten beschreven. Onderzoek met Hif2**α** knock-out muizen heeft aangetoond dat Hif2α nodig is voor de normale longontwikkeling, met name voor de productie van surfactant en het differentiëren van alveolaire type II cellen. Studies met knock-out muizen van een andere isoform van de hypoxia factoren, NEPAS/Hif3α lieten zien dat ook Hif3α een belangrijkerol heeft bij de de longontwikkeling. Deze resultaten, gecombineerd met de mogelijke rol van Hif2α bij pulmonale hypertensie is de basis voor de experimenten gepresenteerd in dit proefschrift.

In hoofdstuk 2 beschrijven wij de rol van Hif2α tijdens de embryonale longontwikkeling, door Hif2α tot overexpressie te brengen specifiek in de cellen van het luchtwegepitheel. Wij hebben aangetoond dat Hif2α een belangrijke regulator is voor alveolaire uitrijping en de productie van phospholipiden door de alveolaire type II cellen. Onze resultaten leveren een bijdrage aan de ontstaanswijze van bepaalde ziekten met een tekort aan surfactant, omdat overexpressie van Hif2α in muizen een surfactant deficiëntie laat zien en neonatale respiratoire stress (hoofdstuk2). Bovendien dragen onze studies bij aan de kennis over de moleculaire spelers, die zijn betrokken in de differentiatie van de distale luchtwegepitheel cellen. Aangezien heterozygote Hif2α muizen beschermd zijn tegen zuurstof geïnduceerde pulmonale hypertensie, hebben wij onderzocht in hoeverre Hif2α betrokken is in de ontwikkeling van pulmonale hypertensie. Wij hebben daartoe het eiwit expressie patroon van Hif2α in longen van humane neonaten met pulmonale hypertensie onderzocht. Uit dit onderzoek bleek dat HIF2α in een bepaalde groep patiënten met pulmonale hypertensie hoog tot expressie komt, terwijl in controle patiënten van dezelfde leeftijd HIF2α expressie afwezig is. Onze resultaten wijzen op een belangrijke associatie tussen HIF2α en pulmonale hypertensie (hoofdstuk 3).

Vervolgens werd de rol van Hif3α tijdens long ontwikkeling onderzocht door Hif3α specifiek tot overexpressie te brengen in luchtweg epitheel cellen tijdens embryonale ontwikkeling. In tegenstelling tot muizen die Hif2α tot overexpressie brengen, werden de Hif3α overexpresserende pups levend geboren en vertoonden geen opvallende ademhalingsproblemen. Echter, deze pups lieten afwijkingen zien in het vertakkingspatroon van de luchtwegen en een verminderd aantal alveoli in de long. De longen van Hif3α overexpresserende muizen toonden een verminderd aantal van de twee voornaamste distale epitheliale cellen zien (Type I en Type II cellen) en een verminderd aantal Clara cellen. De proximale cel marker Sox2 was verhoogd in de Hif3α transgene longen, en Hif3α kan direct de Sox2 promoter activeren. Onze resultaten suggereren dat Hif3α een belangrijke regulator is in de differentiatie van zowel alveolaire type I als type II cellen. (Hoofdstuk 4).

Uit ons onderzoek blijkt dat Hif2α noodzakelijk is voor normale longontwikkeling, met name voor alveolaire epitheliale cel differentiatie en surfactant productie. Bovendien suggereren onze resultaten dat Hif2α een mogelijke rol speelt in bepaalde congenitale longafwijkingen, zoals neonatale pulmonale hypertensie (PPHN), surfactant deficiëntie en neonatale respiratoire stress. Aangezien zowel Hif3α knock-out als overexpressie niet lethaal is, lijkt het erop dat Hif3α een meer subtiel effect heeft op de transcriptionele regulatie tijdens longontwikkeling dan Hif1a en Hif2α. Tevens blijkt dat Hif3α bijdraagt aan de distale epitheliale cel differentiatie tijdens longontwikkeling.

APPENDIX

CELL PROLIFERATION AND APOPTOSIS IN LUNGS OF HUMAN AND RAT CONGENITAL DIAPHRAGMATIC HERNIA

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ABSTRACT

Congenital diaphragmatic hernia (CDH) is a congenital disorder associated with abnormal pulmonary development. During embrogenesis, both cellular proliferation and apoptosis are important processes to control organogenesis. A recent finding indicated that lung fibroblasts isolated from rat CDH lungs showed decreased ability to undergo apoptosis but maintained proliferation. In order to better understand the balance between apoptosis and proliferation processes in the lungs of CDH patients, we examined the expressions of proteins associated with activation (Bax) and inhibition (Bcl-2) of apoptosis in human and rat CDH lungs. We also examined the expression of Ki-67 to investigate the cell proliferation process in these lungs. Overall, we did not find significant differences between the expression patterns of the two apoptotic markers (Bax and Bcl-2) and the single proliferation marker (Ki-67) between normal and CHD lungs in human and rat samples, suggesting CDH patients undergo normal proliferation and apoptosis.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a congenital disorder with an incidence o f 1 in 2500 live births. Newborns with congenital diaphragmatic hernia (CDH) may suffer from severe pulmonary hypoplasia and persistent pulmonary hypertension, which causes major clinical challenges.[1-2] The hypoplastic lung is characterized by a reduction in the number of airways and smaller airspaces, whereas the reduced number of vessels combined with an increased vascular wall thickness causes pulmonary hypertension. Despite innovations in prenatal and postnatal care, mortality and morbidity remains high. The developing fetal lung undergoes dramatic tissue growth and remodeling to achieve the mature structure required for its postnatal role as an air-exchanging organ. Organogenesis of the lung is highly dependent on epithelial-mesenchymal interactions.[3-4] The fetal lung remains densely cellular after completing the majority of bronchial branching during the pseudoglandular stage of development. An effective alveolar-capillary interface is then gradually established by regression of the mesenchyme, accompanied by flattening of the epithelium and integrations of capillaries. This process begins *in utero* during canalicular and saccular stage of lung development and completed during alveolar stage postnatally.

Normal organogenesis requires a fine balance between cell proliferation, cell differentiation, and cell death.[5] Apoptosis, a form of programmed cell death, has been shown to be involved in several processes during embryogenesis, including limb, kidney and heart development.[6,7,8] Several regulatory genes affecting apoptosis have been identified, the most prominent being the Bcl-2 family. Apoptosis is initiated by the transduction or translocation of pro-apoptotic Bcl-2 family members (e.g. Bax) and is prevented by the overexpression of anti-apoptotic molecules such as Bcl-2 or Bcl-X_L.[9] In view of the well-established role of apoptosis in embryonic and fetal modeling processes, it may be that apoptosis is important for normal and abnormal lung development. It has been suggested that apoptosis is another mechanism involved in prenatal and post natal lung remodeling.[10-11] Also, a recent finding showed that lung fibroblasts isolated from nitrofen-induced hypoplastic (CDH) rat lungs showed decreased ability to undergo apoptosis and maintained an overall proliferation.[12] Pulmonary hypoplasia in CDH patients may be the result of a disturbed balance in normal mechanisms of pulmonary organogenesis, such as apoptosis and proliferation, but the data are scarce and inconsistent. Therefore, we examined the expressions of proteins associated with activation (Bax) or inhibition (Bcl-2) of apoptosis in human and rat CDH lungs. In parallel, we examined these lungs for cell proliferation by immunochemical study of Ki-67 expression.

MATERIALS AND METHODS

Lung tissues

Following approval of the experimental design and protocols by the Erasmus MC Ethical Review Board, lung tissues were retrieved from the tissue bank of the Department of Pathology, Erasmus MC, Rotterdam. We obtained 17 CDH lung samples collected from either termination of pregnancy or patients who died within 48 hours after birth (gestational age 18-41 weeks). None of these patients had been subjected to prenatal steroid or extracorporeal membrane oxygenation therapy postnatally. Lung tissue from 20 fetuses and newborns (from elective termination of pregnancy or autopsies) without pulmonary abnormalities served as control material (gestational age 13.5-41 weeks). All samples had been harvested within 24 hours after death.

Nitrofen Rat model:

Timed-pregnant Sprague-Dawley rats (Harlan / Charles River) received either 100 mg nitrofen (2,4-dichlorophenyl-p-nitrophenylehter, Cerilliant) dissolved in 1 ml olive oil or 1 ml olive oil (placebo) by gavage on day 9.5 of gestation. Fetuses were isolated at different gestational age and sacrificed by the intra-abdominal administration of pentobarbital (0.5 mg). Diaphragms of nitrofen-exposed fetuses were inspected to discriminate between CDH and non-CDH fetuses. The lungs of control, CDH and non-CDH fetuses were collected and appropriately processed for different experiments. The animal ethical committee of the Erasmus MC approved all procedures and protocols.

Immunohistochemistry

Antibodies used were Ki-67 (clone MIB-1, 1:150), Bax (1:200), and Bcl-2 (clone 124, 1:100); all antibodies from Dako, Heverlee, Belgium. Immunohistochemistry was performed using a ChemMate™ DAKO EnVision™ Detection Kit, Peroxidase/DAB, Rabbit/Mouse (DakoCytomation B.V., Heverlee, Belgium). Immunohistochemical processes were described in detail in our previous study.[13-14] Negative controls were performed by omission of the primary antibodies.

RESULTS

In order to identify proliferating cells in the lung, the Ki-67 antibody was used, which recognizes proliferating cells during all active phases of cell cycle including G1, S, G2 and mitosis except G0 phase[15] Unambiguous expression was observed in both airway epithelium and mesenchyme cells (figure 1). Remarkably, the cycling cells in the epithelium appeared to be were more prominent at earlier stages of gestation, whilst the positive cells in the mesenchyme were more obvious at later stages of development. The expression of the anti-apoptotic protein, Bcl-2, was detected in the basal layer of the airway epithelium and in mesenchymal cells early in gestation (figure 2 A, B), but at later time points, its expression was also detected in airway epithelial cells (figure 2 C-F).

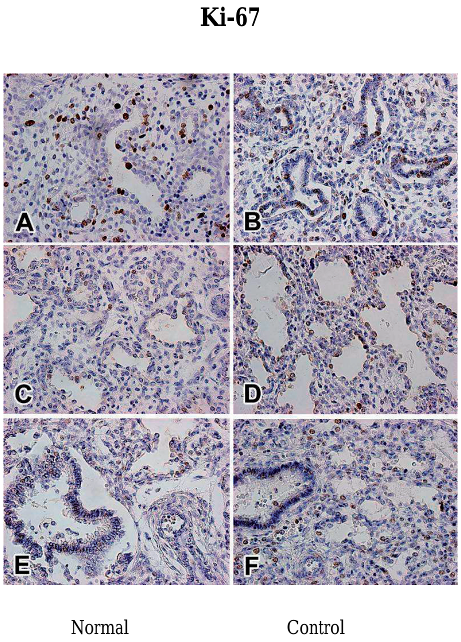

Figure 1: Immunohistochemical study of Ki-67 in normal (A, C, E) and CDH (B, D, F) lungs at different gestational age; 18 weeks (A, B), 23 weeks (C, D), and 35 weeks (E, F). Ki-67 immunoreactivity was detected in both epithelial and mesenchymal cells.

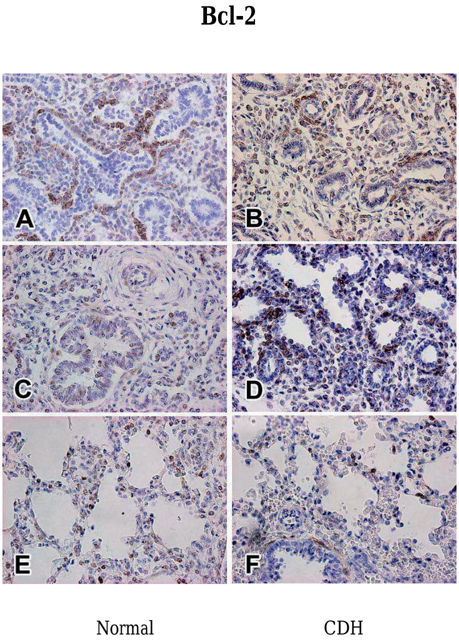

Figure 2: Immunohistochemical study of Bcl-2 in normal (A, C, E) and CDH (B, D, F) lungs at different gestational age; 18 weeks (A, B), 23 weeks (C, D), and 39 weeks (E, F). Bcl-2, one of the anti-apoptotic proteins, was detected at basal layer of airway epithelium and mesenchymal cells earlier in development (A, B). Later on gestation its expression was also detected in airway epithelial cells (figure 2 C-F).

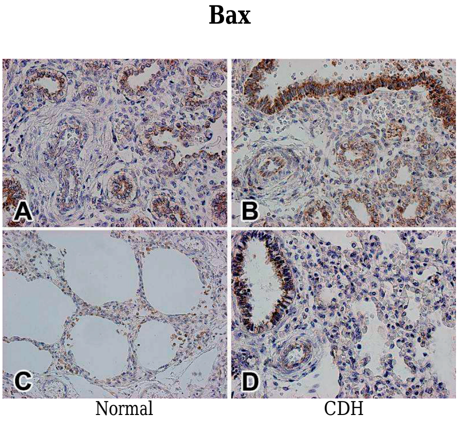

Figure 3: Immunohistochemical study of Bax in normal (A, C) and CDH (B, D) lungs at different gestational age; 23 weeks (A, B), 38 weeks (C, D). Bax immunoreactivity was strongly expressed in cytoplasm of airway epithelial cells. Its expression was also detected in arterial endothelial cells.

The pro-apoptotic protein, Bax, was detected in the cytoplasm of most epithelial cells. Moreover, its expression was also detected in the cytoplasm of pulmonary artery endothelial cells (figure 3). The pattern of Bax protein expression was comparable and consistent throughout gestation. There was no striking difference in the expression pattern of Ki-67, Bcl-2, or Bax between normal and CDH lungs in human samples.

Next, we compared the expression patterns of Bcl2 and Bax in both control and CDH lungs of Rats. Bcl-2 is prominently expressed in the basal layer of epithelium and mesenchymal cells at earlier gestational age week 17 (figure 4, A and B), while at later gestational ages (week 19 and week 21), Bcl2 is expressed less prominent than at week 17 (figure 4, C-F). There is no obvious difference in the expression pattern of Bcl-2 in both control and CDH cases. Bax is also expressed in the cytoplasm of airway epithelial cells and some mesenchymal cells, which show no obvious difference between control and CDH cases (figure 5 A-F).

Thus, comparing the immunostaining patterns of the pro-apoptotic markers Bax and the anti-apoptotic marker Bcl2 between CDH and control lungs from both human and rat samples, suggests that there is no significant contribution of Bax/Bcl mediated apoptosis in the development of the hypoplastic lungs in CDH.

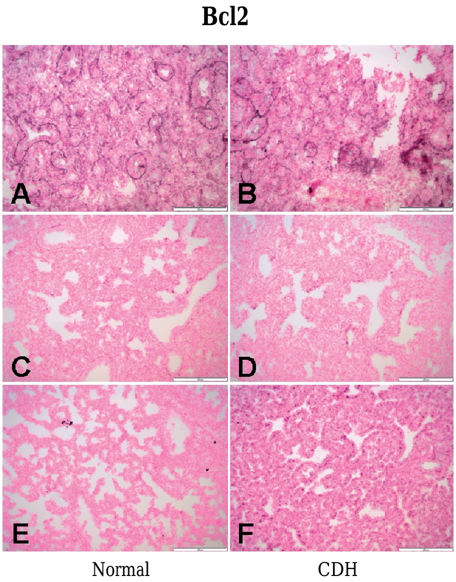

Figure 4: Immunohistochemical study of Bcl-2 in Normal (A, C, E) and CDH (B, D, F) at different gestational age of rat lungs; 17 weeks (A, B), 19 weeks (C, D), 21 weeks (E, F). Bcl-2 immunoreactivity was strongly expressed at basal layer of airway epithelium and mesenchymal cells at developmental age week 17 (A, B); Later on gestation (19 weeks and 21 weeks) its expression was also detected in airway epithelial cells, but not as strong as at week 17. (C, D, E, F)

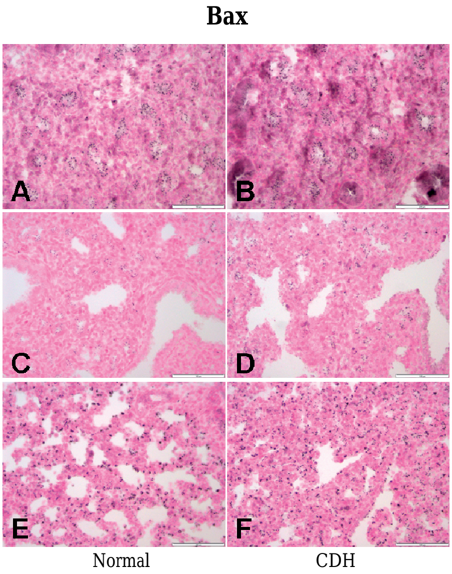

Figure 5: Immunohistochemical study of Bax in Normal (A, C, E) and CDH (B, D, F) at different gestational age of rat lungs; 17 weeks (A, B), 19 weeks (C, D), 21 weeks (E, F). Bax immunoreactivity was detected in the cytoplasm of airway epithelial cells and some mesenchymal cells.

DISCUSSION

In this study, we evaluated cell proliferation and expression of apoptotic related proteins during normal and abnormal lung development in human and rat lungs. Proliferating cells were observed in both epithelial cells and mesenchymal cells, albeit more prominent in the airway epithelial cells. Expression of the anti-apoptotic Bcl-2 protein was observed in basal layer of airway epithelium at early stages of development and was progressively seen in both epithelial and mesenchymal cells. The Bax protein, which is associated with apoptosis, was predominantly expressed in airway epithelial cells. However, we did not observe significant differences between the expression of these proteins between normal and CDH lungs, both in human and rat samples.

Cell death in multicellular organisms occurs by either necrosis or apoptosis, each of which has distinct morphologic and biochemical characteristic. The cell undergoing apoptosis is characterized by cell shrinkage, nuclear condensation, and DNA fragmentation, with the cytoplasmic membrane remaining intact during the early stage.[16] Apoptosis is an active process and proceeds by multistep cascade involving specific membrane receptors (such as Fas), the Bcl-2 protein family, and cystein proteases (caspases).[17] Presence of those signaling protein (Blc2 and Bax) for apoptosis in our study is not surprising in developing lung because these proteins are constitutively expressed in many tissues and cell lines.[5,18-20] The similar expression patterns of Bcl-2 and Bax between normal and CDH lungs suggest that the disturbance of apoptosis in CDH lungs, if any, occurs largely independent of Bcl-2/Bax pathway.

Apoptosis and lung development

The development of organs requires an orchestrated and complex interplay between proliferation, differentiation, and apoptosis.[6,8,21-22] During organ morphogenesis, apoptosis occurs either by direct stimulation or by lack of growth and/or differentiation factors, resulting in removal of unwanted or excessive structures. Although most organogenesis occurs *in utero*, several organ systems do not complete their development and maturation until after birth. In humans, alveolarization and microvascular maturation of the lung continue up to at least a few years after birth.

Apoptosis has only recently been implicated in the process of lung development. Shift in apoptosis was observe from mesenchymal cells during the earlier stage of development [11] to both mesenchymal and airway epithelial cells from the canalicular stage onward.[10] Throughout the embryonic stage of lung development, apoptosis was almost exclusively found in the peripheral mesenchyme in regions of new bud formation or in the mesenchyme underlying branch points that are the site of extensive epithelial branching morphogenesis and remodeling of interstitial tissue, allowing room for outgrowth of the lung bud.[10,23] Interestingly, mesenchymal cells undergoing apoptosis were intermingled with proliferating mesenchymal cells,[23] suggesting that a coordination of these two processes is important in the cell dynamics associated with bronchial branching. One of the mechanisms believed to be involved in regulation of apoptosis during lung development is cellular stretching resulting in mechanical force increases via liquid secretion or fetal breathing movements.[24-25]

In rabbit, it has been demonstrated that normal fetal lung development is associated with a progressive increase in epithelial and mesenchymal apoptotic activity and this process was enhanced by tracheal ligation.[26] Tracheal occlusion (TO) *in utero* is known to accelerate fetal lung development and was used in selected CDH cases to enhance lung growth and maturation before birth.[27] De Paepe et al. have demonstrated synchronous to the onset of TO-induced distension and alveolar type 2 cell apoptosis, the pulmonary FasL protein levels were dramatically higher in TO fetuses, while the expression of Bcl-2 and Bax were similar in control and To lungs at all time point.[20]

Previous studies in the Nitrofen-induced rat model suggested a role for proliferation and apoptosis in both diaphragm and fetal lung development. Enhanced apoptosis was observed in cervical somites that are the precursors of the diaphragm,[28] while decreased proliferation was observed in nitrofen-exposed lung before diaphragmatic closure.[29] Also, fibroblasts isolated from nitrofen-induced hypoplastic CDH lungs show decreased activity of apoptosis.[12] In this study, no striking difference in cell proliferation between normal and CDH lungs was observed, although no quantitative analysis was performed. Future animal and human experiment might reveal the relevance of these studies for further understanding of a role of proliferation and apoptosis during lung development in CDH cases.

Role of apoptosis in development of pulmonary vascular remodeling

Lungs of CDH patients are physically smaller than normal lungs, have fewer airway branches and have vascular abnormalities including medial hyperplasia of the pulmonary arteries, adventitial thickening, and a reduced size of the pulmonary vascular bed.[30-31] Apoptosis has important pathophysiological consequences contributing to the loss of pulmonary smooth muscle cells and therefore in reversing the pulmonary pressure. In recent years, the process of the programmed cell death has gained much interest because of its influence on many pathological states. Increased proliferation and decreased apoptosis of pulmonary artery smooth muscle cells can concurrently mediate thickening of the pulmonary vasculature, which subsequently reduces the inner-lumen diameter of pulmonary arteries, increases pulmonary vascular resistance, and raises pulmonary arterial pressure.[32-34] The balance between apoptosis and cell proliferation is vital for cellular homeostasis, but so far, little is know about the mechanism that coordinate these two mechanism, particularly in the vessel wall. The role of apoptosis in the pathogenesis and treatment of pulmonary hypertension has been reviewed by Gurbanov and Shiliang.[35]

In conclusion, the modulation of expression of proteins in the apoptotic cascades and evidence of on-going cell proliferation during gestation suggest that lung development occurs through a coordinate process of cell proliferation coupled with programmed cell death for structural remodeling. Despite intense investigation into the role of apoptosis in many human diseases, little information is presently available concerning altered pattern of apoptosis in numerous biologic and clinical fields, including pediatric surgery. The role of apoptosis and proliferation in pulmonary epithelial and vascular remodeling needs further investigation to define the mechanism involved in programmed cell death. Therapeutic strategies to enhance or decrease the susceptibility of specific cells to apoptosis might form the basis of many diseases.

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CURRICULUM VITAE
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Seminars in Pediatric Surgery (2010) 19(3) 171-179

Yadi Huang, Marjon Buscop-van Kempen, Anne Boerema-de Munck, Sigrid Swagemakers Siska Driegen, Dies Meijer, Wilfred van IJcken, Peter van der Spek, Frank Grosveld, Dick Tibboel and Robbert J. Rottier.
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Am. J. Respir. Cell Mol. Biol. 2012; 46: 224-232

PHD PORTFOLIO SUMMARY

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		Workload	
PhD training	**Year**	**Hours**	**ETCS**
General academic skills			
Biomedical English Writing and Communication	2010		4 ETCS
Laboratory animal science (art 9)	2007		3 ETCS
Radiation Safety Course (Level 5b)	2007		
“Safety working in laboratory”	2011		
Specific courses (e.g. Research school. Medical Training)			
Experimental Approach to Molecular and Cell Biology	2008	168hrs	
From development to diseases	2009		
Photoshop and Illustrator CS5 workshop	2010		0,3 ETCS
Seminars and workshops			
MGC workshop Brugge, Belguim (Poster Presentation)	2009	32hrs	
MGC workshop Koln, Germany (Oral Presentation)	2010	32hrs	
MGC workshop Maastricht, Netherlands (Oral Presentation)	2011	32hrs	
Monday Morning Meetings	2007-2012	320hrs	
International conferences			
“American Thoracic Society” New Orleans LA (Presentation)	2010		

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