THE PATHOGENESIS OF PULMONARY HYPOPLASIA IN CONGENITAL DIAPHRAGMATIC HERNIA

A continuing quest

Rhiannon B. van Loenhout

The Pathogenesis of Pulmonary Hypoplasia in Congenital Diaphragmatic Hernia:
A continuing quest

Rhiannon Bojoura van Loenhout

The research studies described in this dissertation were conducted at the Lung Development Programme at the Department of Physiology & Experimental Medicine, the Hospital for Sick Children, Toronto, Canada, and, the Department of Pediatric Surgery, Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands.

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The Pathogenesis of Pulmonary Hypoplasia in Congenital Diaphragmatic Hernia:
A continuing quest

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Een voortdurende strijd

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General Introduction & Outline Of The Dissertation

GENERAL INTRODUCTION

Congenital diaphragmatic hernia or CDH is a developmental defect of the diaphragm that allows abdominal organs, such as intestines and liver, to herniate into the thoracic cavity during lung development. CDH has a prevalence of 1 in 2000 – 3000 newborns and accounts for approximately 8% of the known major congenital anomalies [1]. In humans, three different types of hernia can be distinguished: a posterolateral Bochdalek-type (~70% of the cases), an anterior Morgagni-type (~27% of the cases) and a central hernia, septum transversum-type (~2 – 3% of the cases). Eighty-five percent of the hernias occur on the left side, 13% on the right and only 2% bilateral (reviewed in [2-4]). Children with a CDH suffer from a substantial amount of morbidity and mortality due to the associated abnormal pulmonary development resulting in two clinical problems, pulmonary hypoplasia and persistent pulmonary hypertension of the neonate (PPHN). Characteristics of pulmonary hypoplasia in CDH are thickened alveolar walls, an increase in interstitial tissue, reduced alveolar air spaces and reduced gas-exchange surface area. Apart from the gas exchange layer, well-documented changes are present in the vascular components consisting of media hyperplasia, peripheral muscularization of pre-acinar vessels and adventitial thickening. Both conditions are present in patients with CDH to a variable extent and despite the fact that recent progress in the care of these children has resulted in survival rates of up to 90% in some tertiary care centers, these measures have not led to a lower morbidity [5-7]. In contrast, due to the absence of sufficient lung-protective strategies, most of the newer treatment modalities have replaced mortality for a higher morbidity in these babies. The problem with these new treatment modalities, such as high frequency oscillation (HFO) and/or inhaled nitric oxide (NO) and extracorporeal membrane oxygenation (ECMO), is that they are designed for treating the sequelae of CDH, pulmonary hypoplasia and PPHN and do not contribute to the prevention of these conditions. Prenatal modulation by minimal invasive techniques such as tracheal occlusion may potentially lead to diminished need of supportive care postnatally by inducing lung growth [8]. However, a sound understanding of the etiology and pathogenesis of CDH is necessary in order to prevent the severe morbidity or the anomalies altogether. Essential elements required for a better understanding, such as how the different clinical problems relate to each other, are still lacking. A basic understanding of CDH together with pulmonary hypoplasia and PPHN is fundamental in our quest for new answers to protect these children from the sequelae of this anomaly.

Consequently, the aim of this dissertation was to improve our understanding of the pathogenesis of pulmonary hypoplasia in CDH, to eventually aid in finding ways to modulate the natural course in a prenatally diagnosed child.

OUTLINE OF THE DISSERTATION

As stated above, the pathogenesis and etiology of CDH and its associated anomalies are still largely unknown despite all research efforts over the past years. Several animal models are available to study CDH, including the nitrofen rodent model, a surgical lamb or rabbit model and multiple genetic mouse models. In **Chapter 2** we discuss the strengths and limitations of these three main models, and their relevance to the human situation.

Previously, the associated pulmonary hypoplasia was interpreted as a result of the 'compression' of the developing lung by herniated abdominal organs. A decade ago, pulmonary hypoplasia was found to be present before the closure of the diaphragm, which prompted the foundation of the dual-hit hypothesis [9, 10]: pulmonary hypoplasia was not solely caused by the herniated organs but also present due to an intrinsic defect of the lung itself. Subsequently, research focused on the intrinsic defect. One of the main questions of this intrinsic pulmonary defect is which of the two major tissue layers, epithelium or mesenchyme, is affected? In **Chapter 3** we utilize an *in vitro* recombinant model for pulmonary hypoplasia to answer this question.

In order to decipher the exact pathogenesis of developmental diseases, it is necessary to understand the physiology of normal, healthy development. In **Chapter 4** we describe the influence of developmental stage on epithelial-mesenchymal interactions during normal fetal rat lung development.

In **Chapter 3** we established that fibroblasts are the primary malfunctioning tissue layer, which disturbs the normal epithelial-mesenchymal interactions necessary for proper lung development. The origin of these cells however is unknown. Recently, a new theory involving cell transformations has arisen to explain both normal morphogenesis and the development of diseases such as fibrosis and malignancies [11-15]. Epithelial-mesenchymal transition (EMT) is the transformation of an epithelial cell into a mesenchymal (fibroblast) cell. One explanation for the origin of the defective fibroblasts could be this process (pilot study in **Chapter 5**).

Translational research is necessary to validate the results of fundamental research in the clinical setting. While CDH animal models have generated new hypotheses of human CDH and its associated anomalies, tissue availability to test these hypotheses has decreased dramatically [16-19]. Both the improved survival and decrease in permission to perform full autopsy can be accounted for this. In 2001 a new protocol was instituted in our hospital to increase the accrual of CDH lung tissue via a quick and minimal invasive method: postmortem lung biopsy through a mini-thoracotomy. In

Chapter 6 we describe how we were able to quadruple the accrual of human lung tissue for both diagnostic and translational research.

CDH consists of two major defects: the diaphragmatic hernia itself and the associated lung anomalies. It is very likely that these two defects share the same original insult: the 'two defects, one origin'-theory. The retinoic acid pathway has been implicated in the pathogenesis of CDH. Several transcription factors involved in the retinoic acid pathway have been described in both the diaphragm and lungs, such as COUP-TFII and GATA4. In addition, these factors are located on chromosome regions commonly deleted in individuals with CDH, which supports a similar orginal insult [20-22]. In normal organogenesis, vessel development is a prerequisite. The Von Hippel-Lindau (VHL) pathway is a pathway important for angiogenesis and vessel function. In human CDH lungs, disruptions in the VHL pathway were observed previously [23-26]. To investigate this pathway in the human diaphragmatic situation, we investigated the VHL pathway in diaphragmatic tissues from patients with CDH (**Chapter 7**).

In **Chapter 8** we discuss our major findings and future perspectives, and **Chapter 9** summarizes this dissertation in both English and Dutch.

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2

Congenital Diaphragmatic Hernia: A Comparison of Animal Models and Relevance for the Human Situation

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Adapted from Neonatology, 2009 (96): 137 – 149

INTRODUCTION

Congenital diaphragmatic hernia (CDH) occurs in one in 3000 newborns. Mortality and morbidity are due to the amount of pulmonary hypoplasia, the response to artificial ventilation and the presence of therapy resistant pulmonary hypertension. The pathogenesis and etiology of CDH and its associated anomalies are still largely unknown despite all research efforts over the past years. Several animal models have been proposed to study CDH. The main animal models available to study CDH are the surgical rabbit or sheep model, the pharmacological (nitrofen) rat or mouse model, and the genetic (knockout) mouse model. In this chapter we will describe these three models and discuss their strengths and limitations to study pulmonary hypoplasia, and we will address their differences and discuss the relevance for human CDH.

SURGICAL MODELS

One of the hypotheses of the pathogenesis of pulmonary hypoplasia in CDH is that intrathoracic herniation of the abdominal viscera results in compromised pulmonary development. Fetal breathing movements are impaired and, therefore, normal development of the lungs is hampered. Based on this idea, the first surgical animal models were created to study both lung pathogenesis and rescue options. The most commonly used surgical models are in sheep and rabbits.

The sheep model was introduced by Delorimier in 1967 [1]. The hernia is surgically created at gestational day 72 – 75 (term is 145 – 149 days). The abdominal bowel is positioned into the chest to optimally mimic human CDH. Gestational day 72 – 75 in sheep is equivalent to a gestational age of 10 weeks in humans. This is the pseudoglandular stage of lung development: the moment of pleuroperitoneal canal fusion during diaphragmatic development [2]. Later, a similar surgical model was developed in rabbits. Advantages of the rabbit model over sheep are its shorter gestational period (term is 31 days, the hernia is created at day 23), the larger litter size, easy availability and lower costs [3, 4].

Surgical models are mainly suitable to investigate interventional strategies in CDH. Examples of investigated interventions are the administration of corticosteroids, retinoic acid or growth factors, *in utero* repair of the diaphragmatic defect and fetal tracheal occlusion or a combination of the two [5-10]. *In utero* repair has been attempted with either closure of the defect by using a patch (immediate reduction) or by using the slow 'silo' reduction technique in which the opening gradually reduces as the

fetus grows [11-13]. After successful *in utero* repair of induced CDHs in animal models, including in non-human primates, Harrison et al. performed the first human surgical repair *in utero*. Unfortunately, it quickly became clear that there was no improvement in survival and moreover, an increase in premature delivery was observed [12].

Later, tracheal ligation or clipping was developed with the aim to gradually reposition the abdominal viscera back into the abdomen [14-16]. The rationale was based on the observation that children with a prenatal airway obstruction have hyperplastic lungs [17]. Preventing lung fluid efflux exerts a build-up pressure in the thoracic cavity that repositions the abdominal viscera back in the abdomen. Di Fiori et al. demonstrated that tracheal ligation reversed the effects of surgically induced pulmonary hypoplasia in the sheep model [15]. Unfortunately, results of human trials on this ex-utero intrapartum treatment (EXIT) technique with clipping were disappointing even when a minimal invasive approach was used. Again premature delivery appeared to be the problem [18-20]. Later the 'plug the lung until it grows' (PLUG) method was developed in lambs. Endoscopically an inflatable balloon is inserted through the fetal mouth in the trachea through a catheter, which then is filled with saline and kept in place for various days [21-26]. This demonstrated improved lung morphogenesis and function [27]. A tracheal occlusion trial in humans in North-America demonstrated no differences in survival when compared to controls. The authors blamed this on an improvement in survival in the control group due to the increased care in a specialized center [20]. However, Deprest et al. stated that a great part of the enrolled patients were likely to have survived without treatment based on the 'lung area to head circumference ratio' (LHR) risk assessment [28, 29]. Therefore, in the European programme (FETO task force), only the most severe CDH cases (LHR < 1.0) were enrolled and underwent the Fetoscopic Endoluminal Tracheal Occlusion (FETO)-procedure. This study demonstrated up to 64% survival in comparison to 8% survival of non-treated comparable CDH patients, and thus appeared very promising [28, 30-32]. The same group demonstrated similar results in a more homogeneous group a year later [33]. Premature prelabor rupture of the membranes, with the risk of premature delivery, appeared to be a common complication, but in a more recent study it was shown that there was an increase to 69% of deliveries after 34 weeks when FETO was performed due to improved experience [34]. The most recent publication comparing FETO trials demonstrated a survival rate of up to 58%, in a study of 16 patients and, likely more accurate, an overall survival of 49% in the largest study which contained data from 210 FETO-patients [35]. Optimal timing of prenatal removal of the balloon was set at 34 weeks; a longer duration had a negative effect on type II pneumocyte development in the surgical sheep model [25, 36-38]. Prior to FETO, observed-to-expected lung volume was the single most important predictor for survival; post-FETO the contralateral lung vascularization index at four weeks has been the best neonatal outcome predic-

tor in one uncontrolled study [39]. In postmortem studies of non-survivors of CDH with tracheal occlusion increased fetal lung growth has been observed. In contrast, no improvement of parenchymal structure or the muscularization of pulmonary arterioles was observed [40]. Ruano et al. however demonstrated the contralateral lung vascularization index to improve significantly in CDH survivors [39]. At this moment, the Tracheal Occlusion To Accelerate Lung growth (TOTAL)-trial is conducted to investigate late FETO for moderate pulmonary hypoplasia and validate previous results [35].

Tracheal occlusion in surgical models has not only shown to improve pulmonary hypoplasia, but also pulmonary vascular abnormalities benefit from this procedure. For instance, tracheal occlusion studies in fetal sheep with a surgically induced diaphragmatic hernia demonstrated thinning of the pulmonary artery, correction of the abnormal muscularisation of pulmonary arterioles, and a decrease in vessel resistance in the left pulmonary artery with maternal hyperoxia as seen in normal fetal sheep at term [41-43]. In fact, the sheep model has been used on many occasions to study pulmonary vascular abnormalities in CDH, but this is beyond the scope of our review [44, 45]. Similar results of (treatment of) pulmonary vascular abnormalities in CDH have been demonstrated in the rabbit model as well [26, 46, 47].

Surgical models are based on a surgical intervention making a diaphragmatic defect relatively late in gestation in both rabbits and sheep [48, 49]. This model has been proven especially useful in investigating interventional therapies, such as the administration of corticosteroids, *in utero* repair of the diaphragmatic defect and tracheal occlusion, but also to compare different tissue substitutes for hernia repair [10, 50-53]. Due to the fact that the diaphragmatic defect is created late in gestation after a period of normal lung and diaphragm development, we have to take into consideration that certain pathogenetic changes in human CDH might have occurred during lung development before this phase. Therefore, pulmonary hypoplasia in these models might have different characteristics and information about the cause and early pathogenesis of this hypoplasia cannot be obtained. Another disadvantage is that the investigated interventional therapies might not react in a similar manner, as they should in the human situation, because we are comparing a uni-hit *versus* a more severe dual-hit pulmonary hypoplasia [54]. On top of that, a lot of the other associated anomalies (such as heart defects) cannot be studied in this model.

In summary, surgical animal models are useful in investigating interventional therapies but are less instructive in studying the etiology and pathogenesis of CDH. Interventional studies in this model have resulted in the incorporation of new prenatal techniques in human fetuses, with very promising results so far.

NITROFEN MODEL

The nitrofen model has been used for the past two decades to investigate the anomalies in CDH. Originally, nitrofen (2,4-diclorophenyl-p-nitrophenyl ether) was used as a herbicide. In toxicology screens on adult rats no apparent problems were observed, though administration to pregnant dams during midgestation appeared to cause developmental anomalies to the heart, lungs, diaphragm, and skeleton of the embryos [55, 56]. Based on the latter findings, nitrofen has been investigated for its usefulness to simulate the anomalies of CDH in rodents. Numerous groups including ours demonstrated that nitrofen induced diaphragmatic hernias that were strikingly similar to the human condition. The specific location and extent of the diaphragmatic defects were very comparable, but also the similarities in the CDH-associated anomalies, including pulmonary hypoplasia and persistent pulmonary hypertension, and cardiovascular and skeletal defects as well, were impressive [57-60]. When nitrofen is administered to pregnant rat dams on day 9 of gestation when normal lung (day 11 of gestation) and diaphragm development (day 13 of gestation) are just about to begin, approximately 70% of the offspring will have a CDH and 100% pulmonary hypoplasia. Therefore, the nitrofen animal model, taking into account the obvious disadvantages of being a toxicological (animal) model, can serve as a good tool to investigate the pathogenesis and therapeutical options in CDH and its anomalies in rodents. Despite the extensive use of nitrofen as a herbicide in agriculture, its possible teratogenetic effects have never been proven to play a role in human CDH.

In 2000, the dual-hit hypothesis was introduced. It explained pulmonary hypoplasia in CDH as a result of two insults [54]. There is an early bilateral nitrofen-induced pulmonary hypoplasia observed prior to closure of the diaphragm (first insult) [61, 62]. The second insult is caused by herniation of the abdominal viscera into the thorax due to disrupted closure of the diaphragm and affects the ipsilateral lung only by interference with fetal breathing movements. The etiology of both human CDH and nitrofen-induced CDH in rodents has been connected to perturbations in the retinoid signaling pathway (Figure 1), although the exact underlying mechanism remains to be elucidated. The first evidence that CDH could be connected to perturbations in the retinoid signaling pathway was obtained already in 1941 by Andersen who noted diaphragmatic hernias in embryos of pregnant rats on a vitamin A-deficient diet [63]. This effect of maternal vitamin A deficiency was confirmed by Wilson et al. in 1953 [64]. More modern approaches using genetic manipulation in mice have shown that ablation of retinoic acid receptor (RAR) signaling during development indeed results in diaphragmatic hernias, pulmonary hypoplasia and/or lung agenesis [65]. In humans, newborns with CDH had lower levels of plasma retinol and retinol binding protein (RBP) in cord blood than controls [66, 67]. Nitrofen-treated rats demonstrated

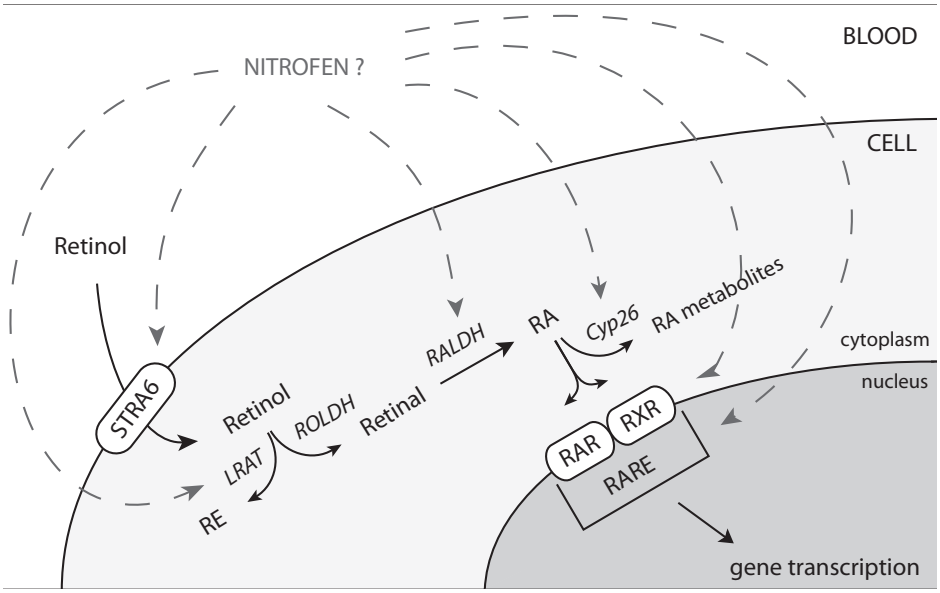

Figure 1. Schematic representation of the retinoid signaling pathway. Retinol binds STRA6 and is transferred into the cytoplasm. Retinol can either be stored as retinyl ester (RE) by lecithin : retinol acyltransferase (LRAT) or be converted into retinal by retinol dehydrogenase (ROLDH). Retinal is converted into retinoic acid (RA) by retinal dehydrogenase (RALDH). RA can either remain in the cytoplasm to be metabolized by cytochrome p450 (Cyp) 26 enzymes, or bind to retinoic acid receptor (RAR) or retinoid X receptor (RXR) to activate the retinoic acid response element (RARE) and thereby alter gene transcription. Pathways that might be influenced by the effects of nitrofen are indicated by dashed arrows.

decreased protein levels of both transport proteins RBP and transthyretin in lungs [68]. In addition, CDH has been observed in patients with deletions on the 15q chromosome, which contains the encoding gene for a cellular retinoic acid binding protein (CRABP1), although so far mutation analysis in isolated CDH cases are negative [69-71]. Administration of retinoic acid (RA) to nitrofen-treated lung explants demonstrated an increase in lung growth and partially rescued the hypoplasia [72]. This observation was supported by Thebaud et al. who demonstrated an improvement in lung maturation and growth in nitrofen-treated embryos when the pregnant dam was treated with vitamin A either before, during or after the nitrofen administration [73, 74]. In addition, survival of the fetuses improved in the vitamin A-treated group [74]. Subsequently, administration of RA and vitamin A were compared in their effectiveness to reduce the number of hernias. The untreated group had 54% hernias. With vitamin A treatment, the number of hernias was reduced to 32%. RA demonstrated a reduction to 15%, and with continuation of the RA feedings up to 5 days, a percentage less than 10% was even reached [75]. This supports the concept that both the diaphragmatic hernia and the pulmonary hypoplasia are resulting from a disruption in the retinoid signaling pathway. *In vivo* prenatal treatment of RA to nitrofen-treated rats

improved alveolization [76]. In these fetuses, acceleration of type I alveolar epithelial cell proliferation was demonstrated, a process important for lung development [77]. The improvement in alveolization after RA treatment could be explained by the upregulation of several genes involved in this process [78-82].

Retinal dehydrogenase (RALDH) 2 is perceived to be the key enzyme in the RA synthetic pathway [83-85]. *In vitro* experiments have demonstrated that several agents (including nitrofen) responsible for the induction of diaphragmatic hernias, inhibit RALDH2 activity [86, 87]. In contrast, Kling et al. demonstrated the decline in RA in nitrofen-treated Hek-293 cells to be independent of RALDH inhibition [88]. The authors attributed the decline in RA to nitrofen-induced cell toxicity, as they observed increased cell death. Others have proposed that nitrofen interferes with the uptake of retinol by lung cells. Nitrofen-treated lungs have lower retinol levels while circulating retinol levels are increased in comparison to controls, in agreement with the idea of a disturbed uptake of retinol [89]. Recently, STRA6 has been identified as the membrane receptor for serum retinol and mutations in *STRA6* result in diaphragmatic hernias and pulmonary hypoplasia amongst a variety of other anomalies [90-92]. However, nitrofen does not block the uptake of retinol by STRA6 [87]. Nitrofen treatment has been reported to downregulate the pulmonary retinol storage enzyme, lecithin : retinol acyltransferase (LRAT), and the RA-degrading enzyme Cyp26, while not affecting RALDH2 (42). Since vitamin A deficiency experiments have shown similar decreases in RA-degrading enzymes and -storage enzymes [93, 94], it is thought that this downregulation is due to low pulmonary retinol levels. The exact mechanism by which nitrofen influences RA signaling remains to be elucidated. Recently, Goumy et al. proposed *STRA6*, *LRAT*, *COUP-TFII*, and nine other retinoic-related genes as potential candidate genes for human CDH [95]. It has also been suggested that nitrofen may compete with RA to bind to the RAR during embryogenesis, thereby impairing lung and diaphragm development [74, 96]. In a two-hybrid yeast-assay nitrofen inhibited RAR and RXR association only when very high, embryonic lethal dosages were used [87]. In contrast, Chen et al. demonstrated that nitrofen inhibits the activation of retinoic acid response elements (RARE) in lacZ mice [97]. RAR expression is not affected in CDH. Rajatapiti et al. reported that it was normal in human CDH lungs and in nitrofen-induced rat CDH lung tissue [98].

In summary, the retinoid signaling pathway is complex and it appears that a disruption anywhere in the pathway might be responsible for the morphological changes including pulmonary hypoplasia seen in CDH. An interesting novel perspective to investigate the RA pathway in patients with CDH is the use of fetal skin fibroblasts, in which the *RALDH2* and *Cyp26* gene expression were altered [99].

Besides the retinoid signaling pathway, another pathway implicated in CDH is the thyroid hormone signaling pathway (Figure 2) [100]. Nitrofen has a similar chemical structure as triidothyronine (T3) and thyroxine (T4). All three are halogenated diphenyl ethers [101-103]. Thyroid hormones are important in lung morphogenesis [104-106]. Thyroid hormone receptors (TRs) are mostly expressed after gestational day 13 in the rat [107], but it has been demonstrated that very low levels of message are present at day 11 in the embryo [108]. Both T3 and T4 can cross the placenta during embryo morphogenesis in the rat from gestational day 9 onwards [109-111]. Therefore, it is possible that nitrofen influences both diaphragm formation and lung development by

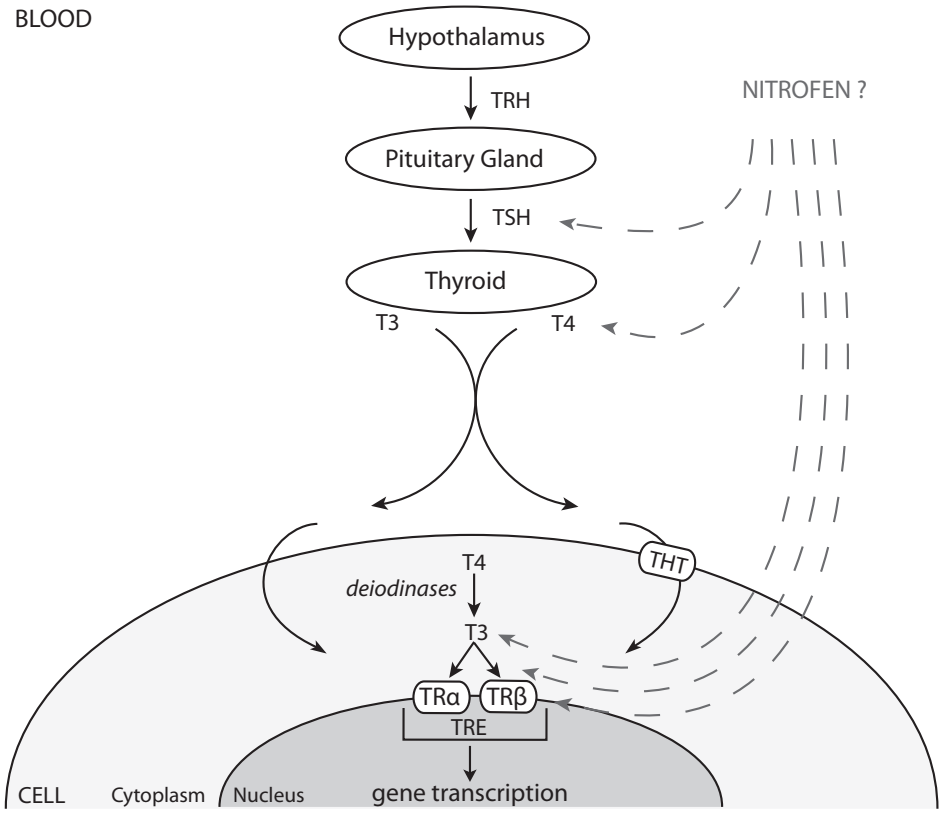

Figure 2. Schematic representation of the thyroid signaling pathway. Thyroid hormones thyroxine (T4) and the triiodothyronine (T3) are produced in the thyroid gland. The hypothalamus produces thyrotropin-releasing hormone (TRH), which stimulates the pituitary gland to release thyroid-stimulating hormone (TSH). TSH directly acts on the thyroid gland to stimulate T4 and T3 synthesis. At the target cells, membrane passage is either carrier-mediated by thyroid hormone transporters (THT) or by diffusion. In the cell T4 is converted to T3 by deiodinases and binds to the nuclear thyroid hormone receptors (TRs) TRα and TRβ. Through activation of the thyroid response element (TRE) gene expression is altered. Pathways that might be influenced by the effects of nitrofen are indicated by dashed arrows.

interfering with the thyroid hormone signaling pathway [87]. However, nitrofen-treated adult mice have decreased T4 levels while T3 levels remain normal [101]. In addition, fetuses of pregnant rats treated with nitrofen have lower circulating T3 and T4 levels, but pulmonary levels of T3 and T4 are not changed when compared to control fetuses [102, 112, 113]. If nitrofen exerts its action due to structural similarities with thyroid hormones (thyromimetica), it would be in a competitive manner. Nonetheless, Brandsma et al. demonstrated that nitrofen inhibits binding of T3 to TRα1 and TRβ1 in a non-competitive manner by reducing the maximal binding capacity *in vitro* [103]. In contrast, Noble et al. found no perturbation in TR binding in the presence of nitrofen [87]. When nitrofen and T4 were administered simultaneously to thyroidectomy pregnant rats, the incidence of congenital anomalies in embryos dropped with 70% [102]. Despite this observation, co-administration of T4 and nitrofen did not reduce the percentage of CDHs [87].

Similar to RAR, TRα1 and TRβ1 belong to the steroid/thyroid/retinoid receptor superfamily. Noble et al. demonstrated that TRα1 and thyroid response element (TRE) activity were not influenced by nitrofen *in vitro* [87]. *In vivo*, however, nitrofen reduced the expression of TRα1 and TRβ1 in CDH rat lungs [114] without changing their cellular localization [98]. A similar decrease in TRα1 expression was noted in human CDH related hypoplastic lungs [98]. This decrease in TRα1 could lead to a diminished response to maternal thyroid hormone (TH) and later on (from gestational day 18 in rats) to the hormones produced by the fetus itself. In this way lung morphogenesis might be affected by nitrofen [114]. As described earlier, Montedonico et al. demonstrated a partial rescue by RA treatment in nitrofen-induced hypoplastic lung explants. The authors suggested that this might be only partial because downregulation in the thyroid hormone signaling pathway might also contribute to the pulmonary hypoplasia [72]. However, we should bear in mind that the role of thyroid hormones appears to be limited since in TR null mutant mice no apparent lung and diaphragm problems were observed and, therefore, antenatal lung growth does not seem to be impaired by a lack of thyroid hormones [115]. Van Tuyl et al. demonstrated that both maternal and fetal hypothyroidism in transgenic mice did not alter prenatal lung development [116]. However, Pei et al. showed repression of TR by knock-in mutations of the nuclear co-repressor Silencing Mediator of Retinoid and Thyroid hormone receptors (SMRT) was linked to a respiratory distress syndrome with premature type I pneumocytes [117].

In summary, TRα1 expression is reduced in both CDH lungs of nitrofen treated rats as in human hypoplastic CDH lungs, which might suggest a role of TRs in the lung pathogenesis of CDH. However, the lack of both lung and diaphragm defects in TR null mice makes this less likely. Even though nitrofen has a similar structure as the

thyroid hormones, a clear relation between the thyroid signaling pathway and lung or diaphragm defects has not been demonstrated so far.

Recently the scope of the nitrofen model has broadened from purely pathogenetic research to a combination with surgical treatment. Tracheal occlusion was investigated in the nitrofen model, which is necessary to investigate the influence of this prenatal treatment onto the 'dual-hit'-affected lung. Tracheal occlusion improved lung branching, as observed in the surgical sheep and rabbit model [118, 119].

Not only pulmonary hypoplasia has been investigated in the nitrofen model but, as in the surgical models, vascular development in CDH has also been used for this purpose. An example is the study of vascular muscle tone *in vitro* by Belik et al. [120]. However, this is beyond the scope of our review.

The nitrofen model is based on the administration of the herbicide before the onset of lung and diaphragm formation. Although this model appears to be the best model available since the timing of the developmental insult is similar to that in humans, a large disadvantage is that the significance of the potential teratogenic effects of nitrofen in rodents has never been proven in humans. Increasing evidence about the etiology of CDH is pointing towards a disturbance in the retinoid signaling and/or thyroid signaling pathway, but this model has not yet led to a clear explanation of the pathogenesis of CDH and the associated pulmonary hypoplasia.

GENETIC MODELS

Since the first knockout mice were produced at the end of the eighties, they have been widely employed by molecular biologists to investigate the function of the gene that is made inoperable. In this way several expected and unexpected genes have been linked to CDH.

Wilm's Tumor 1 (Wt1)

The original paper describing the phenotype of Wilm's Tumor 1 (*Wt1)* null mutant mice focused on the role of this tumor suppressor gene in urogenital development [121]. In the same paper the authors briefly describe the incomplete formation of the diaphragm in the mutants resulting in the herniation of the lungs into the abdominal cavity, whereas in human CDH abdominal contents normally herniate into the thorax. However, in a recent publication Clugston et al. describe a more classical picture of abdominal contents herniating into the thorax in a comparative study on diaphragm development in three animal models for CDH including *Wt1* null mutant

mice [122]. In addition, the authors observe a 'real' posterolateral (Bochdalek) hernia based on the malformation of the pleuroperitoneal folds (PPFs) as opposed to different diaphragmatic defects observed in other knockout mice. An accurate indication of the incidence of CDH could not be calculated because of the small numbers of fetuses investigated. In addition, in the nitrofen rat model a downregulation of *Wt1* gene and protein expression was observed in the PPF of diaphragms [123]. Despite the 'true' Bochdalek phenotype of the *Wt1* null mutant mice, a translation to the human situation of CDH has not been made. Besides a few reports of mutations of *WT1* in human case reports on syndromic CDH such as WAGR and Denysh Drash, no relationship between the presence of the *WT1* mutation and isolated CDH was found [124-127]. In a Swedish series of 27 children with isolated CDH no *WT1* gene mutations could be detected [128].

Sonic Hedgehog (Shh) and Gli2/Gli3

Sonic Hedgehog (Shh) and *Gli2* and *Gli3* are members of a highly conserved morphogenetic family known as the *Shh*-signaling pathway [129]. In the original publications on the functions of *Shh* and *Gli2* and *Gli3* no mention was made on the diaphragmatic defects some of the null mutant mice displayed. These papers focused on the foregut anomalies, such as abnormal branching morphogenesis of the lungs, tracheal-esophageal fistula and esophageal atresia, respectively [130-132]. In *Shh* null mutants there is a failure of tracheo-esophageal separation and, in addition, the lungs have undergone less branching morphogenesis, making them hypoplastic [130, 132]. Nitrofen-treated mice however, did not demonstrate any differences in spatial transcript distribution and gene expression levels in early lung development compared to controls [133]. In addition, in wildtype mice *Shh* was not expressed in normal primordial PPF diaphragms but was strongly present in developing E11.5 lungs. Dispatched Homolog 1 (*Disp1*), which is required for *Shh* function, was specifically expressed in the PPF [134]. Interestingly, *Shh* expression is decreased in human hypoplastic lungs of CDH patients [135], and recently, *DISP1* sequencing revealed a de novo mosaic point mutation in a patient with non-isolated left-sided Bochdalek diaphragmatic hernia [134].

Studies from *Gli2* and *Gli3* double knockout mice demonstrated a similar phenotype of foregut abnormalities, but a more severe phenotype of disturbed branching morphogenesis. *Gli2*$^{-/-}$ mice have only one lobe on the right side (instead of four) indicating that lungs are formed, but primary branching is affected. In *Gli2*$^{-/-}$/*Gli3*$^{-/-}$ mice no lungs are formed at all. In *Gli2*$^{-/-}$/*Gli3*$^{+/-}$ mice there is ectopic branching and fusion of lung lobes [131]. However, none of the first studies described a CDH in these mice. In a more recent publication, the same group demonstrated that the single null mutant mice for both *Gli2* and *Gli3* as well as the double mutant *Gli2*$^{-/-}$/*Gli3*$^{+/-}$ mice have

diaphragmatic defects [136]. No description of the type of hernia was given for either *Shh* or *Gli* null mutant mice. No relationship between a mutation in *GLI* genes and CDH has been demonstrated in humans so far.

Slit3

Slit3 belongs to the family of Slit guiding proteins that are highly conserved throughout evolution. Especially *Slit1* and *Slit2* have been investigated for their role in axon guidance and cell migration (reviewed in [137]). Approximately 70% of *Slit3* null mutant mice have a CDH [138, 139]. In contrast to other animal models for CDH, most of these mice do not die after birth. The mice display a defect in the central tendon of the diaphragm, which fails to detach from the liver on the right side, thereby making it a good model of the human central (septum transversum) type of hernia. The origin of the defect lies in a defective connective tissue formation in the central septum transversum. The innervation of the phrenic nerve to the diaphragm was found to be normal in the knockout mice. No primary lung phenotype was observed in the mice. However, mice with end stage CDH were short of breath due to atelectasis and intrapulmonary hemorrhage. In addition, hypoplastic lungs of nitrofen-treated rats exhibit increased *Slit3* expression [140]. Altogether, these data suggest that *Slit3* might play a role in a small subgroup of CDH cases. Recently, a case report of a newborn with CDH with a hernia sac attached to the liver was published [141]. The left side of the liver formed a part of the sac of the CDH. Liver tissue including vessels were seen in the sac, as seen in *Slit3*$^{-/-}$ mice [139]. This is the first time that presence of hepatocytes within the sac itself was observed in a patient with CDH.

Fog2 (Friend of GATA2)

Employing an elegant approach of high throughput mutagenesis analysis using the chemical mutagen N-ethyl-N-nitrosourea (ENU), Ackerman et al. identified a mouse line with respiratory failure at birth [142]. This phenotype was based on pulmonary hypoplasia, disturbed heart development and a diaphragmatic defect and could be linked to a mutation in the gene *Fog2 (Friend of GATA2)*. In contrast to the other models these mice displayed severe pulmonary hypoplasia in the absence of a hole in the diaphragm. Instead a posterolateral muscularization defect was observed. As such, this phenotype correlates better to the human situation of CDH with regards to the existence of pulmonary hypoplasia. Interestingly, the authors demonstrated that pulmonary hypoplasia occurs independently from deficient diaphragm development. This is a situation that can also be observed in unilateral agenesis of the lung with an intact diaphragm. We and others have demonstrated that the same holds true for lung development in the nitrofen model of CDH [54, 143, 144]. However, a clear relationship between disturbed lung development and diaphragmatic development

has not been shown. It is more likely that both lungs and diaphragm are disturbed in their development separately.

Subsequently, the authors searched for *FOG2* mutations in a series of 30 autopsy specimens of CDH patients and found a nonsense mutation in a female patient with severe bilateral pulmonary hypoplasia and a posterior diaphragmatic eventration on the left side. The authors suggested that this was the first reported gene mutation in a patient with CDH and pulmonary hypoplasia. More recently, Bleyl et al. reported novel *FOG2* sequence variants in two isolated CDH patients, but could not identify them as mutations [145].

Gata4 and Gata6

Fog2 can interact with many different transcription factors such as the *Gata* zinc finger transcription factors *Gata4* and *Gata6*. Null mutant mice for both *Gata4* and *Gata6* die early in embryonic development because of their essential roles in ventral morphogenesis (including heart development) and differentiation of visceral endoderm, respectively [146-148]. Therefore, these models could not be used to evaluate their roles in lung or diaphragm development that occur later in gestation. However, we and others demonstrated that *Gata6* is essential for normal branching morphogenesis of the lung and late epithelial cell differentiation using a chimeric mouse mutagenesis approach [149-151]. So far *Gata6* has not been implicated in diaphragm development. Jay et al. recently observed a role for *Gata4* in lung or diaphragm development [152]. They noticed disturbed heart, lung and diaphragm development in approximately 70% of heterozygous *Gata4* knockout mice that were generated in a different genetic background [152]. The mutation resulted in a mortality of up to 40%. The defect in the diaphragms consisted of a ventral hernia covered with a sac that was not attached to the liver, but allowed abdominal viscera to protrude. The incidence of CDH was approximately 30%. Pulmonary development in the mutant mice was not very disturbed, although the authors describe some airway dilatation and altered expression of certain genes in the most affected mice. In addition, another study recently demonstrated that *Gata4* is important for normal pulmonary lobar development [153]. Interestingly, a microdeletion on human chromosome 8p23.1 that includes the *GATA4* gene, has been linked to isolated human cases of CDH especially in combination with cardiac anomalies [154-157]. A recent report discussed a patient with an 8p23.1 deletion with pulmonary hypoplasia and an anterior diaphragmatic defect, similar to the location of the defect reported in heterozygous *Gata4* mice [152, 158].

COUP-TFII

Another transcription factor that is a binding partner of *Fog2* is *COUP-TFII*, which belongs to a nuclear steroid/thyroid/retinoid hormone receptor superfamily and

has been shown to be essential for embryonic mouse development [159]. Mice lacking *COUP-TFII* show defects in cardiovascular development and die around day 10.5 of gestation. Conditional mutagenesis of *COUP-TFII* in mice using the Cre-lox conditional knockout system to ablate *COUP-TFII* function in the mesenchyme only resulted in a Bochdalek-type diaphragmatic defect on the left side [160]. However, the authors did not find a deficient lung phenotype in these mice, although this might be due to the tissue-specific ablation of the gene in the mesenchyme. In line with the latter idea, the authors describe that *COUP-TFII* expression is markedly decreased in the structures contributing to the developing diaphragm such as the PPF, but the expression was only slightly reduced in the developing lung. However, in lungs and the PPF of diaphragms of nitrofen-treated rats, gene expression of *COUP-TFII* was significantly increased [161, 162]. These results are even more interesting in the light of the location of *COUP-TFII* on human chromosome 15q26. Our group has identified this region as a potential candidate region for human patients with isolated CDH [163, 164]. However, following an evaluation of over 130 cases of isolated CDH from different hospitals, no mutations in the coding regions of *COUP-TFII* have yet been identified.

PDGFRα

Very recently, the platelet-derived growth factor receptor alpha (*PDGFRa*) gene has been identified as an important factor in the formation of the diaphragm and lung development [145]. This gene is known for its role in tumorigenesis of gastrointestinal and neural tumors [165-168]. In *Pdgfra* null mice, Bleyl and colleagues observed pulmonary hypoplasia and a range of diaphragmatic defects including posterolateral diaphragmatic hernias [145]. Interestingly, Dingemann et al. demonstrated upregulated gene and protein expression in nitrofen-treated fetal rat lungs [169]. This and the other phenotypical characteristics observed are similar to the human Fryns syndrome (non-isolated CDH) [170]. Hence *PDGFRa* might be a candidate gene for non-isolated CDH. Moreover, in one patient with non-isolated CDH a novel sequence variant of *PDGFRa* was identified. The authors did not prove the variant to be a mutation [145]. Interestingly, successful treatment of a patient with CDH-associated pulmonary hypertension with a PDGFR-antagonist was recently described [171].

Retinoid signaling pathway in knockout mice

Increasing evidence from data obtained with the nitrofen model and knockout mice points towards perturbations in the retinoid signaling pathway as we described above. For example, *COUP-TFII* has been shown to be a downstream target of retinoid signaling (reviewed in [172]), and different *Gata* transcription factors have been demonstrated to interact with RA receptors [173]. In addition, prenatal treatment of RA in nitrofen-treated fetal rats upregulated *COUP-TFII, Fog2,* and *Gata4* gene expression [161]. Therefore, we also want to review the role of members of the retinoid

signaling pathway in knockout mice. The first evidence from knockout mice that RA is involved in the pathogenesis of CDH came from retinoic acid receptor (RAR) double knockout mice, as described earlier. Single RAR null mutant mice did not show the expected anomalies that were observed in the vitamin A deficient rats indicating that there is a high redundancy between the different types of receptors [174-177]. However, when the function of multiple receptors was abolished, multiple congenital abnormalities were observed including right-sided CDH in RARαβ2 and left-sided CDH in RARαβ2$^{+/-}$. In addition, these mice displayed severe pulmonary hypoplasia [65]. A more recent study of a pan-RAR antagonist (BMS493) demonstrated a very high induction rate of CDH, including the observation of apparent smaller ipsilateral lungs [178]. Despite the convincing data from animal studies, the results in humans have been limited. Until now the only described mutations in CDH patients related to the RA pathway are in *STRA6* and *CRABP1* on chromosome 15 [69-71, 90-92]. Our group demonstrated significantly lower retinol and retinol-binding protein levels in cord blood of CDH newborns in a case-control study of 56 patients [67].

Despite the growing amount of evidence that certain genes are involved in the pathogenesis of different types of CDH, until now only a mutation in FOG2 has been demonstrated in a single patient with non-syndromic CDH. This might be due to several factors. First, as described for the *Gata4* gene, the genetic background of the species carrying the mutation is of importance to the phenotype that is related to the mutation. The diaphragmatic defects were only observed in heterozygous C57Bl/6 *Gata4* mutant mice. This is also true for the rodent model based on the teratogenic effects of nitrofen. When nitrofen is administered to Sprague-Dawley rats, the percentage of the offspring having CDH is higher than following administration to Wistar rats. The same phenomenon has been observed in different mouse strains. Second, the pathogenesis of CDH might be explained by the necessity of multiple developmental insults to happen during development of the diaphragm and the lung. We and others demonstrated this scenario for pulmonary hypoplasia in the nitrofen model for CDH [54, 144]. We named this the dual-hit hypothesis. Finally, the observed phenotype in CDH is so diverse that it is potentially not due to a single gene mutation, but the result of multiple gene mutations. Different genes involved in different signaling pathways that have been shown to be important for normal embryonic development might be involved. In addition, as has been suggested for the nitrofen model, there maybe a disturbed interaction of certain genes with environmental factors.

CONCLUSIONS

Surgical models have been of great importance for validating new interventions in CDH. Many new approaches used today in CDH patients, for example the fetoscopic endoluminal tracheal occlusion, were first optimized in surgical animal models. However, these models are less suitable than either nitrofen or genetic models in elucidating the pathogenesis of CDH, since the diaphragmatic defect is created rather late in fetal development. Combining two models might improve our understanding [179]. Nitrofen has never been demonstrated to cause CDH in humans, despite its massive use as a herbicide. Nevertheless, the nitrofen rodent model has great similarities with human CDH. The most plausible pathogenetic explanation for CDH and its associated anomalies is a general genetic defect that causes the cardiovascular, lung and diaphragm defects. However, this particular genetic defect has not been identified, although some mice models come close to resembling human CDH, as discussed previously. A new approach to discover changes in the genetics of CDH in human cases is the genome-wide array. Genome-wide arrays are useful to compare the genetic changes between CDH patients to search for the existence of CDH-related genes. Until now this has only been done in non-isolated (syndromic) human cases, mainly for the Fryns sydrome [164, 180, 181].

Although none of the animal models have been perfect in mimicking human CDH and its associated anomalies, they have all shed a light on the underlying pathogenesis and pathology, and eventually prenatal modulation, of the disease. Increasing evidence from studies in both human CDH and animal models of CDH (nitrofen and knockout mice) suggest that a disturbance in the retinoid signaling pathway might be responsible for the anomaly. However, not all findings can solely be explained by disturbances in this pathway. Based on the spectrum of defects in heart, lungs and diaphragm, it is likely that there is a general defect in mesenchymal signaling in all involved organs in CDH with a variable genetic involvement in individual patients. This changing effect between epigenetic and genetic factors contributes to the highly variable phenotypic expression in the newborn. Our understanding of normal and abnormal diaphragm and lung development in relation to CDH is still incomplete. Eventually, such investigations will help in the design of new treatment modalities to better treat, modulate the natural course or even prevent this anomaly.

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3

The Pulmonary Mesenchymal Tissue Layer is Defective in Nitrofen-Induced Pulmonary Hypoplasia

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ABSTRACT

Background Despite modern treatments, congenital diaphragmatic hernia (CDH) remains associated with variable survival and significant morbidity. The associated pulmonary hypoplasia is a major determinant of outcome. To develop better treatment modalities, improved comprehension of the pathogenesis of pulmonary hypoplasia is warranted. We developed an *in vitro* cell recombinant model to mimic pulmonary hypoplasia and specifically investigate epithelial-mesenchymal interactions and decipher which tissue layer is primarily defective in nitrofen-induced CDH-associated pulmonary hypoplasia.

Methods Epithelial cells (E) and fibroblasts (F) were isolated from E19 control ($_C$) and nitrofen-induced hypoplastic rat lungs ($_N$). Cells were recombined and cultured as either homotypic [(F_C)(E_C) and (F_N)(E_N)] or heterotypic [(F_C)(E_N) and (F_N)(E_C)] recombinants.

Results Recombinants containing F_N fibroblasts had a thickened fibroblast tissue layer and there were fewer organized alveolar-like epithelial structures compared to control (F_C)(E_C) recombinants. These F_N recombinants exhibited a decrease in TUNEL and cleaved caspase-3-positive cells. Cell proliferation was arrested in recombinants containing F_N fibroblasts, which also exhibited increased $p27^{Kip1}$ and $p57^{Kip2}$ expression.

Conclusions Fibroblasts, and not epithelial cells, appear to be the defective cell type in nitrofen-induced hypoplastic lungs due to a decreased ability to undergo apoptosis and maintain overall proliferation. This may explain the characteristic pulmonary interstitial thickening and hypoplasia observed in both nitrofen-induced hypoplastic lungs as well as human hypoplastic CDH lungs.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a developmental defect of the diaphragm that allows abdominal organs to herniate into the thoracic cavity during lung development. CDH occurs in 1 in 2500 live births [1, 2]. Despite modern treatments CDH remains associated with variable survival and significant morbidity, mainly due to the associated anomalies of the lung [3, 4]. Although the exact pathogenesis is unknown, pulmonary hypoplasia is a major associated anomaly. Characteristics of human pulmonary hypoplasia in CDH are thickened alveolar walls, an increase in interstitial tissue, reduced alveolar air spaces and reduced gas-exchange surface area [2, 5-7]. Apart from the gas exchange layer, well documented changes are present in the vascular components consisting of medial hyperplasia, peripheral muscularization of preacinar vessels and adventitial thickening [8]. It has been demonstrated that the lung in itself is defective, and that pulmonary hypoplasia is not caused by compression of the lungs by herniated abdominal organs alone [9]. However, which of the two major tissue layers (epithelial or fibroblast/mesenchyme) in the lung is defective has never been addressed in detail. Such knowledge is required to design tissue-specific treatment modalities that can exclusively target the defective tissue layer in pulmonary hypoplasia, and thereby modulate or potentially prevent pulmonary hypoplasia in CDH.

Several animal models have been utilized to study CDH and/or the associated pulmonary hypoplasia such as the nitrofen rodent model, a surgical lamb or rabbit model and multiple genetic mouse models. The nitrofen model however, is the preferred model to study the pathogenetic aspects of CDH. This model was demonstrated to interfere with the retinoic acid (RA) signaling pathway (RA hypothesis), which was recently proven relevant in human CDH patients as well. [10-12]. Previously we utilized this nitrofen model to demonstrate that CDH-associated pulmonary hypoplasia is a result of two hits: an intrinsic problem in the hypoplastic lungs itself before development of the diaphragmatic defect, and interference with fetal breathing movements and competition of space of the lungs due to herniation of abdominal organs through the diaphragmatic defect [9]. In the present study, we were interested in the primary cause of the pulmonary hypoplasia, and therefore merely focused on 'the first hit', the intrinsic lung defect of the nitrofen model. Previous studies have demonstrated abnormal patterns in proliferation, apoptosis and cell differentiation in hypoplastic nitrofen-lungs, but the defective tissue layer has not been identified [9, 13].

Already 40 years ago it was shown that interactions between the different tissue layers are crucial for proper embryonic lung development [14]. Since then, many stud-

ies have contributed to the analysis of molecular determinants of lung growth [15, 16]. Previously we have demonstrated that fetal lung epithelial cells recombined with fetal lung fibroblasts reorganize in alveolar-like structures *in vitro* and that fibroblasts direct epithelial morphogenesis [17]. In addition, distal lung embryonic mesenchyme has been shown to induce expression of distal epithelial markers in proximal (tracheal) lung epithelial cells [18]. Thus, lung fibroblasts are essential for proper lung organogenesis. Consequently, a defective fibroblast layer could result in abnormal lung formation.

Knowing the defective tissue layer in CDH-related pulmonary hypoplasia is critical for designing improved treatment modalities specifically targeted at this defective tissue layer. To determine which lung tissue layer is defective in CDH, we used the above mentioned *in vitro* cell recombinant model [17]. Since the access to human (hypoplastic) lungs to perform such experiments is at best very limited, we utilized the nitrofen rodent model to develop a novel *in vitro* model for pulmonary hypoplasia to address this question.

Epithelial cells and fibroblasts isolated from control and nitrofen-treated lungs were recombined as either homotypic (control epithelial cells plus control fibroblasts or nitrofen epithelial cells plus nitrofen fibroblasts) or heterotypic (nitrofen epithelial cells or fibroblasts with healthy control fibroblasts or epithelial cells, respectively) recombinants (Figure 1). This approach enabled us to investigate the actual tissue interactions and the effects of a healthy opposing layer on a 'diseased' tissue layer, thereby gaining new insights into the pathogenesis of pulmonary hypoplasia in CDH and the potential role for epithelial-mesenchymal interactions. These recombination studies demonstrated that the fibroblast (mesenchymal) layer is the defective tissue layer in hypoplastic lungs due to a decreased ability to undergo apoptosis and

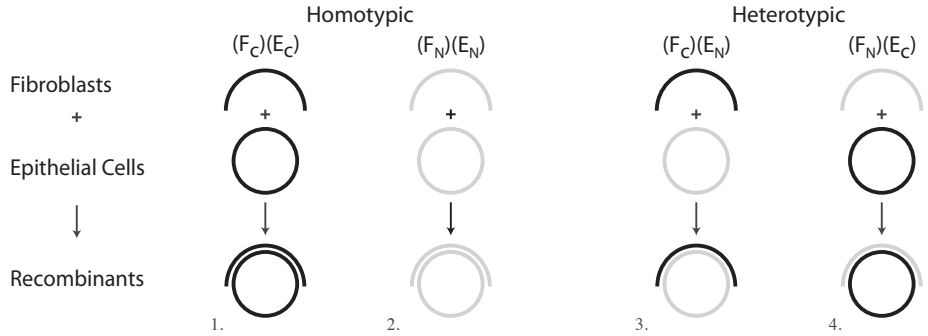

Figure 1. Schematic representation of the formation of the homotypic and heterotypic recombinants. Black represents control cells (C), and grey nitrofen-treated cells (N). The arcs denote fibroblasts (F), and the circles represent epithelial cells (E). Model 1 (F_C)(E_C) (control) and model 2 (F_N)(E_N) (nitrofen) are homotypic recombinants, while model 3 (F_C)(E_N) and model 4 (F_N)(E_C) are heterotypic recombinants.

maintain overall proliferation. This may explain the characteristic nitrofen-induced pulmonary interstitial thickening and hypoplasia as well as similar features noted in hypoplastic lungs in children with CDH.

METHODS & MATERIALS

Animals

The animal care committee of the Hospital for Sick Children approved all experimental procedures. Timed-pregnant Sprague-Dawley rats (*Rattus Norvegicus*) were ordered from Charles River (St. Constant (QC)).

Nitrofen treatment

Congenital diaphragmatic hernia and pulmonary hypoplasia were induced in pregnant rats using 2,4-dichlorophenyl-*p*-nitrophenyl ether (nitrofen) (Cerilliant, Round Rock (TX)) as described previously [9]. E19 embryos (term = 22 days) were collected by Caesarian section under aseptic conditions. Thoracic contents were removed and collected per group in ice-cold Hanks' Balanced Salt Solution (HBSS) (Invitrogen, Burlington (ON)). Lungs were microscopically separated from all other tissues including removal of the major airways. In nitrofen-treated embryos, lungs from both hernia-positive and hernia-negative embryos were used, as pulmonary hypoplasia is present in 100% of these embryos. In a control experiment, lungs from embryos with a hernia were separated from embryos without a hernia to investigate differences in the severity of hypoplasia in our recombinant model. We did not observe any obvious differences between recombinants of lung cells isolated from hernia-positive or hernia-negative embryos (results not shown). E19 whole lungs from nitrofen-treated and control rats served as *in vivo* controls for the recombinants.

Cell isolation and recombinant culture

Fetal epithelial cells and fibroblasts were isolated by primary culture as described previously [19]. After overnight culture, cells were washed, trypsinized and collected by centrifugation. Cells were counted and recombined ($3.0x10^6$ epithelial cells with $3.0x10^6$ fibroblasts in solution) in four different combinations, as depicted in Figure 1. Subsequently, recombined cells were spun down, and supernatant was removed. Cells were lightly stirred and incubated for one hour, and transferred onto inserts (Millipore, Etobicoke (ON), Canada). The recombined cells were cultured according to Deimling et al. [17]. After five days of culture, recombinants were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated and embedded in paraplast, and 5μm sections were cut. To ensure that isolated cells did not differ between the groups

prior to recombination with respect to differentiation markers and number of apoptotic cells, freshly isolated cells were grown on coverslips for immunocytochemical analysis.

Hematoxylin and Eosin (H&E) staining

Sections were rehydrated and stained with hematoxylin (Sigma, Oakville (ON)). Slides were rinsed with warm tap water for 30 minutes and dehydrated to 95% ethanol. Subsequently, the sections were stained with 0.5% eosin (Sigma, Oakville (ON)) in 95% ethanol, dehydrated, and mounted with 70% permount (Fisher, Pittsburgh (PA)) in xylene.

Immunofluorescence

Immunofluorescence (IF) analysis was performed as described previously [17]. Briefly, tissue sections were rehydrated and antigen retrieval was performed in 10mM pH 6.0 sodiumcitrate using a pressure cooker in a microwave for 15 minutes at maximum wattage. Slides were incubated with blocking solution consisting of 10% (w/v) normal goat serum (NGS) and 1% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) for one hour. Subsequently, the primary antibody in blocking solution was added and sections were incubated overnight at 4°C. Following 3 washes with PBS containing 0.05% (v/v) Tween-20 (PBST), slides were incubated with a secondary antibody in blocking solution for one hour. The slides were then washed and the samples were mounted with 4,6-diamidino-2-phenylindole (DAPI) hard mounting medium (Vector, Burlington (ON)). Primary antibodies were: 1:500 rabbit anti-cytokeratin (Dako, Mississauga (ON)), 1:50 mouse anti-vimentin (Dako, Mississauga (ON)), 1:400 mouse anti-α-smooth muscle actin (αSMA) (NeoMarkers, Fremont (CA)), 1:200 rabbit anti-pro-surfactant protein-C (pro-SFTPC) (Abcam, Cambridge (MA)), 1:200 rabbit anti-clara cell secretory protein (CCSP) (SantaCruz, Santa Cruz (CA)), 1:1000 rabbit anti-platelet endothelial cell adhesion molecule (PECAM) (SantaCruz, Santa Cruz (CA)). Secondary antibodies (dilution of 1:200) were fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Calbiochem, San Diego (CA)) for vimentin and αSMA, rhodamine-labelled anti-mouse IgG (Invitrogen, Eugene (OR)) for vimentin, rhodamine-labelled anti-rabbit IgG (Invitrogen, Eugene (OR)) for cytokeratin, pro-SFTPC, CCSP and PECAM. Whole lungs of E19 rat embryos were used as positive controls for immunofluorescence.

Immunohistochemistry

Following antigen retrieval, slides were treated with 3% (v/v) hydrogenperoxide in methanol to block endogenous peroxidase activity. Blocking solution, containing avidin (Vector, Burlington (ON)), was added for one hour according to the manufacturer's instructions. Primary antibody in blocking solution containing biotin (Vector,

Burlington (ON)) was added and slides were incubated overnight at 4°C. The following day a biotinylated secondary antibody was added for one hour in blocking solution. Subsequently, ABC-complex (Vector, Burlington (ON)) was added for 30 minutes. Slides were developed using ImmPACT 3,3'-Diaminobenzidine (DAB) (Vector, Burlington (ON)), and counterstained with hematoxylin. Slides were mounted with 70% permount in xylene. Primary antibodies used for DAB staining were 1:100 rabbit anti-cleaved caspase-3 (Cell signaling, Danvers (MA)), 1:2000 rabbit anti-ki67 (Dako, Mississauga (ON)), 1:1000 mouse-anti-cyclin D3 (Abcam, Cambridge (MA)), 1:1000 rabbit anti-cyclin E (Abcam, Cambridge (MA)), 1:400 rabbit anti-phosphohistone H3 (pH3) (Upstate, Atlanta (GA)) and 1:100 rabbit anti-thyroid transcription factor-1 (TITF-1) (NeoMarkers, Fremont (CA)) in blocking solution. Biotinylated anti-mouse IgG (Vector, Burlington (ON)) (for cyclin D3) and anti-rabbit IgG (Calbiochem, San Diego (CA)) (for cleaved caspase-3, ki67, cyclin E, pH3, TITF-1) were used as secondary antibodies in a dilution of 1:200. Whole lungs of E19 rat embryos were used as positive controls for immunohistochemistry.

TUNEL

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-assay (Roche, Toronto (ON)) was carried out according to the manufacturer's instructions. Rehydration and antigen retrieval were performed as described above. Slides were washed twice with PBS. TUNEL solution was added to the slides and the slides were incubated for an hour at 37°C. Slides were washed three times with PBS and counterstained with DAPI mounting medium.

EdU-uptake

5-ethynyl-2'-deoxyuridine (EdU)-incorporation assay was carried out according to the manufacturer's instructions (Invitrogen, Burlington (ON)). Briefly, recombinants were incubated for two hours with 10μM EdU component A on day five of culture prior to fixation. Sections were incubated with 0.5% (v/v) Triton X-100 for 20 minutes to permeabilize the samples. EdU detection was performed by incubation with a freshly prepared reaction cocktail of FITC-labelled anti-EdU antibody for 30 minutes. Slides were washed with 3% (w/v) BSA in PBS and counterstained with vimentin and DAPI.

Immunocytochemistry

Cells on coverslips were permeabilized with 0.2% Triton-X100 (Sigma Life Science, St Louis (MO)) in 1% BSA for five minutes. Subsequently, IF analysis for cytokeratin, vimentin, pro-SFTPC, αSMA, and PECAM was performed and apoptosis measured using the TUNEL-assay as described above.

Quantification

Mitotic index was quantified by counting pH3-positive cells for each type of recombinant. Per recombinant five different randomly selected areas with alveolar-like structures were counted. Simultaneously, we determined cell origin (epithelial cell or fibroblast). Volocity4 software (Quorum Technologies Inc., Guelph (ON)) was used to quantify the surface area of cytokeratin (epithelial cells) and vimentin (fibroblasts)-positive cells in all four types of recombinants. In each group, recombinants from at least four separate experiments were analyzed with an average of eight pictures per recombinant.

Western blot analysis

Fibroblasts from nitrofen-treated and control lungs were isolated at E19 and cultured for 24 hours. Cells were collected in RIPA buffer and sonicated. Western blot analysis was performed as previously described [20]. Primary antibodies (1:1000 dilution) were rabbit-anti-$p27^{Kip1}$, rabbit-anti-$p57^{Kip2}$, and mouse-anti-$p21^{Waf/Cip1}$ (all from Cell signaling Technology, Danvers (MA)). Anti-rabbit and anti-mouse secondary antibodies (Vector, Burlington (ON)) were used in a concentration of 1:5000.

Data analyses and statistics

All recombination experiments were repeated at least four times. Data are presented as mean ± standard error of the mean. For statistical analyses we used an ANOVA and Bonferroni multiple comparisons test to compare fibroblast and epithelial cell area fractions and ratios, and the pH3-positive cells per field. Subsequently, a Student's t-test was used for pairwise comparison of the four recombinant groups and the p-values were adjusted for multiple comparison error. Significance was defined as p-value < 0.025.

RESULTS

Organogenesis

In all four types of recombinants (Figure 1), cells spontaneously organized in alveolar-like structures (Figure 2A – D). IF staining for cytokeratin (epithelial cell marker) and vimentin (fibroblast marker) revealed fewer organized alveolar-like epithelial structures and a thickened fibroblast (mesenchymal) tissue layer in F_N containing recombinants *versus* F_C containing recombinants (Figure 2F,H *vs* 2E,G). Area measurements of the four types of recombinants demonstrated significant differences between F_C containing recombinants and $(F_N)(E_N)$ recombinants in the epithelial and fibroblast surface areas (Figure 3A). Fraction of fibroblasts were significantly different between $(F_C)(E_C)$ and $(F_N)(E_N)$ recombinants (Figure 3B).

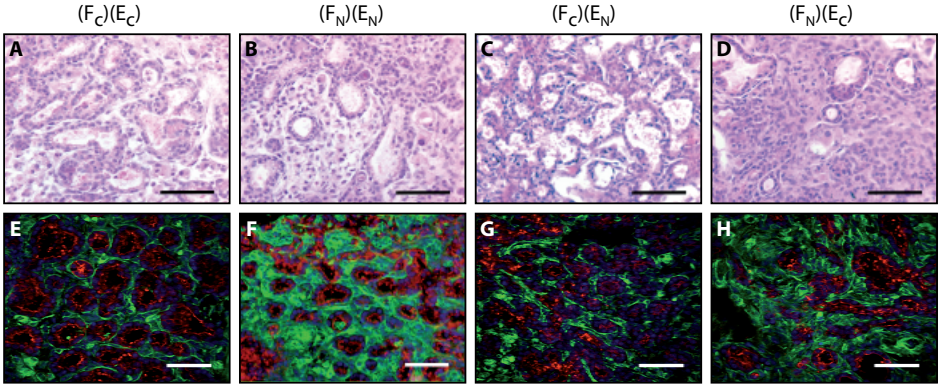

Figure 2. Organogenesis in homotypic and heterotypic recombinants. Alveolar-like structures developed spontaneously in all four types of recombinants (A – D). IF staining of the recombinants for cytokeratin (epithelial cells; red) and vimentin (fibroblasts; green) and counterstained with DAPI (nuclei; blue) (E – H) revealed tissue layer-specific morphological differences. In F_N containing recombinants (F,H) the fibroblast layer appeared thickened and epithelial structures were less organized in comparison to F_C containing recombinants (E,G). The scale bar represents 50μm.

Freshly isolated epithelial cells and fibroblasts from nitrofen-treated and control lungs separately grown on coverslips did not differ in differentiation markers and number of apoptotic cells (results not shown).

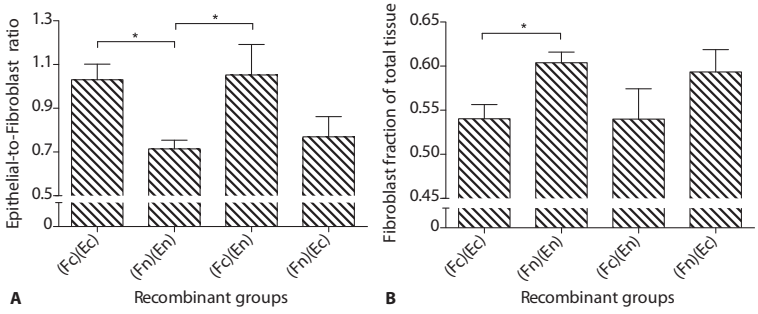

Figure 3. Quantitative analysis of epithelial cells versus fibroblasts surface area in recombinants. To quantify the observed differences in Figure 2E – H, the surface area of epithelial cells and fibroblasts was measured using Volocity software. The epithelial : fibroblast (E : F)-ratio (A) and fraction of fibroblasts (B) in the recombinants were determined. The E : F ratio was significantly decreased while the number of fibroblast increased in $(F_N)(E_N)$ recombinants in comparison to F_C containing recombinants. Both the E : F ratio and number of fibroblasts trended to either decrease or increase in $(F_N)(E_C)$ recombinants, respectively. *Indicates a significant difference (p-value < 0.025); the bars represent the standard error of the mean.

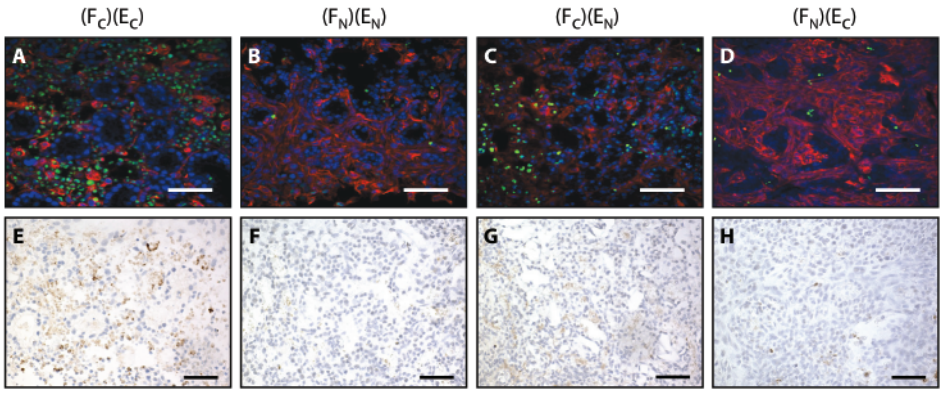

Figure 4. Nitrofen-treated fibroblasts exhibit reduced apoptosis in recombinants. Immunofluorescent TUNEL (green)-analysis on recombinants counterstained with vimentin (fibroblasts; red) and DAPI (nuclei; blue) showed less TUNEL-positive fibroblasts of both FN containing recombinants in comparison to FC containing recombinants (B,D vs A,C). Cleaved caspase-3 (brown) immunoreactivity was less in both FN containing recombinants in comparison to FC containing recombinants (F,H vs E,G) as well. Scale bar represents 50mm.

Apoptosis

To determine if the observed differences were due to apoptotic changes we performed TUNEL-assays and immunohistochemistry for cleaved caspase-3. Both analyses revealed less apoptosis, primarily in the fibroblast (mesenchymal) layer, in F_N containing recombinants when compared to F_C containing recombinants (Figure 4B,D,F,H *vs* 4A,C,E,G). Freshly isolated F_N and F_C cultures were analyzed for TUNEL-positive cells, but no differences between the cultures were observed prior to recombination (results not shown). E19 nitrofen-treated rat lungs also demonstrated less apoptosis compared to E19 control rat lungs (results not shown).

Proliferation

We then assessed cell proliferation by immunohistochemical analyses of ki67 (general proliferation marker), cyclin D3 ('first gap' G_1-phase marker), cyclin E (G_1/S-phase marker) and pH3 (mitosis marker). In addition, we measured the uptake of EdU into DNA (S-phase). Immunoreactivity for cyclin D3 (Figure 5E – H) and cyclin E (results not shown) were similar in all four types of recombinants. However, EdU-incorporation assays showed less cells in the S-phase of the cell cycle in $(F_N)(E_N)$ recombinants compared to $(F_C)(E_C)$ recombinants (Figure 5J *vs* 5I). Ki67 (Figure 5A – D) and pH3 (Figure 5K – N) immunohistochemistry corroborated the finding of less proliferating/dividing cells in F_N containing recombinants *versus* F_C containing recombinants (Figure 5B,D,L,N *vs* 5A,C,K,M, Figure 6). The ratio of proliferating cell types (epithelial *versus* fibroblast) remained the same in all four groups. No differences in total cell amount for each type of recombinant were observed. Western blot analysis demonstrated increased protein levels of cyclin-dependent kinase (Cdk)-inhibitors $p27^{Kip1}$ and

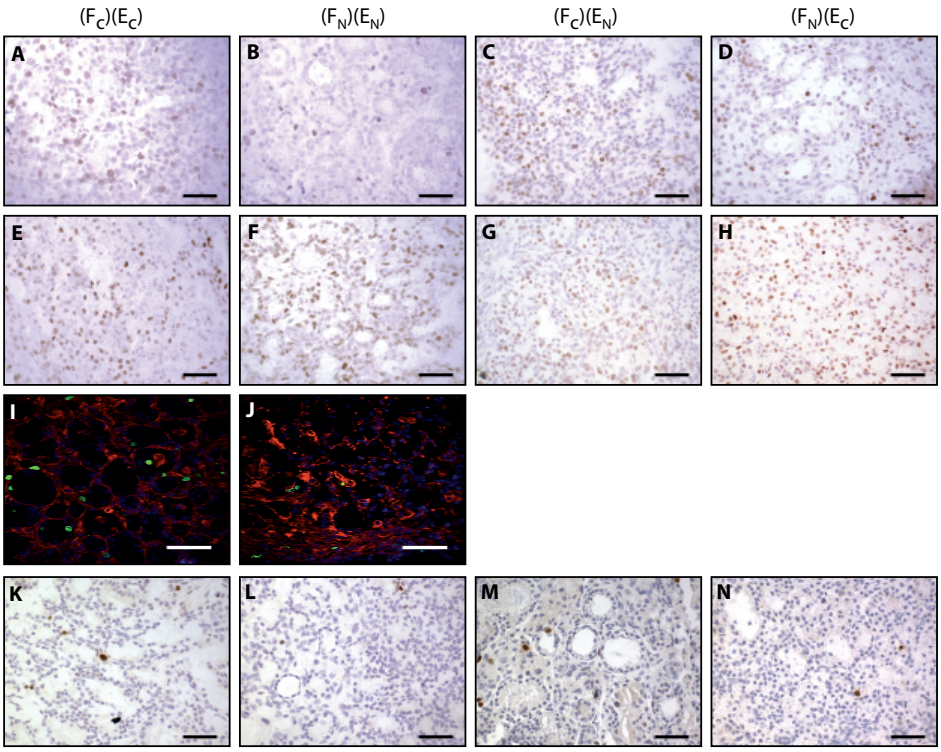

Figure 5. Arrest in proliferation induced by nitrofen-treated fibroblasts in recombinants. Immunohistochemistry for ki67 (brown) demonstrated less proliferation in F_N containing recombinants (B,D) in comparison to F_C containing recombinants (A,C). Cyclin D3 (brown) demonstrated no significant differences between all four types of recombinants (E – H). EdU-uptake (green) by recombinants counterstained with vimentin (fibroblasts; red) and DAPI (nuclei; blue) showed a decrease in cells in the S-phase in (F_N)(E_N) recombinants (J) in comparison to (F_C)(E_C) recombinants (I). PH3 (brown) staining showed less cells undergoing mitosis in F_N containing recombinants (L,N) in comparison to F_C containing recombinants (K,M). The scale bar represents 50μm.

p57^{Kip2} in nitrofen-treated fibroblasts in comparison to control fibroblasts (Figure 7). These inhibitors are known to inhibit the transition from the G1- to S-phase of the cell cycle. The other Cip/Kip family member of Cdk-inhibitors, p21$^{Waf1/Cip1}$, was not detectable in either fibroblast population (results not shown). E19 nitrofen-treated rat lungs had less ki67- and pH3-positive cells but equal numbers of cyclin D3-positive cells when compared to E19 control rat lungs (results not shown).

Cell differentiation

To determine if the recombinants demonstrated differences in cell differentiation besides the reduced proliferation and apoptosis *in vitro*, we performed IF for αSMA (marker for myofibroblasts), TITF-1 (marker for lung epithelial cells), pro-SFTPC (marker for epithelial type II cells), CCSP (marker for clara cells) and PECAM (marker

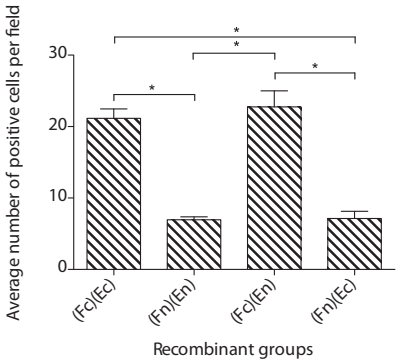

Figure 6. Decrease in mitosis in affected recombinants. To quantify the mitotic index of Figure 5K – 5N, pH3-positive cells were counted in 20 slides per type of recombinant. The number of pH3-positive cells was significantly decreased in $(F_N)(E_N)$ and $(F_N)(E_C)$ recombinants in comparison to both $(F_C)(E_C)$ and $(F_C)(E_N)$ recombinants. *p-value < 0.0001.

for endothelial cells). αSMA IF staining was similar in myofibroblasts in all four types of recombinants (Figure 8A – D). In addition, IF staining for TITF-1 (results not shown), pro-SFTPC (Figure 8E – H), and CCSP staining (results not shown) was comparable in all types of recombinants. No PECAM-positive cells were present in recombinants. Freshly isolated epithelial cells and fibroblasts from nitrofen-treated and control lungs separately grown on coverslips did not differ in differentiation markers (results not shown).

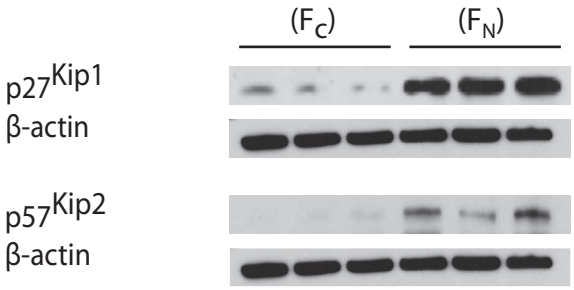

Figure 7. Increased expression of Cdk inhibitors in nitrofen-treated fibroblasts. Western blot analysis demonstrated that cultured fibroblasts from nitrofen-treated embryonic lungs have increased amounts of p27Kip1 and p57Kip2 proteins compared to fibroblasts from control embryonic lungs.

DISCUSSION

Combining the well-established rodent model for CDH based on the teratogenic effects of nitrofen with our previously developed cell recombinant model, we created a new pulmonary cell recombinant *in vitro* model that resembles human CDH-associated pulmonary hypoplasia. This cell recombinant model enabled us to investigate

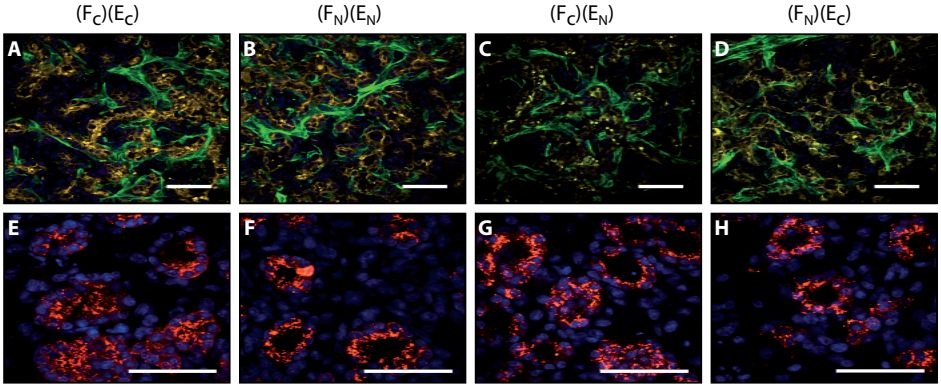

Figure 8. Nitrofen exposure does not alter cell differentiation in recombinants. IF for αSMA (smooth muscle cells; green) and cytokeratin (epithelial cells; orange) in recombinants counterstained with DAPI (nuclei; blue) showed a similar pattern of myofibroblast staining in all four types of recombinants (A – D). Pro-SFTPC (type II epithelial cells; red) counterstained with DAPI (nuclei, blue) demonstrated also a similar pattern of staining in all four types of recombinants (E – H). The scale bar represents 50μm.

epithelial-mesenchymal interactions in nitrofen-induced pulmonary hypoplasia into more detail and resulted in the following observations. Recombinants containing *in vivo* nitrofen-treated fibroblasts (F_N) exhibited decreased apoptosis and a late G_1 cell cycle arrest. We therefore postulate that fibroblasts are the defective cells in CDH-associated hypoplastic lungs.

Adequate *in vitro* model for pulmonary hypoplasia

Obvious morphological differences between recombined normal lung cells ((F_C)(E_C) recombinants) and recombined nitrofen-treated lung cells ((F_N)(E_N) recombinants) were a thickened fibroblast (mesenchymal) layer and fewer organized epithelial structures (alveolar-like structures) in the (F_N)(E_N) recombinants. Similar structural abnormalities have been observed in hypoplastic lungs of nitrofen-treated rodents and children with CDH [5-7, 21-24]. Hence, we believe the pulmonary cell recombinant model is an adequate *in vitro* model for pulmonary hypoplasia in CDH. More importantly, the *in vitro* model enabled us for the first time to recombine a 'healthy' with a 'diseased' tissue layer and investigate the unique crosstalk between the two tissue layers. After establishing a functional model for homotypic recombinants (recombining epithelial cells and fibroblasts from the same treatment lung group), we created heterotypic recombinants (recombining epithelial cells and fibroblasts from opposing treatment lung groups) to decipher which layer was malfunctioning and, simultaneously, to investigate potential rescue of the malfunctioning layer, by evaluating cell-cell interactions. Morphologically, the heterotypic recombinants containing control fibroblasts (F_C) mimic the control (F_C)(E_C) recombinants whereas recombinants containing nitrofen fibroblasts (F_N) are similar to the nitrofen (F_N)(E_N)

recombinants, suggesting a defect in the nitrofen-treated fibroblast layer. Previous studies hypothesized that the mesenchymal tissue layer was defective in nitrofen-treated diaphragm primordia, based on the observed morphology of a thickened fibroblast tissue layer [25]. We hereby, for the first time, provide evidence that the fibroblast tissue layer is also the defective tissue layer in nitrofen-induced pulmonary hypoplasia. More importantly, this defect was intrinsic to the fibroblasts and not influenced by epithelial-mesenchymal interactions.

Decrease in apoptosis of fibroblasts

Apoptosis is one of the developmental entities essential to the formation of a healthy lung [26]. Immunohistochemical (TUNEL and cleaved caspase-3) analyses demonstrated less apoptosis in F_N containing recombinants in comparison to F_C containing recombinants. In addition, E19 whole lungs of the nitrofen-treated embryos demonstrated less apoptosis *versus* control embryos. Apoptotic cells were mainly fibroblasts, as expected from previous studies [26, 27]. Diminished apoptosis of fibroblasts could explain the thickened fibroblast tissue layer seen in F_N containing recombinants. Diminished apoptosis could also explain the fewer alveolar-like structures observed due to this increase in fibroblasts. Furthermore, inhibition of apoptosis of the fibroblast layer has been reported to reduce epithelial branching, which could also contribute to decreased formation of alveolar-like structures [27]. To our knowledge, this is the first time a decrease in apoptosis has been demonstrated in nitrofen-treated lung cells and E19 whole lungs. In previous reports, TUNEL analysis did not demonstrate differences between control and nitrofen-exposed explants [9] nor in whole lungs after nitrofen treatment *in vivo* [13, 28]. Similar to our recombinant findings, TUNEL-positive cells were mainly detected in the fibroblast layer of lung explants [9]. *In vitro* exposure to nitrofen increased apoptosis of NIH 3T3 and HEK-293 cells, and rat fetal lung explants [28, 29]. An explanation for these conflicting results could be that in our study whole lungs and lung cells from the recombinant experiments were exposed to nitrofen *in vivo* at E9 and harvested at E19. All other studies investigated E12 – E16.5 rat lungs and it appears that pulmonary apoptosis is more pronounced later in gestation [26]. In addition, E19 lungs of nitrofen-treated rats are subjected to mechanical compression and lack proper fetal breathing movements (due to herniation of abdominal contents into thorax), which negatively impacts cell growth [9, 30] and may have an influence on apoptosis as well. It is also possible that exposure to nitrofen *in vitro* as opposed to exposure *in vivo* could generate different results. An overview of the literature is shown in Table 1.

Proliferative arrest

In the present study, we found a late arrest in the G_1-to-S-phase transition of the cell cycle in F_N containing recombinants. Similar amounts of cyclin D3- and E-positive

cells were seen in all four types of recombinants. In contrast, significantly less ki67- and pH3-positive cells were detected in F_N containing recombinants. Also, fewer EdU-positive cells were noted in $(F_N)(E_N)$ recombinants (heterotypic recombinants were not assessed). Thus, F_N containing recombinants appear to have a late G_1 arrest and are unable to continue the proliferation cycle. The exact mechanism of this G_1 arrest remains to be elucidated. Similar to our observation with E19 lungs, previous studies have reported decreased proliferation in nitrofen-treated rat lungs [9, 13]. Jesudason et al. (2000) showed reduced lung cell proliferation 24 hours prior to diaphragm closure (E15.5) in nitrofen-treated rats, while we found a diminished fibroblast proliferation in nitrofen-exposed lung explants [9, 13]. In addition to lung, Clugston et al. (2009) demonstrated a decrease in cellular proliferation of the pleuroperitoneal fold (diaphragm) in nitrofen-treated rat embryos [28]. None of these studies addressed the specific phase of the cell cycle involved and the present study is the first one to report a proliferative arrest in the G_1-to-S-phase transition in nitrofen-induced pulmonary hypoplasia. Quantitative analyses of pH3-positive cells demonstrated a more than two-fold decrease of proliferation in both epithelial and fibroblast tissue layers. Western blot analysis demonstrated increased expression of Cdk inhibitors, $p27^{Kip1}$ and $p57^{Kip2}$, in nitrofen-treated fibroblasts. Although the increase in Cdk inhibitors agrees with the proliferative arrest in the late G_1-phase in the F_N containing recombinants, it does not explain the overall increase in fibroblasts. One possibility is that the reduction in apoptosis of fibroblasts in the F_N containing recombinants outbalances the decrease in proliferation. The decrease in proliferating epithelial cells likely explains the fewer alveolar structures observed in the F_N containing recombinants. An intriguing insight into the late G_1-phase arrest is the link to RA. RA has been connected to the etiology of nitrofen-induced abnormalities and the human CDH lung since 1941 by Andersen [11, 12, 31] reviewed in [11]. RA treatment has demonstrated to reduce the number of hernias and to induce lung growth in nitrofen-treated embryos [23, 32, 33]. It has been shown that RA protects alveolar epithelial cells from a late G_1-phase arrest induced by hyperoxia [34]. Cells exposed to oxygen did form less cyclin E-Cdk complexes but these complexes regained normal values when oxygen-exposed cells were pretreated with RA. Interestingly, we observed a late G_1-phase arrest in the F_N containing recombinants. Several treatment options have been aimed at increasing proliferation in CDH-associated pulmonary hypoplasia but none has specifically targeted a G_1-phase proliferative arrest [35, 36]. An overview of the literature is shown in Table 1.

Differentiation not altered

Four investigated cell differentiation markers (TITF-1, CCSP, αSMA, pro-SFTPC) were present and not differently distributed among isolated cells and the recombinants. PECAM was absent in freshly isolated cells prior to recombination and in the recom-

binants, which indicates no endothelial cells were isolated during primary culture. The standard primary culture techniques we utilized to obtain epithelial cells and fibroblasts did not allow us to isolate endothelial cells. In addition, differentiation into endothelial cells during the culture period did not occur. Endothelial cells in human fetal hypoplastic lungs did not appear to be different from healthy lungs [37]. αSMA was present in all four groups, but no apparent differences were observed. Smaller pulmonary vessels, which are normally not muscularized, contain smooth muscle cells in CDH hypoplastic human lungs [38, 39]. Furthermore, the hypoplastic CDH human lung contains a decrease in interstitial and bronchial smooth muscle cells [37]. In previous studies, differences between normal lungs and nitrofen-treated lungs have been reported for these differentiation markers [9, 21, 24, 40-43]. This discrepancy could be due to the use of isolated cells in our experiments instead of using whole lungs. Whole lungs are exposed to mechanical forces from lung liquid and fetal breathing movements that contribute to lung growth and maturation by inducing stretch and expansion [30, 44]. Due to a defect in the diaphragm in CDH and herniation of abdominal organs into the thorax, contractions for fetal breathing movements are disturbed, which makes the lung liquid expanding forces in nitrofen-induced lungs less efficient than in normal fetal lungs. This may lead to a disturbance in cell differentiation *in vivo* that is too subtle (or not present at all) to be detected in isolated lung cells *in vitro*. An overview of the literature is shown in Table 1.

Fibroblasts are the defective tissue layer in nitrofen-induced pulmonary hypoplasia

Based on our findings, we postulate that lung fibroblasts are the primary malfunctioning layer in the nitrofen model (Figure 9). As cell markers were similar in nitrofen-treated and control cells just after isolation of the cells and prior to making the recombinants, all observed differences in recombinants are considered to be due to the intrinsic qualities of the cells and their cellular interactions. Interestingly, various CDH rodent models have shown a defective fibroblast (mesenchymal) compartment of the pleuroperitoneal fold as the underlying mechanism resulting in diaphragmatic defects [25, 45, 46]. The fact that we observed similar structures in F_C containing recombinants can be explained by the postulated hypothesis, as there are no malfunctioning F_N cells in these recombinants present which makes them appear like normal lungs. The abnormal structures of F_N containing recombinants may be explained by the presence of a malfunctioning F_N tissue layer. 'Rescue' of diminished apoptosis through recombination with a healthy epithelial layer E_C did not occur. (F_C)(E_C) and (F_C)(E_N) recombinants had a comparable phenotype, supporting the concept of E_N being unaffected. The observed thickening of the fibroblast tissue layer in F_N containing recombinants could be the result of the observed diminished apoptosis in this tissue layer. Epithelial cells E_C and E_N were unable to rescue this defect, imply-

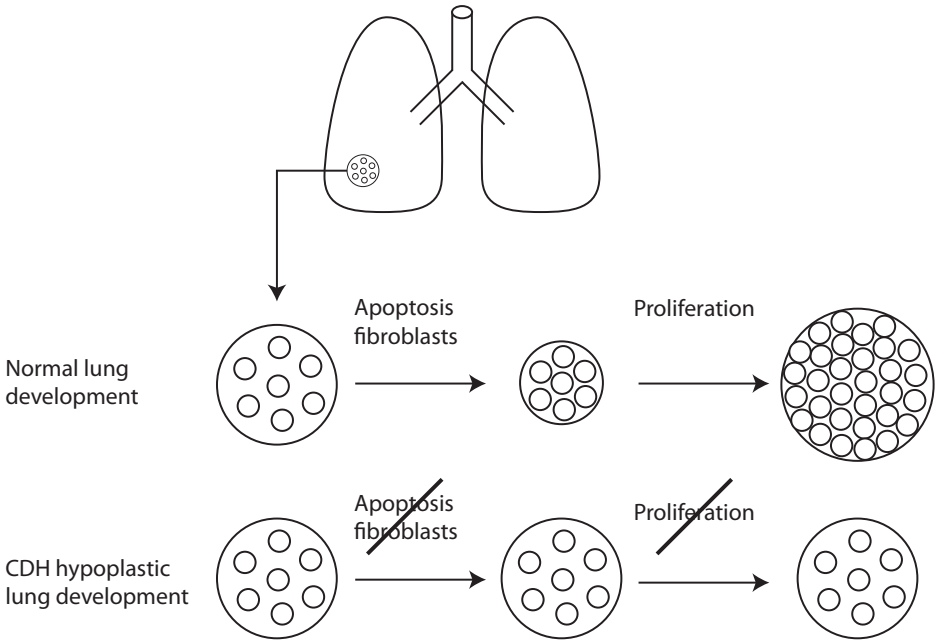

Figure 9. Schematic representation of the hypothesis. A schematic representation of our hypothesis of the pathogenesis in pulmonary hypoplasia: the lung fibroblasts are the malfunctioning layer. Both a defect in mesenchymal apoptosis and a proliferative arrest generate the characteristic image seen in hypoplastic CDH lungs such as thickening of the fibroblast (mesenchymal) tissue layer and reduced alveolar spaces.

ing that the diminished apoptosis was intrinsic to the nitrofen-exposed fibroblasts. The decrease in proliferation in F_N but not F_C containing recombinants was equally notable in epithelial cells and fibroblasts. Therefore the diminished proliferation in the epithelial cells must be due to the influence of the defective F_N fibroblasts since we did not observe such an effect in the F_C containing recombinants. Taken together, the diminished proliferative capacity probably reflects in the smaller size of the lungs (pulmonary hypoplasia), while the diminished apoptosis of the fibroblasts causes the thickening of the fibroblast tissue layer. Identification of the timing and, eventually, targeted modulation of the defect in the fibroblasts during lung development warrants more research.

Limitations of our model

We are aware of the limitations of our study. First of all, we are dealing with an animal model for pulmonary hypoplasia in CDH based on the teratogenic effects of the herbicide nitrofen; a role of herbicides such as phenyl ether compounds in the etiology of human CDH has never been found. Nitrofen was found to inhibit RALDH2, an enzyme involved in the RA pathway [47], and, recently, lower levels of retinol and retinol binding protein were documented in human umbilical cord blood [12]. This

suggests a possible epigenetic role of RA metabolism in the pathogenesis of human CDH. However, despite its shortcomings, the pharmacological nitrofen model is the preferred model to investigate pathogenetic aspects of (pulmonary hypoplasia in) CDH as it has very similar characteristics to human CDH [22, 48]. The surgical model, another model for human CDH, is mainly suitable to investigate interventional strategies in CDH. In this model a hernia is surgically created in the diaphragm and abdominal organs are positioned in the thoracic cavity of either fetal sheep or rabbits to optimally mimic human CDH. Limitations of this model are the creation of pulmonary hypoplasia by mechanical interference while lungs in itself are normal, the relatively late creation of the defect during lung development, and the almost completed alveolization in sheep while in humans only 20% of adult alveolization is achieved at that timepoint. Genetic mouse models have also been used to study pulmonary hypoplasia in CDH. However, no knockout mouse model is presently available that mimics the human phenotype with isolated left posterior CDH (Bochdalek's hernia) and pulmonary hypoplasia sufficiently. Recently, common expression of transcription factor (regulator)s such as CoupTFII, GATA-binding protein 4 (GATA4), and Friend of GATA2 (FOG2) have been identified in both the developing lung and diaphragm, and are located on chromosome regions commonly deleted in individuals with CDH [46, 49, 50]. However, no single gene mutation has been identified to date, and, as the CDH phenotype is so variable, it is unlikely to be caused by a single gene mutation but more a result of multiple gene defects. Therefore the use of genetic mice models was deemed unfeasible to address our study objective. An overview of CDH models has been elaborately reviewed in [10, 11]. Another argument that supports the significance of the nitrofen model is the link to the RA signaling pathway. Nitrofen has been demonstrated to interfere with the RA pathway in several ways and consequently with the before mentioned transcription factor (regulator)s (GATA4, CoupTFII, etcetera) [10, 11]. The so-called retinoic acid hypothesis was recently supported by our human studies, which showed that human newborns with CDH had significant lower retinol and retinol binding protein levels in umbilical cord blood [12].

Secondly, our recombination model is an *in vitro* cell culture model based on an experimental approach. Even though the hernia and pulmonary hypoplasia were induced *in vivo*, the recombinants were cultured *in vitro*. We realize that our recombinants mimic lung development under unnatural conditions, and we cannot exclude that this influences certain developmental processes. For example, in our experiments we recombined equal numbers of fibroblasts with epithelial cells. We did not take into consideration that hypoplastic (nitrofen) lungs contain less cells, or that the ratio of fibroblasts and epithelial cells might not be 1:1 during *in vivo* lung development. However, as was previously demonstrated, alveolar-like structures

did form and important structural differences between recombinant groups became apparent following recombination with different sources of fibroblasts [17]. In addition, previous experiments by our group also demonstrated no differences between using recombinants in a 1:1 or 1:3 (Fibroblasts : Epithelial cells) ratio (results not shown). Consequently, since our goal was to investigate the interactions between different tissue layers during (ab)normal lung development, this *in vitro* model was deemed appropriate. In order to translate the obtained results of this nitrofen rat-model to human CDH, the obvious next step would be to use this *in vitro* model using human CDH lung samples. However, given the limited availability of fresh pulmonary tissues from terminated pregnancies, such an approach is nearly impossible due to ethical and logistical problems.

Relevance for human pulmonary hypoplasia in CDH

Our hypothesis of fibroblasts being the defective tissue layer in hypoplastic CDH-lungs becomes more interesting when we relate it to the abnormalities observed in human CDH-associated pulmonary hypoplasia. Characteristics of human pulmonary hypoplasia in CDH are thickened alveolar walls, an increase in interstitial tissue, reduced alveolar air spaces and reduced gas-exchange surface area [2, 5-7]. A malfunctioning fibroblast layer could basically explain all these features. A decrease in apoptosis of fibroblasts prevents the thinning of the interstitial fibroblast layer normally seen during later lung development [51, 52]. The increase in alveolar wall thickness likely results in more primitive alveolar saccules. In addition, the proliferative arrest of epithelial cells may contribute to reduced air spaces and, therefore, a reduction in the gas-exchange area [5-7, 21]. Our findings do not support an immature state of CDH lungs, as reported by others [53, 54]. The idea that the CDH hypoplastic lung is immature remains controversial since to date no studies have reported primary surfactant deficiencies in human and nitrofen-induced CDH lungs [55, 56], neither a decreased pool size of surfactant [57]. Oligohydramnios due to obstructive uropathy, another cause of human pulmonary hypoplasia, has different morphological features and might therefore not be associated with the pathogenesis for pulmonary hypoplasia in CDH [58]. Further research is warranted to investigate whether this defect in the fibroblast tissue layer is present in human CDH lungs as well. Unfortunately, as mentioned earlier, the recombinant approach cannot easily be applied to human CDH lungs. Even in whole human postnatal CDH lungs experimental results should be interpreted carefully, as it is difficult to distinguish if observed differences are due to the underlying cause of the hypoplastic CDH lungs or secondary to treatment modalities such as ventilation and extracorporeal membrane oxygenation (ECMO).

Future directions

Utilizing this cell recombinant *in vitro* model, we demonstrated the malfunctioning fibroblast tissue layer to exhibit decreased apoptosis and an arrest in proliferation. Future experiments need to address the mechanism by which these processes are influenced. Several fibroblast growth factors (FGFs) are known to play an important role in lung development [59, 60]. For instance FGF2 plays a role in lung cell apoptosis, while FGF1 is an example of a growth factor with proliferative qualities [61, 62]. In addition, in the nitrofen model both FGF7 and FGF10, and FGF receptor L1 (FGFRL1) were downregulated, while recently LopezJimenez et al. demonstrated a CDH patient with a 4p16.3 deletion that included the FGFRL1 region [63-65]. The next step to elucidate the pathogenesis and work towards innovative therapeutic strategies would be to investigate FGFs and their receptors in this model. Also the effect of RA given the strong link with the pathogenesis in humans as documented before is worth investigating [23].

CONCLUSIONS

We demonstrated that the fibroblast tissue layer is malfunctioning in nitrofen-induced pulmonary hypoplasia. Knowing the malfunctioning tissue layer will aid to develop targeted treatments inducing apoptosis in fibroblasts and annulling the proliferative arrest. An example of a treatment modality that could induce these two components is tracheal occlusion. In both surgically- and nitrofen-induced diaphragmatic hernia models, tracheal occlusion induces fibroblast apoptosis and accelerates cell proliferation [35, 66-69], the main defects according to our recombinant model. Thus, this treatment modality appears to have great potential.

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4

Apoptosis of Fibroblasts is Regulated by Epithelial Cells during Fetal Rat Lung Development in a Cell Recombinant Model

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Submitted

ABSTRACT

Background It is well established that lung morphogenesis is dependent on epithelial-mesenchymal interactions; however, the exact molecular mechanisms guiding lung organogenesis have yet to be determined. Cell proliferation has been recognized as a crucial modulator of lung development. Apoptosis is another important mechanism involved in remodelling of both the prenatal and postnatal lung; for instance late gestational thinning of the fibroblast tissue layer through apoptosis is important to optimize postnatal gas exchange in the lungs. The aim of our study was to investigate the influence of gestational stage on epithelial-mesenchymal interactions through cell proliferation and apoptosis during lung morphogenesis utilizing an *in vitro* cell recombinant model.

Methods Embryonic day (E)13 endodermal and mesenchymal cells and E19 epithelial cells and fibroblasts were isolated from fetal rat lungs using standard primary culture techniques. The cells were then recombined in various combinations and cultured. In these recombinants, proliferation and apoptosis were investigated using PCNA immunohistochemistry and TUNEL-assay, respectively.

Results We observed more proliferating cells in homotypic E13 recombinants than in homotypic E19 recombinants. The proliferation characteristics of the different tissues appeared to be intrinsic to those tissues, and were not altered by heterotypic recombination. Homotypic E19 recombinants and E19 fetal lungs demonstrated more apoptosis in fibroblasts than homotypic E13 recombinants and E13 fetal lungs, respectively. To our surprise, apoptosis of E19 fibroblasts was abolished, when the fibroblasts were recombined with E13 endodermal cells.

Conclusions Our results suggest that apoptosis of the fibroblast tissue layer late in pulmonary development is regulated by the surrounding epithelial cells, and consequently is not intrinsic to the fibroblasts itself. With our study we are a step closer to understanding the physiology of normal lung development, which will aid in deciphering the pathogenesis of, and ultimately design targeted treatments for, developmental lung diseases such as pulmonary hypoplasia in CDH and oligohydramnios.

INTRODUCTION

Organogenesis of the lung is highly dependent on epithelial-mesenchymal interactions as well as cell-matrix interactions [1-3]. Several tissue recombination studies have demonstrated that for normal branching morphogenesis of the lung to occur, interaction between lung epithelium and lung mesenchyme is a prerequisite [4-7]. It has long been recognized that branching morphogenesis of lung epithelium is guided by instructions from the surrounding mesenchyme. Interestingly, proximal tracheal epithelium, which is normally not able to branch, can in this manner be forced to undergo branching morphogenesis when recombined with distal lung mesenchyme [8]. In contrast, recombining proximal tracheal lung mesenchyme with distal lung epithelium, which is normally undergoing extensive branching morphogenesis during pulmonary development, inhibits branching [9]. Additional experiments demonstrated that epithelial differentiation is dependent on the type of mesenchyme used for recombination [6, 10] (for review see [5]). These epithelial-mesenchymal interactions are modulated by a well-orchestrated interplay between hormones, growth factors and extracellular matrix proteins (for review see [1-3, 5, 11]). Various factors have been revealed by experiments with isolated mesenchyme-free pulmonary epithelium. In this system isolated epithelium could be stimulated to undergo branching morphogenesis when it was cultured in the presence of a specified mix of certain growth factors and extracellular matrix proteins [12]. All these experiments have demonstrated an important role for the mesenchyme in instructing the developing epithelium in lung development. However, the influence of epithelium on development of the mesenchyme is less well understood.

The aim of our study was to investigate the influence of gestational stage on epithelial-mesenchymal interactions through cell proliferation and apoptosis during lung morphogenesis utilizing an *in vitro* cell recombinant model. Apoptosis and proliferation are a crucial part of epithelial-mesenchymal interactions in lung development. Cell proliferation has been recognized as a crucial modulator not only for lung growth, but also for branching morphogenesis [3, 13-16]. Outgrowth of terminal lung buds is associated with higher numbers of proliferating cells. Programmed cell death, apoptosis, is another important mechanism involved in remodelling of both the prenatal and postnatal lung [17-20]. Apoptosis was demonstrated to occur predominantly in the mesenchyme, in particular during the pseudoglandular phase and perinatally [18-20]. Perinatally a huge thinning of the pulmonary mesenchyme has to occur in order to enable gas exchange over the pulmonary epithelium. During this process, apoptosis is known to play a significant role [17-19]. Taken together, a balanced interplay between cell proliferation and apoptosis, is required to help the primary lung bud to become the postnatal gas exchanging organ. Herein, we investigated apoptosis and

proliferation in normal fetal rat lungs and, more specifically, studied the influence of gestational stage on epithelial-mesenchymal interactions utilizing a previously established *in vitro* cell recombinant model [10, 21]. We demonstrate that apoptosis of the fibroblasts late in pulmonary development is regulated by surrounding epithelial cells in this *in vitro* cell recombinant model.

METHODS & MATERIALS

Animals

The animal care committee of the Hospital for Sick Children approved all experimental procedures. Female (200 - 250 g) and male (250 - 300 g) Wistar rats were obtained from Charles River (St. Constant (QC)). The animals were kept in a controlled light-dark cycle and food and water were supplied ad libitum. Rats were mated overnight and the finding of a sperm-positive vaginal smear was designated embryonic day (E)0. At E13 and E19 (term = E22), timed-pregnant rats were sacrificed. The fetuses were delivered by Caesarian section using aseptical surgical techniques.

Cell isolation

Using microsurgical techniques, lungs were dissected from embryos at E13 and transferred to Hanks Balanced Salt Solution (-) from Gibco (Grand Islands (NY)). E13 lungs were divided into two groups: to be used as whole fetal lungs or as isolated cells for the cell recombinants. To obtain E13 isolated cells, we treated E13 lungs with 20% (v/v) dispase and 100mg/ml DNAse for 45 minutes at 37°C in order to aid in the surgical separation of the tissue layers. Following neutralization of enzyme activity with fetal calf serum (FCS), the epithelial and mesenchymal components were separated under a dissection microscope, using microsurgical techniques. Single cells were isolated from the endodermal and mesenchymal cell layers by gentle mechanical agitation.

Simultaneously, lungs were isolated from E19 embryos. E19 lungs were divided into two groups for the use of E19 whole fetal lungs and to isolate E19 cells to be used for recombinants. To obtain E19 isolated cells, epithelial cells and fibroblasts were isolated using standard primary culture techniques, as previously described [10]. Isolated epithelial cells and fibroblasts were evaluated for purity as described previously [21]. E13, E16, E19, E21 and postnatal day (P)1 lungs were isolated to investigate the spatial and temporal changes of proliferation and apoptosis during development.

Formation of recombinants

Recombinants were formed as previously described [10, 21]. Briefly, E13 endodermal cells were recombined with either E13 mesenchymal cells or E19 fibroblasts; E19 epithelial cells were recombined with either E13 mesenchymal cells or E19 fibroblasts (Figure 1), and transferred to 4mm porous membranes (Millipore, Bedford (MA)). The cell recombinants were cultured at 37°C in an atmosphere of 5% CO_2 in air for five days. During the culture period, medium was changed daily. E13 endodermal and mesenchymal cells as well as E19 epithelial cells and fibroblasts were cultured alone as well.

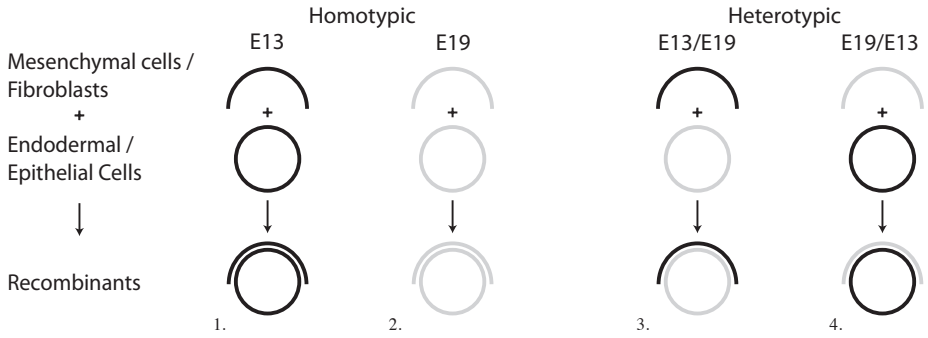

Figure 1. Schematic representation of recombination experiments. Schematic representation of the various combinations that were used in the recombination experiments. Following separation of lung cell layers, E13 endodermal cells (black circles) and E13 mesenchymal cells (black half circles) were recombined in homotypic recombinants (model 1). The same procedure was used in making the homotypic recombinants of E19 epithelial cells (grey circles) and E19 fibroblasts (grey half circles) (model 2). In heterotypic recombinants, E13 endodermal cells (black circles) were recombined with E19 fibroblasts (grey half circles) (model 3) and E19 epithelial cells (grey circles) were recombined with E13 mesenchymal cells (black half circles) (model 4).

Tissue preparation

All lungs, epithelial cells and fibroblasts alone, and the recombinants were fixed in 4% (v/v) paraformaldehyde in phosphate buffered saline (PBS) overnight at 4°C, dehydrated and embedded in paraplast (Oxford Labware, St. Louis (MO)). Sections of 5µm were cut and mounted on Superfrost slides (Fisher Scientific, Unionville (ON)).

Immunohistochemistry

Immunohistochemistry was carried out as described previously [22]. Following pretreatments including antigen retrieval, sections of the recombinants were incubated with primary antibodies at 4°C overnight. Primary antibodies used were 1:5 mouse anti-pan cytokeratin (Boehringer Mannheim, Laval (QC)), undiluted mouse anti-vimentin (Serotec, Missisauga (ON)) and 1:1000 mouse anti-proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz (CA)). Sections were washed

and incubated for one hour at room temperature with 1:200 biotinylated anti-mouse IgG (Calbiochem, LaJolla (CA)). Sections were washed and incubated for two hours at room temperature with Vectastain (avidin-biotin peroxidase-kit) (Vector Laboratories, Burlingame (CA)). Subsequently, sections were washed, and developed in 3,3'-diaminobenzidine. Hematoxylin was used to counterstain sections. Sections were dehydrated and mounted with Permount. Terminal Deoxyribonucleotidyl Transferase dUTP Nick-End labeling (TUNEL)-assay was carried out as described previously [22]. 4',6-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei of cells.

RESULTS

Morphogenesis of the recombinants

E13 and E19 lungs were used for the recombination studies. At the saccular stage (E19) of lung development there is a peak in prenatal apoptosis and E13 lungs are the smallest lungs to isolate cell layers with a reasonable yield [19, 20]. After approximately 72 hours of culture of the recombinants, morphogenesis started to occur spontaneously in all previously unorganized clumps of cells (results not shown). After five

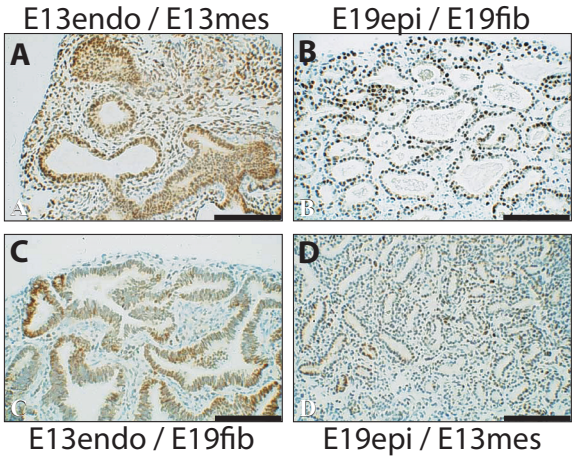

Figure 2. Cell proliferation in recombinants. An antibody against PCNA was used to investigate cell proliferation in the recombinants. Highest numbers of proliferating cells (endodermal and mesenchymal cells) were observed in E13 homotypic recombinants (A). In E19 homotypic recombinants (B) proliferating cells were mainly observed in the epithelial cell layer and much less in the fibroblast tissue layer. In the heterotypic recombinants of E13 endodermal cells and E19 fibroblasts, proliferating cells were predominantly localized in the endodermal cell lining (C). In the heterotypic recombinants of E19 epithelial cells and E13 mesenchymal cells, proliferation was less pronounced, but evenly distributed over both cell layers (D). The scale bar represents 100μm.

days of incubation, the E13 homotypic recombinants (E13 endodermal cells with E13 mesenchymal cells) developed large airspaces with a lining of cuboidal endodermal cells surrounded by mesenchymal cells. These resembled the E13 fetal lungs (Figure 3A). In the E19 homotypic recombinants (E19 epithelial cells with E19 fibroblasts), alveolar-like structures formed containing a circle lining of epithelial cells and a thin layer of fibrobasts. Again, this resembled the situation observed in the E19 fetal lungs (Figure 3B).

In the heterotypic recombinants with E13 endodermal cells and E19 fibroblasts large airspaces with a thick lining of cuboidal endodermal cells were formed (Figure 2C). Fibroblasts surrounded the endodermal lining. The structures of these recombinants resembled the structure of the E13 homotypic recombinants (Figure 2A) and E13 fetal lungs (Figure 3A).

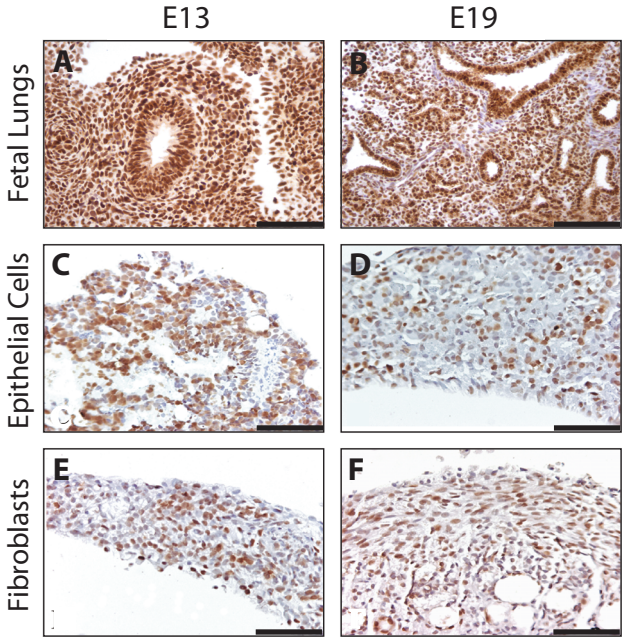

Figure 3. Cell proliferation in fetal lungs and cells alone. In a series of control experiments, cell proliferation (PCNA) was investigated in fetal lungs, cells of endodermal / epithelial origin alone and mesenchymal cells or fibroblasts alone. In E13 fetal lungs extensive proliferation was observed in both the endodermal cell layer and mesenchyme (A). As in the E19 homotypic recombinants, in E19 fetal lungs more proliferating cells were observed in the epithelial cell layer than in the fibroblast layer (B). In isolated E13 endodermal (C) and mesenchymal (E) cells high amounts of proliferating cells were present. Isolated E19 epithelial cells (D) had more proliferating cells than E19 fibroblasts (F). The scale bar represents 100μm.

In the heterotypic recombinants of E19 epithelial cells and E13 mesenchymal cells more alveolar-like structures were observed (Figure 2D), albeit smaller than observed in the E19 homotypic recombinants (Figure 2B) or the E19 fetal lungs (Figure 3B).

Proliferation

Using an antibody against PCNA, a cell cycle associated protein, we investigated patterns of proliferation in the different types of recombinants [23]. Extensive cell proliferation was observed in endodermal and mesenchymal cells in E13 homotypic recombinants (Figure 2A) as well as in the E13 lungs (Figure 3A). In contrast, in the E19

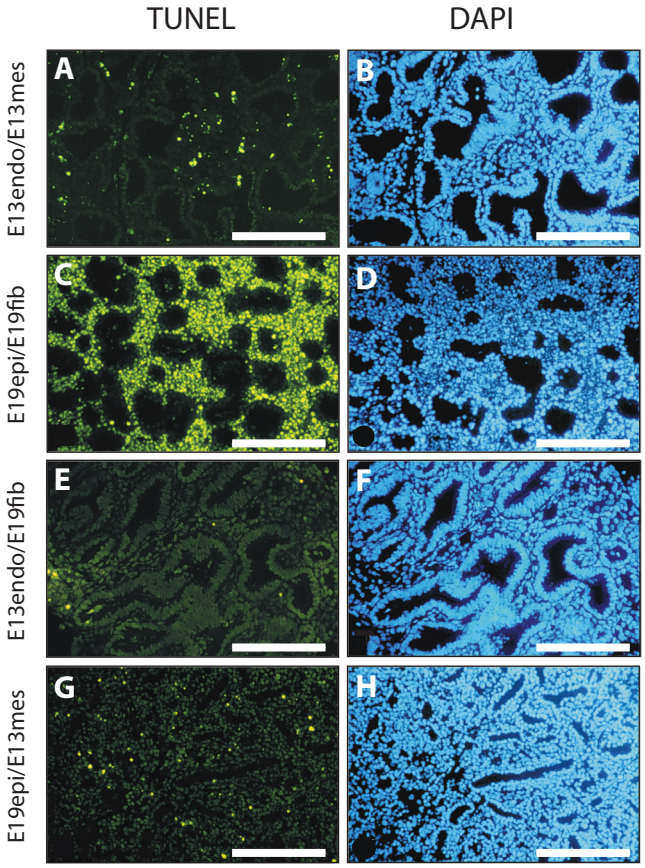

Figure 4. Apoptosis in recombinants. Apoptosis was determined in the recombinants using TUNEL-assay (A,C,E,G). Nuclei were stained with DAPI to visualize the structure of the recombinants (B,D,F,H). In the E13 homotypic recombinants only a few TUNEL-positive cells were observed in the mesenchymal cells (A). The same holds true for both the heterotypic recombinants of E13 endodermal cells and E19 fibroblasts (E) and the heterotypic recombinants of E19 epithelial cells and E13 mesenchymal cells (G). In contrast, extensive apoptosis was observed in the E19 fibroblasts in the E19 homotypic recombinants (C). The scale bar represents 100μm.

homotypic recombinants proliferating cells were mainly observed in the epithelial lining and much less so in the fibroblasts (Figure 2B). A similar pattern was observed in the E19 lungs (Figure 3B). The distribution of PCNA-positive cells changed from being abundantly present in both tissue layers at E13 to mainly present in endodermal/epithelial lining at P1 (Figure 6: left column).

In the heterotypic recombinants, a similar pattern of proliferation was observed. In the recombinants of E13 endodermal cells and E19 fibroblasts (Figure 2C), proliferating cells were predominantly localized in the endodermal cells, whereas proliferation appeared to be attenuated in the fibroblasts when compared to the E13 homotypic recombinants (Figure 2A). In the recombinants of E19 epithelial cells and E13 mesenchymal cells, proliferation appeared to be less pronounced, but evenly distributed between epithelial cells and mesenchymal cells (Figure 2D). In isolated E13 endodermal and mesenchymal cells high amounts of proliferating cells were present (Figure 3C,E). Isolated E19 epithelial cells had more proliferating cells than E19 fibroblasts (Figure 3D *vs* 3F). Results are summarized in Table 1.

Apoptosis

Using the TUNEL-assay, apoptosis was investigated in all recombinants. Apoptotic cells were only observed in the mesenchymal cells and fibroblasts. In the E13 homotypic recombinants TUNEL-positive cells were sporadically observed in the fibroblast tissue layer (Figure 4A). In contrast, almost every fibroblast was TUNEL-positive in the E19 homotypic recombinants (Figure 4C). Similarly, we observed more apoptosis in the fibroblast tissue layer in E19 fetal lungs in comparison to E13 (Figure

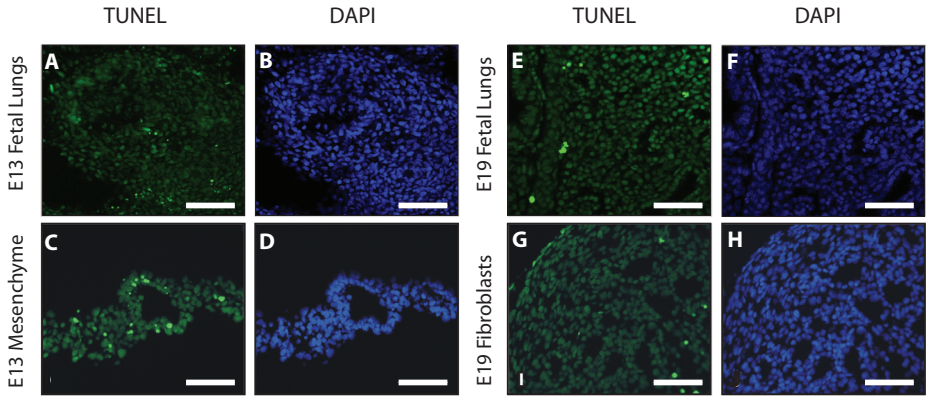

Figure 5. Apoptosis in fetal lungs and cells alone. In control experiments, apoptosis was investigated in fetal lungs and isolated cell cultures. In E19 fetal lungs (E) more apoptosis was observed compared to E13 fetal lungs (A). E13 mesenchymal cells (C) and E19 fibroblasts (G) had a minimal amount of TUNEL-positive cells. The scale bar represents 50μm.

5E *vs* 5A), E16 and E21 fetal and P1 postnatal lungs (Figure 6: right column). In both heterotypic recombinants, a similar pattern as in the E13 homotypic recombinants, was observed: hardly any mesenchymal cell or fibroblast was TUNEL-positive, and no TUNEL-positive endodermal or epithelial cells were observed (Figure 4E,G). The

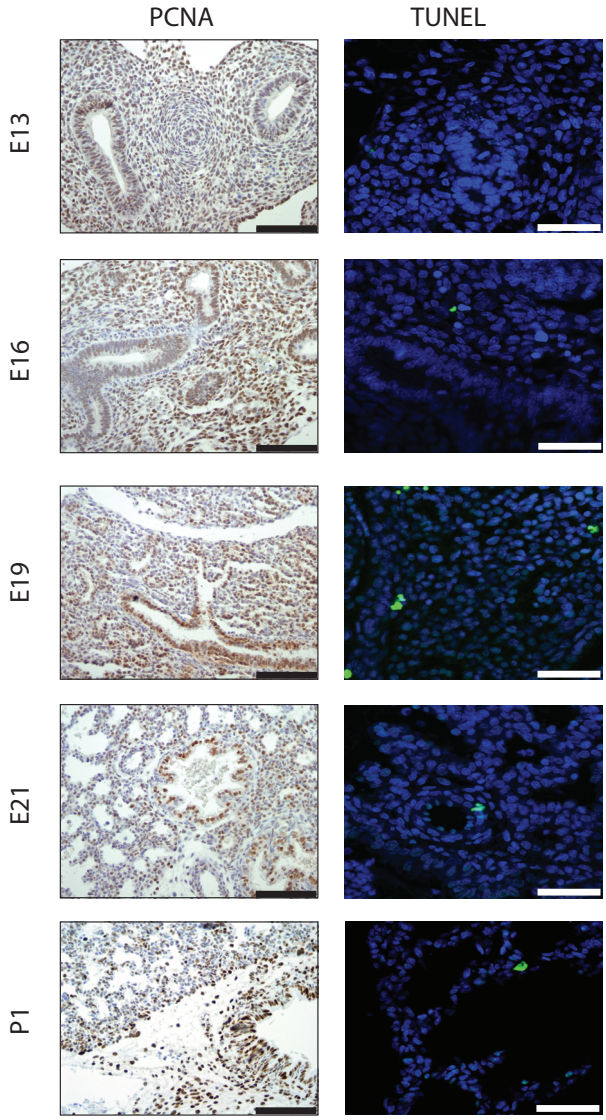

Figure 6. Proliferation and apoptosis during fetal lung development. In a control experiment, spatial and temporal changes of proliferation (PCNA: left column) and apoptosis (TUNEL-assay: right column) during fetal rat lung development were assessed. Proliferation changed from abundantly present in both tissue layers to mainly present in the epithelial lining of the lungs. Apoptosis was most pronounced in the saccular phase (E19) of fetal rat lung development. The black scale bar represents 100mm; the white scale bar represents 50μm.

same was seen in the E13 mesenchymal cells (Figure 5C) and E19 fibroblasts (Figure 5G) when cultured alone: only a few cells were TUNEL-positive. Only non-specific background staining was observed in the E13 endodermal cells and E19 epithelial cells (results not shown). DAPI was used to visualize the nuclei (Figure 4B,D,F,H and Figure 5B,D,F,H). Results are summarized in Table 1.

Table 1. Summary of proliferation and apoptosis in all cultures

	Proliferation		Apoptosis	
Type of culture	Endodermal or epithelial cells	Mesenchymal cells or fibroblasts	Endodermal or epithelial cells	Mesenchymal cells or fibroblasts
E13 fetal lungs	+++	+++	-	+
E13 endo only	+++	n/a	-	n/a
E13 mes only	n/a	+++	n/a	++
E13 endo/E13 mes	+++	+++	-	+
E19 fetal lungs	+++	+	-	++
E19 epi only	++	n/a	-	n/a
E19 fib only	n/a	+	n/a	+
E19 epi/E19 fib	++	+	-	+++
E13 endo/E19 fib	+++	+	-	+
E19 epi/E13 mes	++	++	-	+

+++ indicates high number of cells; ++ indicates medium number of cells; + indicates low number of cells; - indicates no cells; n/a indicates not applicable. E indicates embryonic day; endo indicates endodermal cells, epi indicates epithelial cells, fib indicates fibroblasts, mes indicates mesenchymal cells.

DISCUSSION

Morphogenesis of the lung has long been recognized to be highly dependent on epithelial-mesenchymal interactions [1, 3, 24]. Most studies focussed on branching morphogenesis and demonstrated epithelial-mesenchymal interactions in one direction: pulmonary epithelial branching morphogenesis is regulated by factors produced by its surrounding mesenchyme [1, 3, 11]. Using a cell recombinant model of fetal rat lung we provide evidence for regulation of mesenchymal morphogenesis by its surrounding epithelium in a gestational stage-dependent manner.

Apoptosis of E19 fibroblasts was demonstrated to be developmentally regulated by epithelial cells. Apoptosis of E19 fibroblasts occurred when they were recombined with E19 epithelial cells, but not when E19 fibroblasts were recombined with E13 endodermal cells. On the other hand, E13 mesenchymal cells could not be forced to undergo apoptosis by recombining them with E19 epithelial cells. Therefore, we

believe that thinning of the pulmonary mesenchymal tissue layer through apoptosis (as observed in E19 fetal lungs) is regulated by its surrounding epithelial cells, and is not intrinsic to the fibroblast tissue layer itself. The distribution of apoptotic cells is in accordance with what has been reported *in vivo* during rat pulmonary development, and what we observed in E19 fetal rat lungs [18]. Apoptotic cells were only observed in the mesenchymal cell layer, and predominantly during the later stages of pulmonary development [18, 19].

Apoptosis characteristics appeared to be extrinsic to the cells, since they changed depending on the gestational age of the cells that was used for recombination. An increase in apoptosis occurs during normal prenatal lung development, as confirmed in the E19 fetal lungs. This could have several explanations. Logically, this could be the result of a cell's intrinsic induction of apoptosis. However, in our experiments, E19 fibroblasts did not undergo apoptosis when recombined with E13 endodermal cells or when cultured alone, which rules out this possibility. Consequently this means that surrounding epithelial cells regulate the apoptosis in the fibroblast tissue layer observed late in gestation. Epithelial cells could do this in different ways. First, E19 epithelial cells could directly send pro-apoptotic signals to E19 fibroblasts to induce apoptosis. E13 mesenchymal cells are most likely too immature –lacking pro-apoptotic signal receptors– and therefore do not respond to these apoptosis-inducing signals of the E19 epithelial cells. This possibility is in agreement with the minimal apoptosis observed when E19 fibroblasts were cultured alone. However, we cannot rule out another possible explanation, namely that lung fibroblasts intrinsically have an anti-apoptotic phenotype due to a balance favouring pro-survival over death-inducing molecules. This balance maybe disturbed due to paracrine (soluble, matrix or cell contact) signals from E19 epithelial cells later in gestation. This second possibility would also agree with the minimal apoptosis seen in E19 fibroblasts cultured alone.

Apoptosis is considered to be a physiological phenomenon, which is required for normal thinning of mesenchymal tissue layer in late pulmonary development. In addition, apoptosis was demonstrated to play an important role during the postnatal structural maturation of the lung [25]. The amount of apoptosis observed in homotypic E19 recombinants, however, was more extensive than what we observed in the control experiment using E19 fetal rat lungs. We and others observed much less apoptosis *in vivo* than *in vitro* during late gestation [18-20]. This can be explained by the unnatural conditions under which the cells in our experiments were cultured. We recombined equal amounts of epithelial cells and mesenchymal cells whereas *in vivo* there are much less mesenchymal cells than epithelial cells at the investigated stage of pulmonary development (E19). Another '*in vitro*'-limitation is the

two-dimensional growth of the cells. Several groups have demonstrated that fetal breathing movements (and thereby stretching of the alveolar wall) are a vital part of lung development processes, such as late term apoptosis [26-28]. However, despite the use of our two-dimensional model, we did observe obvious differences between the four groups in terms of apoptosis. As all other circumstances remained equal between groups, we feel that the differences we observed are attributable to the epithelial-mesenchymal interactions.

Our results indicate that proliferation in both pulmonary epithelial cells and mesenchymal cells occurs in a cell autonomous manner during pulmonary development. Highest numbers of proliferating cells were observed among E13 endodermal and mesenchymal cells. Recombining these cells with E19 epithelial cells or fibroblasts did not alter the distribution and number of proliferating cells. Proliferation appeared to be an intrinsic feature of both epithelial and mesenchymal cells when they were cultured in close contact to each other. E13 endodermal cells remained in a high proliferative state when recombined with E19 fibroblasts that proliferated much less. On the other hand, the E19 fibroblasts could not be induced to proliferate more when recombined with the E13 endodermal cells. Proliferation characteristics of heterotypic recombinants of E19 epithelial cells and E13 mesenchymal cells also did not change when compared to their homotypic counterparts. Thus, E13 endodermal and E19 epithelial cells and E13 mesenchymal cells and E19 fibroblasts all displayed intrinsic proliferation characteristics, which were not influenced by heterotypic recombination. This result is in contrast to results obtained from studies using conditioned medium from epithelial cells, which were demonstrated to elaborate a hydrophobic polypeptide that inhibits fetal and adult lung mesenchymal cell proliferation *in vitro* [29, 30]. However, in those studies mesenchymal cells were not in close contact to the epithelial cells, and this might be an essential condition for normal proliferation to occur.

Future research is warranted to determine which epithelial paracrine signals (soluble, matrix or contact) regulate fibroblast apoptosis. For instance, prostaglandin E2 (PGE2) has been shown to induce fibroblast apoptosis [31]. Both epithelial cells and fibroblasts as well as other cells produce PGE2 [32-34]. Therefore, it is conceivable that epithelial cells regulate late term fibroblast apoptosis by excreting such prostanoids or other soluble factors. However, the exact mechanism remains to be determined.

CONCLUSIONS

Our results indicate that epithelial cells regulate the thinning of the surrounding mesenchymal (fibroblast) cell layer late during pulmonary development. To our knowledge this is the first study providing direct evidence of modulation of mesenchymal morphogenesis (through apoptosis) by its surrounding epithelium. This adds to the already extensive literature on the influence of mesenchyme on branching morphogenesis of the epithelial cell layer [2-4, 6-9, 24]. In addition, our study demonstrated once more the essential role of epithelial-mesenchymal interactions in lung morphogenesis through a balanced interplay between cell proliferation and mesenchymal apoptosis. With our study we are a step closer to understanding the physiology of normal lung development, which will aid in deciphering the pathogenesis of, and ultimately design targeted treatments for, developmental lung diseases such as pulmonary hypoplasia in CDH and oligohydramnios. Future studies revealing the signals involved in these epithelial-mesenchymal interactions, in particular in modulating mesenchymal apoptosis by its surrounding epithelium, are warranted.

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5

Epithelial-Mesenchymal Transition in Nitrofen-Induced Pulmonary Hypoplasia in Congenital Diaphragmatic Hernia: A Pilot Study

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ABSTRACT

Background Despite years of research, the pathogenesis of pulmonary hypoplasia in congenital diaphragmatic hernia (CDH) remains elusive. Previously, we demonstrated fibroblasts to be the 'defective' pulmonary tissue layer in the nitrofen model for CDH. Epithelial-mesenchymal transition (EMT) is involved in both normal organogenesis as well as the pathogenesis of several diseases, such as lung fibrosis. In this preliminary study we investigated EMT in nitrofen-induced pulmonary hypoplasia.

Methods Epithelial cells and fibroblasts were isolated from lungs of nitrofen-treated rats at embryonic day (E)19, and analyzed with immunocytochemistry for the simultaneous presence of pancytokeratin (epithelial cells) and vimentin (fibroblasts). Transgenic mice containing an infinite fluorescent labeling of epithelial cells (GNZ ROSA26-SPCrtTA-tetO-cre mice) were treated with nitrofen at E8.5 to induce pulmonary hypoplasia and CDH. At E18 lungs were isolated and immunohistochemical analysis was performed for GFP and mesenchymal markers vimentin and aSMA. Co-localization was determined by confocal microscopy.

Results Isolated cells from nitrofen-treated lungs contained both cytokeratin and vimentin. Control cells from untreated lungs were either cytokeratin- or vimentin-positive. In nitrofen-treated transgenic mice we observed cells positive for both GFP and mesenchymal markers; these were not present in control mice.

Conclusions These preliminary results suggest that EMT might be (partially) responsible for the thickened fibroblast layer observed in nitrofen-induced hypoplastic lungs.

INTRODUCTION

Despite years of research, the mystery around the pathogenesis of congenital diaphragmatic hernia (CDH) and its associated pulmonary anomalies remains unraveled. The main concern of CDH is the severe morbidity due to the associated lung anomalies pulmonary hypoplasia and persistent pulmonary hypertension of the neonate (PPHN). To develop preventative and new treatment strategies, detailed knowledge of the pathogenesis is a requirement.

Previously, utilizing an *in vitro* cell recombinant model, we demonstrated that the fibroblast tissue layer is the primary affected tissue layer in nitrofen-induced pulmonary hypoplasia in CDH [1]. Based on these results, we hypothesized that a similar defect is present in human CDH-associated pulmonary hypoplasia as well. An intrinsic defect in the fibroblasts could explain the morphological characteristics observed in hypoplastic lungs of both the nitrofen model as human CDH. However, the origin of the affected fibroblasts remains elusive.

Epithelial-mesenchymal interactions have been elaborately described as a prerequisite for proper lung development [2]. More recently, the concept of transitional processes has been speculated to be involved in normal lung development. In many organs, transitional processes are essential during development [3, 4]. Epithelial mesenchymal transition (EMT) is the transformation of an epithelial cell into a mesenchymal cell (fibroblast). EMT, and its opposite process mesenchymal-epithelial transition, have been described in many organs (eg. the kidney and the lung), both as a normal part of (embryonic) development as well as a pathological process such as carcinogenesis and fibrosis [3-7]. In lungs, EMT has been demonstrated in both the bleomycin rodent model for lung fibrosis and idiopathic pulmonary fibrosis in patients [8, 9]. In this pilot study we hypothesized that the origin of the affected fibroblast tissue layer descents from an epithelial origin.

To test our hypothesis we first isolated *in vivo* nitrofen-treated fibroblasts and epithelial cells and observed a concomitant expression of cytokeratin (epithelium) and vimentin (fibroblasts / mesenchyme), which was lacking in control lung cells. The gold standard to test whether EMT is present in nitrofen-induced pulmonary hypoplasia involves a method of infinite labeling of epithelial cells (and thereby, consequently, their progeny). We created transgenic mice that contained green fluorescent protein (GFP)-labeled epithelial type II cells. As these cells and their progeny will indefinitely keep their GFP label, it is possible to establish whether the increase in fibroblast cells descends from an epithelial origin. Embryonic day (E)18 lungs of nitrofen-treated

mice were analyzed for the presence of cells containing both the GFP signal (cells from epithelial origin) and fibroblast markers such as vimentin and αSMA.

METHODS & MATERIALS

Animals

The study was conducted according to the guidelines of the Canadian Council for Animal Care and with approval of the Animal Care Committee of the Hospital for Sick Children (protocol #6834 (transgenic mice) and #4245 (rats)). The animals were kept in a controlled light-dark cycle and food and water were supplied ad libitum.

Rats

Timed-pregnant Sprague-Dawley rats were ordered from Charles River (St. Constant (QC)). The finding of a sperm-positive vaginal smear was designated E0.

Transgenic mice

The conditional ROSA26 GNZ knock-in mice (The Jackson Laboratory, Bar Harbor (ME)) contain a nuclear-localized GFP / beta-galactosidase fusion protein (LacZ) (GNZ) inserted into the *Gt(ROSA)26Sor* (ROSA26) locus. Expression of GNZ is blocked by an upstream *loxP*-flanked STOP sequence (in the absence of Cre-recombinase no GNZ is expressed). When bred to *cre* expressing mice, offspring will have the STOP sequence deleted in tissues containing Cre recombinase. ROSA26 GNZ mice are on a C57BL/6J;129 background. Lung epithelial specific recombination between *loxP* sites was achieved by using the *tetO-cre* transgene and the surfactant protein C promotor-reverse tetracycline transactivator transgene (*SPC-rtTA*). All strains were backcrossed to a pure CD1 background to optimize the influence of nitrofen [20]. Mice containing two copies of ROSA26 and at least one copy of *tetO-cre* and *SPC-rtTA* were obtained by breeding and were genotyped by polymerase chain reaction. The finding of a sperm-positive vaginal smear was designated embryonic day E0. Timed-pregnant females received doxycyclin-containing water and food on embryonic days E9 – 12.

Nitrofen treatment

At E9 congenital diaphragmatic hernia and pulmonary hypoplasia were induced in pregnant rats by oral gavage of 100mg of 2,4-dichlorophenyl-*p*-nitrophenyl ether (nitrofen) (Cerilliant, Round Rock (TX)) in 1ml olive oil. Control pregnant rats were gavaged with 1ml of the vehicle. At E19 (term = E22) rats were sacrificed. Mice were gavaged at E8 with 25mg of nitrofen in 250µl olive oil. Control pregnant mice were gavaged with 250µl of the vehicle. At E18 (term = E19) mice were sacrificed.

Isolation of fetal rat pulmonary cells

To investigate whether these pulmonary cells from nitrofen-treated fetal rat lungs were positive for both cytokeratin (epithelial cell marker) and vimentin (mesenchymal cell marker), we isolated epithelial cells and fibroblasts from E19 fetuses as previously described [21]. Briefly, lungs were obtained and microscopically dissected from all other tissues including the major airways. Subsequently, lungs were minced, treated with collagenase and DNAse, and incubated at 37°C twice for one hour to isolate fibroblasts by differential adherence. Next, the remaining cells were incubated overnight to isolate epithelial cells.

Isolation of *in vivo* transgenic mouse lungs

We isolated nitrofen-treated lungs from transgenic mice to investigate whether these contained pulmonary cells positive for both epithelial and mesenchymal markers. The mouse fetuses were delivered by Caesarian section using aseptical surgical techniques. Lungs were removed from the thorax and fixed in 4% paraformaldehyde overnight at 4°C, dehydrated and embedded in paraplast, and 5µm sections were cut.

Immunofluorescence

Immunofluorescence protocols were previously described [1, 22]. Pulmonary cells isolated from nitrofen- and vehicle-treated fetal rats were double-stained for cytokeratin and vimentin. Primary antibodies were: 1:500 rabbit anti-pancytokeratin (Dako, Mississauga (ON)), 1:50 mouse anti-vimentin (Dako, Mississauga (ON)). Secondary antibodies (dilution of 1:200) were fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG (Calbiochem, San Diego (CA)) for vimentin, and rhodamine-labelled anti-rabbit IgG (Invitrogen, Eugene (OR)) for cytokeratin.

Lungs from transgenic mice were triple-stained for 1:500 rabbit anti-E-Cadherin (Cell Signaling, Danvers (MA)), 1:1000 chicken anti-GFP (Novus Biologicals, Cambridge (United Kingdom)), and 1:50 mouse anti-vimentin (BD Pharmingen, Breda (the Netherlands)), or 1:1000 mouse anti-α-smooth muscle actin (αSMA) (Biogenex, The Hague (the Netherlands)). Secondary antibodies (dilution of 1:400) were FITC-labeled anti-mouse IgG (Invitrogen, Bleiswijk (the Netherlands)), Cy3-labeled anti-rabbit IgG (Invitrogen, Bleiswijk (the Netherlands)), and Cy5-labeled anti-chick IgY (Abcam, Cambridge (United Kingdom)). Nuclei were stained with Hoechst-33258, a kind gift from Alex Nigg (Department of Pathology, Erasmus MC, Rotterdam (the Netherlands)), in Vectastain (Vector Laboratories, Peterborough (United Kingdom)).

Confocal microscopy

To analyze co-localization of epithelial / endothelial and mesenchymal markers in lungs, we used confocal microscopy (LSM 700 Zeiss, Sliedrecht (the Netherlands)). Z-stacks were made to visualize the complete cell.

RESULTS

Immunofluorescence of isolated cells

We isolated E19 epithelial cells and fibroblasts from nitrofen-treated and vehicle-treated fetal rat lungs to investigate whether these pulmonary cells were positive for both cytokeratin (epithelial cell marker) and vimentin (mesenchymal cell marker). Nitrofen-treated lungs contained epithelial cells with co-localization for cytokeratin and vimentin (Figure 1C and 1D). In contrast, vehicle-treated lungs did not contain epithelial cells with co-localization for cytokeratin and vimentin (Figure 1A and 1B).

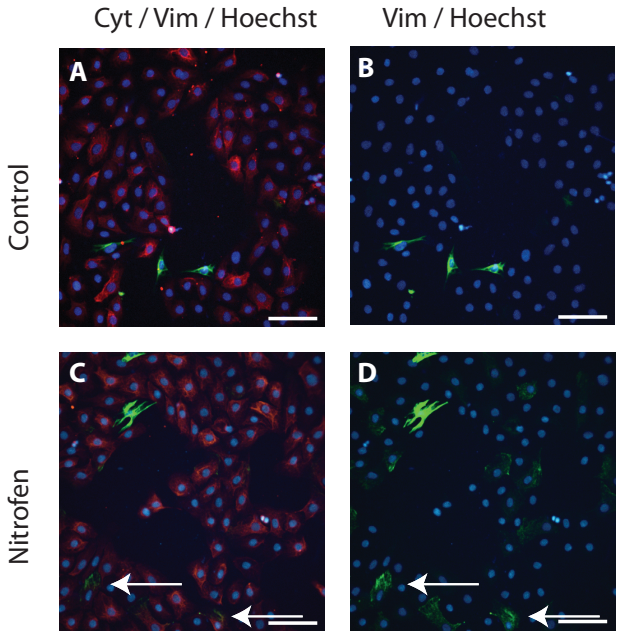

Figure 1. Co-localization of epithelial and mesenchymal markers in nitrofen-treated cells. IF staining of isolated epithelial cells from control (A,B) and nitrofen-treated (C,D) fetal rat lungs. Nitrofen-treated lungs contain epithelial cells with co-localization of cytokeratin (Cyt) (epithelial cell marker; red) (C) and vimentin (Vim) (fibroblast marker; green) (D). The arrows point towards cells positive for both markers. No co-localization was observed in control epithelial cells (A,B). Nuclei were stained with Hoechst (blue). The scale bar represents 100μm.

Isolated fibroblasts did not demonstrate co-localization for cytokeratin and vimentin (results not shown).

Immunofluorescence of *in vivo* lungs

To investigate whether nitrofen-treated lungs from transgenic mice contained cells positive for both epithelial and mesenchymal markers, we performed a triple-stain for GFP (cells from an epithelial origin), E-Cadherin (epithelial cells), and a mesenchymal marker. Mesenchymal markers that we investigated were vimentin (fibroblasts) and αSMA (myofibroblasts).

In nitrofen-treated lungs we observed cells triple-positive for GFP, E-Cadherin, and vimentin (Figure 2G – 2K). In vehicle-treated lungs we observed co-localization of GFP and E-Cadherin, but no co-localization of either of these epithelial markers with mesenchymal marker vimentin (Figure 2A – 2E).

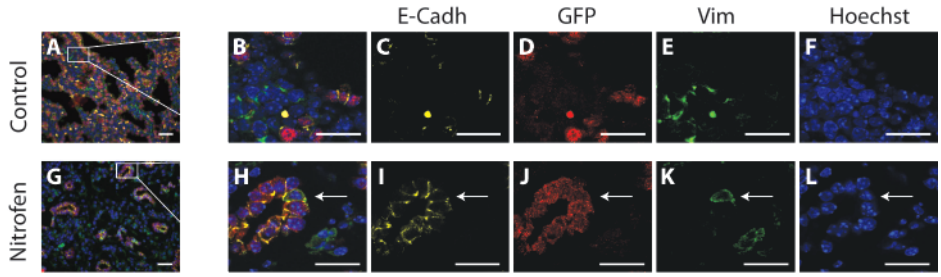

Figure 2. Co-localization of epithelial markers and vimentin in nitrofen-treated lungs. In nitrofen-treated lungs we observed cells triple-positive (G,H) for GFP (red) (2J), E-Cadherin (yellow) (I), and vimentin (green) (K). The arrow points towards a cell positive for all three markers. In vehicle-treated lungs (A,B) we observed co-localization of GFP (red) (D) and E-Cadherin (yellow) (C), but no co-localization of either of these epithelial markers with mesenchymal marker vimentin (green) (E). Nuclei were stained with Hoechst (blue) (F,L). The scale bar represents 25μm.

In nitrofen-treated lungs we observed cells triple-positive for GFP, E-Cadherin, and αSMA (Figure 3G – 3K: upper arrow). In addition, we observed pulmonary cells that contained co-localization of GFP and αSMA, but no E-Cadherin (Figure 3G – 3K: lower arrow). These pulmonary cells from an epithelial descent might have lost their E-Cadherin expression (or might not have adopted their E-Cadherin expression due to reduced mesenchymal-epithelial transition). In vehicle-treated lungs we observed co-localization of GFP and E-Cadherin, but no co-localization of either of these epithelial markers with mesenchymal marker vimentin (Figure 3A – 3E).

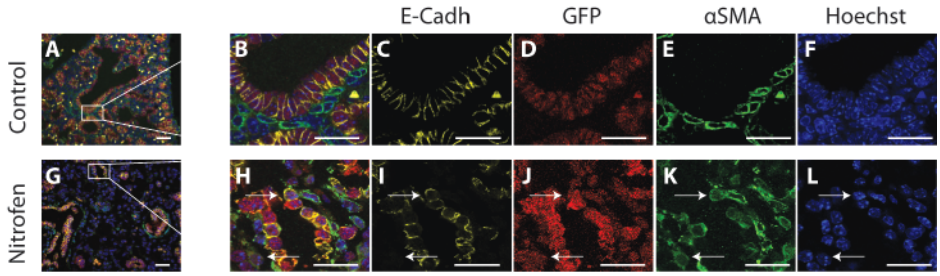

Figure 3. Co-localization of epithelial markers and a-smooth muscle actin in nitrofen-treated lungs. In nitrofen-treated lungs we observed cells triple-positive (G,H) for GFP (red) (J), E-Cadherin (E-Cadh) (yellow) (I), and a-smooth muscle actin (aSMA) (green) (K) (upper arrow). In addition, we observed cells positive for GFP and α-smooth muscle actin, but not E-Cadherin (lower arrow). In vehicle-treated lungs (A,B) we observed co-localization of GFP (red) (D) and E-Cadherin (yellow) (C), but no co-localization of either of these epithelial markers with mesenchymal marker α-smooth muscle actin (green) (3E). Nuclei were stained with Hoechst (blue) (F,L). The scale bar represents 25μm.

DISCUSSION

This pilot study demonstrated that nitrofen-treated lungs contain mesenchymal cells from an epithelial descent. These preliminary results suggest that EMT is a process that occurs in nitrofen-treated lungs *in vivo*, and may contribute to the CDH-associated pulmonary hypoplasia.

EMT has been described in pulmonary diseases such as lung cancer and lung fibrosis [8, 9, 23]. So far, in pulmonary morphogenesis transitional processes have not been described. However, during kidney branching morphogenesis, mesenchymal-epithelial transition (MET), the opposite process of EMT, is present. During kidney morphogenesis, a mutual induction of the (ureteric bud) epithelium and (nephrogenic) mesenchyme occurs. As the ureteric bud grows, the nephrogenic mesenchyme induces the ureteric bud to branch. In addition, the ureteric bud induces the nephrogenic mesenchyme to form around the bud and undergo MET to form the renal epithelium [24, 25]. As pulmonary branching morphogenesis resembles kidney branching morphogenesis, it seems likely that the process of MET plays a role in pulmonary development as well. During late pulmonary development, thinning of the mesenchymal tissue layer is a physiological process in order to establish an optimal cell layer for postnatal gas exchange. MET could play a role in this process during normal pulmonary development. Nitrofen disturbs normal pulmonary development by an unknown mechanism. An explanation for nitrofen to disturb this process would be to interfere with (physiological) interplay between MET and EMT.

Additional experiments should be performed to confirm the presence of EMT in nitrofen-induced pulmonary hypoplasia. Fetuses with a CDH must be included as these contain the most severe form of pulmonary hypoplasia. Subsequently, it would be interesting to quantify the number of cells co-localizing epithelial and mesenchymal markers. So far, we have only investigated lungs of nitrofen-treated fetuses at a late gestational age. Investigating earlier lungs might increase the amount of double-positive cells in close proximity. If the process of EMT starts in early gestation, the double-positive cells might become more scattered later on. The time point at which EMT occurs in this model is unknown. To eventually design a treatment modality specifically aimed at this process it is necessary to decipher the optimal time point to start treatment. Furthermore, EMT can be induced through several pathways, such as the Wnt/beta-catenin pathway. Also the exact role of transforming growth factor-beta (TGF-β) in the nitrofen model should be established. TGF-β has been recognized as an important inducer of EMT [10-12]. There is increasing evidence that TGF-β is involved in negatively regulating fetal lung branching morphogenesis [13-16]. TGF-β has been shown to be involved in the CDH lung in both the nitrofen model and human lungs, which encourages the hypothesis that EMT is involved in the pathogenesis of CDH lungs [17-19]. To develop these targeted treatment strategies to prevent pulmonary hypoplasia, it is necessary to decipher which pathway is responsible.

Besides EMT, MET could have been an explanation for the co-localization of epithelial and mesenchymal cell markers in the isolated cells. The presence of EMT in nitrofen-induced lungs does not necessarily exclude MET. Both EMT and MET could be present simultaneously, especially if one process attempts to regenerate the damaged lung tissue by counteracting the other.

In our transgenic mouse model, cells become fluorescent when the SPC-promotor is switched on. An explanation for co-localization of epithelial and mesenchymal cell markers, other than EMT, is that these cells are pluripotent (progenitor) cells. Previously, cells at the bronchoalveolar junction were identified as bronchoalveolar stem cells (BASCs) [26]. These cells demonstrated co-localization of epithelial cell and clara cell markers *in vivo*. *In vitro* BASCs were capable of differentiation and self-renewal. In addition, these cells had a proliferative response to naphthalene injury instead of cell death. In this study, cells in nitrofen-treated lungs demonstrated the presence of two markers from a different lineage, and could therefore be progenitor cells. To test this hypothesis, co-localizing cells would have to be sorted with fluorescence-activated cell sorting (FACS). Subsequently, these sorted cells should be cultured and tested to observe whether cell differentiation into multiple lineages such as chondrocytes and neural cells could be induced. The limitation of FACS analysis is that it can only detect cell surface markers, and not cytoplasmic or nuclear markers

such as GFP in our transgenic mice. Therefore, only cells double-positive for epithelial and mesenchymal cell markers can be FACS-sorted and cultured, which will lead to the loss of cells that have fully transitioned to a mesenchymal cell.

Another interesting direction for future research would be live imaging of cells in fetal (transgenic mouse) lung explants. Nitrofen has been demonstrated to induce pulmonary hypoplasia *in vitro* as well [27]. Live cell imaging is used to study cell dynamics. Frequent snapshots of cells are taken and can be turned into moving pictures of dynamic processes. The influence of nitrofen on the behavior of healthy fetal pulmonary explants could be examined when cells from an epithelial origin are labeled. The process of EMT could be visualized as epithelial cells would disintegrate from the matrix, and subsequently transform to a spindle-shaped mesenchymal cell in the interstitium. Limitations of live cell imaging are phototoxicity and -bleaching of the cells, but this would be equally present in nitrofen-treated and control fetal lung explants.

CONCLUSIONS

EMT is present in nitrofen-induced pulmonary hypoplasia, and might (partially) explain the thickening of the mesenchymal tissue layer in this model. Further experiments need to be performed to confirm these results.

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6

Postmortem Lung Biopsy for Obtaining Lung Tissue in Congenital Diaphragmatic Hernia

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Submitted

ABSTRACT

Background Accrual of human tissues from autopsies for diagnostic and translational research has decreased significantly over the last decades. The objective of this study was to evaluate our experience with lung biopsy through a mini-thoracotomy as an alternative for obtaining postmortem tissue when full autopsy is refused in congenital diaphragmatic hernia (CDH) patients.

Methods Within two hours of death we routinely asked parents for permission to perform an autopsy. Starting in 2001, parents who refused autopsy were asked permission for a postmortem lung biopsy. Pathology autopsy and biopsy reports were compared to clinical records.

Results Between 2001 and 2009, 46 patients died from CDH. Permission for autopsy was granted in five patients (11%). The parents of fifteen (33%) of the remaining 41 patients agreed to postmortem lung biopsy. In all cases, additional findings were reported from the autopsy or biopsy, without changing the originally reported cause of death. In one case, we isolated fibroblasts from the lung biopsy using standardized cell culture techniques. Parents were able to take their child home with a minimal delay following biopsy.

Conclusions Parents refusing a full autopsy frequently agree to postmortem organ biopsy. The quick procedure of biopsy might be helpful in the mourning process of parents as it prevents delay of transferring their deceased child home. This approach should therefore be considered as a valuable alternative to obtain human tissues for both diagnostic and research purposes and is potentially applicable to other anomalies.

INTRODUCTION

Permission for autopsies has been declining for decades [1-4]. This decline has been elaborately discussed by others and the main reasons are concerns from the next of kin about disfigurement of the body, exposing the child to additional invasive procedures or the feeling that the patient has 'suffered enough', and the possible delay of taking their child home [5-8]. In addition, media coverage of certain scandals has negatively influenced the public opinion on autopsies [9, 10]. Autopsies are not merely necessary to obtain or confirm the cause of death, but can also be instrumental for translational research to improve our understanding of diseases. As a result, we may create new therapeutic strategies and/or evaluate the effects of new treatment strategies [1, 2, 11-14]. Due to the decline in permission for autopsies, less translational research based on human tissue samples investigating the pathogenesis of diseases can be performed. In this study we describe our experience with a minimally invasive method of obtaining postmortem lung biopsies through a mini-thoracotomy of patients with congenital diaphragmatic hernia (CDH). This approach has potential relevance for a broader spectrum of pediatric diseases that result in death.

CDH is an example of a major congenital anomaly with an unknown pathogenesis. The associated lung abnormalities (pulmonary hypoplasia and persistent pulmonary hypertension) observed in CDH are the major cause of either death or severe morbidity. Modern-day treatment modalities such as ventilation and extracorporeal membrane oxygenation (ECMO) may have pathological effects on lungs such as hemorrhage (this study), interstitial fibrosis, and alveolar calcifications and as a result of ECMO, secondary inflammatory changes are frequently observed in these lungs [15, 16]. Understanding the pathogenesis of this disease is warranted to develop alternative (prenatal) therapeutic interventions such as tracheal occlusion to prevent or decrease its severe morbidity [17]. However, human tissue samples for investigation are not readily available. Obtaining a lung biopsy from a living newborn with CDH for research is unethical. The burden of such a biopsy in a very ill child is too high in terms of pain, risk of infection and bleeding, and loss of the already small amount of lung tissue due to pulmonary hypoplasia. Autopsies of children that deceased from the sequelae of CDH are the second best option to obtain human lung tissue samples. However, with permission declining for autopsies in children due to religious or other reasons such as negative public opinion, it is difficult to obtain these tissues for diagnostic and translational research purposes [1, 2, 18, 19]. New minimally invasive autopsy procedures through a mini-thoracotomy might help to deter this trend.

In 2001 we instituted a new protocol for postmortem lung biopsies through a mini-thoracotomy in our center to increase the accrual of lung samples from deceased CDH

children. By increasing the amount of lung tissue available for diagnostic and research purposes we aspired to potentially obtain better insight in the pathology of the lungs by performing a microscopic examination and to potentially facilitate translational research into the pathogenesis of CDH [20, 21]. The main objective of this study was to evaluate our experience with minimally invasive postmortem lung biopsy as an alternative for obtaining postmortem tissue when full autopsy is refused in CDH patients.

PATIENTS & METHODS

The Research Ethics Board of the Erasmus MC – Sophia Children's Hospital approved the protocol for this study. We included all children with CDH admitted to the intensive care unit between 2001 and 2009 that died during their initial admission. Parents are routinely informed during prenatal counseling given the potential risk of dying in CDH about the procedures in case their child would die. One of the aspects mentioned was that permission would be asked for a postmortem investigation (without specifying full autopsy or mini-thoracotomy). After the patients died, parents were first asked to give consent for a full autopsy. If parents refused the autopsy, they were asked for permission to perform a postmortem lung biopsy through a mini-thoracotomy for both diagnostic and research purposes. If parents agreed, one of the pediatric surgeons or pediatric surgical fellows performed a mini-thoracotomy at the level of the fourth or fifth intercostal space with an incision of approximately two centimeters through which the lung biopsy was taken. Biopsies were performed at the ipsilateral side unless the lung was too small to obtain tissue. In that case, the contralateral lung was used. Previously, we and others have failed to observe clear differences in histology between ipsilateral and contralateral lungs of CDH patients, so this approach was not considered to be a bias for the outcome [22, 23]. The specimen was divided in two equal parts and snap-frozen using isopentane in liquid nitrogen and processed for paraffin embedding within two hours after death. In one case we attempted and succeeded in isolating fibroblasts from the lung biopsy using standard cell culture techniques as previously described [24]. Clinical diagnoses and clinical causes of death were obtained from prospective and routinely available electronic medical patient records. Findings from autopsy and postmortem lung biopsy were obtained from pathology reports, and morphology of all lung samples was re-evaluated by a specialized pediatric pathologist (Ronald R. de Krijger) unaware of the treatment given before death. At full autopsy pulmonary hypoplasia was defined as a lung weight : body weight-ratio < 0.012. When not all information was available from the pathology reports or if patients were described as severely edematous at autopsy, hypoplasia was determined by lung weight compared to normal values based on gestational age. In lung biopsies, determining pulmonary hypoplasia based on weight was not feasible as only a part of the lung tissue was available.

RESULTS

From 2001 to 2009 46 newborns died of CDH in the intensive care unit of our hospital. Parents of five patients (11% of all deceased children) gave consent for a full autopsy. Fifteen of the remaining 41 patients (33% of all deceased children and 37% of the remaining parents) underwent a postmortem lung biopsy through a mini-thoracotomy. In total, we were able to quadruple the accrual of lung tissue from 11% from autopsies alone to 44% from autopsies and biopsies combined.

Results from full autopsies

Table 1 provides an overview of the clinical findings and findings at full autopsy of the five patients in which a full autopsy was performed. Two of these patients were female, the median gestational age (GA) at birth was 36^{+5} (27^{+5} – 38^{+2}) weeks $^{+\text{ days}}$, the median time of death was 6 days (1 – 16 days) postnatally, and all hernias were left-sided. Four patients died from therapy-resistant pulmonary hypertension of the neonate (PPHN, diagnosed by repeated Doppler cardiac ultrasound), and one patient died from thrombo-embolic complications and hemorrhage, according to the clinical information. The cause of death according to autopsy reports was denoted as 'due to lung abnormalities' and thrombo-embolic as well as bleeding complications, respectively. Two of five patients did not receive ECMO therapy. In one patient abstaining from ECMO treatment was decided based on a combination of prolonged hypoxia, therapy-resistant pulmonary hypertension and severe pulmonary hypoplasia. The other patient did not receive ECMO treatment due to prematurity, which is an exclusion criterion to start ECMO treatment. In three cases, an increased wall thickness of the pulmonary arterioles consistent with PPHN was reported. In two patients, associated anomalies observed in the clinical setting were infant respiratory distress syndrome (IRDS) and aortic coarctation. In all patients, pulmonary hypoplasia was confirmed. Additional findings in the lungs were hemorrhage (4 patients), congestion (3), hyaline membrane disease (3), amniotic fluid and/or meconium aspiration (fetal distress) (2), extramedullary hematopoiesis (sign of hypoxia), fibrosis of the alveolar septa (2), lungs immature-for-age (2), thrombi in the arteries and arterioles (1), type II pneumocyte hyperplasia (1), and dilated lymph vessels at the pleural surface (1). (Extensive) hemorrhage was present in all ECMO patients, and in one of two non-ECMO patients. Additional findings of other organs were cyst formation in kidneys and testes, asphyctic hemorrhages of the kidneys, and liver, heart, and thymus abnormalities as denoted in Table 1.

Results from postmortem lung biopsies through a mini-thoracotomy

Table 2 gives an overview of the clinical findings and the postmortem lung biopsy findings of the 15 patients. Ten of these patients were female, the median GA at

Table 1. Comparison of clinical and full autopsy findings.

Pt	Gender	GA	Death at	Side	Associated anomalies/ morbidity	Clinical cause of death	Full autopsy cause of death	Additional findings at full autopsy
1	female	36^{+5}	16	left	-	PPHN hypotension possible infection	lung abnormalities	lungs: - hypoplastic left lung (LHW) - congestion - extensive intra-alveolar hemorrhage - extensive hyaline membranes - signs of amniotic fluid inspiration - stage: unable to determine extramedullary hematopoiesis liver, heart, spleen
2*	male	34^{+3}	1	left	-	PPHN	extensive lung abnormalities	lungs: - hypoplastic left lung (LHW) - slight congestion - hyaline membranes - thickened wall of arterioles - extramedullary hematopoiesis - dilated lymph vessels at pleural surface - signs of amniotic fluid and meconium aspiration - stage: alveolar kidneys: multiple tubular cysts testes: cystic dysplasia
3	male	38^{+2}	10	left	-	PPHN therapy-resistant hypotension	therapy-resistant PPHN	lungs: - hypoplastic left lung (LHW) - intra-alveolar hemorrhage - early septal fibrosis - thickened wall of arterioles, mainly left lung - type II pneumocyte hyperplasia - stage: unable to determine kidneys: small asphyctic hemorrhages

Pt	Gender	GA	Death at	Side	Associated anomalies/ morbidity	Clinical cause of death	Full autopsy cause of death	Additional findings at full autopsy
4*	male	27+5	1	left	IRDS	PPHN	ARDS (immature hypoplastic lungs)	lungs: - hypoplastic lungs (LHLBR) - intra-alveolar and interstitial hemorrhage - hyaline membranes grade III - thickened wall of arterioles - stage: canalicular heart: perimembraneous VSD, bicuspid pulmonary valve kidneys: multiple small tubular cysts liver: ductal plate formation
5	female	38	6	left	coarctatio aorta (prenatal PLUG)	thrombo-embolic complications and hemorrhage	thrombo-embolic complications and hemorrhage	lungs: - hypoplastic lungs (LHW) - congestion - extensive septal and intra-alveolar hemorrhage - fibrotic aspect - multiple thrombi arteries/arterioles - stage: saccular heart: several thrombi; several infarctions due to thrombi; open ductus; coarctatio aorta liver: extensive congestion, cholestasis, zone 3 necrosis kidneys: extensive hemorrhage and congested left kidney

* denotes this patient did not receive ECMO treatment.

Pt = patient anonymous code GA = gestational age at birth (in weeks + days) Death at = time of death in postnatal days Side = side of congenital diaphragmatic hernia LHW = lung hypoplasia based on lung weight *versus* GA normal values LHLBR = lung hypoplasia based on lung weight : body weight-ratio (< 0.012) PPHN = persistent pulmonary hypertension of the neonate IRDS = infant respiratory distress syndrome ARDS = acute respiratory distress syndrome VSD = ventricular septal defect

birth was 38^{+2} (36^{+2} – 41^{+1}) weeks $^{+\text{days}}$, the median time of death was 16 days (1 – 29 days) postnatally, and 13 (87%) of the hernias were left-sided. Clinical causes of death were pulmonary hypertension or sepsis and multi-organ failure (MOF). Fourteen of 15 patients had therapy-resistant pulmonary hypertension following ECMO-treatment. Two of 15 patients did not receive ECMO therapy. In one patient, phenotypic characteristics of Cornelia de Lange syndrome (confirmed by genetic analysis) resulted in withdrawal of treatment. The other patient had a hypoplastic left ventricle in addition to CDH, resulting in a dismal prognosis, which was the reason to withdraw further treatment. Four patients had no associated anomalies. Associated clinical anomalies observed in the remaining 11 patients were cerebral hemorrhage/infarction (3 patients), pneumothorax (3), sick euthyroid syndrome (2), severe congenital cardiac anomalies (1), phenotypic Cornelia de Lange syndrome (1), hypospadias (1), hemivertebra T12 (1), and right-sided pulmonary hypoplasia (1). An experienced pediatric pathologist (Ronald R. de Krijger) evaluated the morphology of the tissue samples (Figure 1A). Arteriolar wall thickening due to PPHN was always confirmed. Additional findings in the lung biopsy specimens were hemorrhage (14 patients), hyaline membrane disease (7), septal fibrosis (5), thickened alveolar septa (6), extramedullary hematopoiesis (sign of hypoxia) (4), thrombi (5), congestion (9), type II pneumocyte hyperplasia (6), centroacinary unfolding and/or atelectasis (11), amniotic fluid and meconium aspiration (1), pneumonia (1), and immature parenchyma (10). (Extensive) hemorrhage was present in 12 of 13 ECMO patients, and in one of two non-ECMO patients. The morphological characteristics of lung biopsies resembled findings observed in lungs in full autopsies. Pulmonary fibroblasts were successfully isolated from one of the biopsies (Figure 1B).

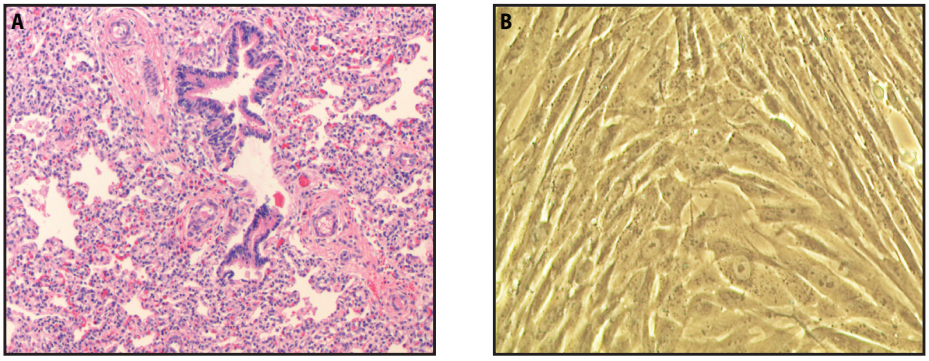

Figure 1. Histology of a postmortem lung biopsy and an image of isolated fibroblasts. Microscopic image of hematoxylin and eosin stain of a postmortem lung biopsy through a mini-thoracotomy (A) and an image of fibroblasts isolated from a postmortem lung biopsy through a mini-thoracotomy (B).

DISCUSSION

Although full autopsy remains the 'gold standard' for postmortem investigations as organs can be investigated entirely and in relationship to other organs, our experience with postmortem lung biopsies through a mini-thoracotomy demonstrates that this can be a good alternative to obtain tissue for diagnostic and translational research purposes if consent for a full autopsy is refused. Herein, we demonstrate that permission for obtaining postmortem lung tissue increased from 11% for conventional autopsies to 44% for the combination of conventional full autopsies and lung biopsies through a mini-thoracotomy.

We speculate that the main reason for the high permission rate for a postmortem lung biopsy through a mini-thoracotomy is that disfigurement of the body and additional invasive procedures are not as severe as compared to a full autopsy, which are common reasons for declining full autopsy [5-8]. Secondly, the opportunity for parents to take their child home within hours after demise of the child might add to this. From a psychological point of view, taking the child home within hours seems favorable as well, as it may support the mourning process. In addition, Sullivan et al. described that in 42% of parents full autopsy added to their grief [25].

Alternative minimally invasive autopsy approaches have previously been described in literature. Non-invasive techniques such as MRI or CT scan were demonstrated to lack accuracy and cost-effectiveness compared to full autopsy, although further evaluation is warranted [26-28]. Non-invasive techniques in combination with organ needle core biopsies varied in accuracy. Depending on the study population some studies demonstrated a 100% false-negative rate while others demonstrated optimistic results of 90% sensitivity [29-31]. Obvious limitations of such approaches are the logistics (as MRI or CT are often not universally available for postmortem purposes) and additional costs of the MRI and CT scans. Laparoscopic postmortem investigations have been described as well. Fan et al. reached a high accuracy with this method when compared to full autopsy [32]. Limitations to this method are again the significant costs and logistics, with an additional limitation of the possible spread of infectious diseases by CO2 leakage from the insertions [32]. A common limitation of all of the alternative methods mentioned is the delay in performing the procedure.

Postmortem lung biopsies through a mini-thoracotomy appear to be a good alternative to increase lung tissue available for diagnostic purposes. In up to 40% of full autopsies additional information is uncovered that was not available in the clinical setting [1, 13, 33, 34]. In our study, the pathology reports and morphological evaluation of all autopsies and biopsies demonstrated additional findings to the clinical reports.

Table 2. Comparison of clinical and lung biopsy findings.

Pt	Gender	GA	Death at	Side	Assoc Anomalies/ Morbidity	Clinical cause of death	Additional findings at lung biopsy
1	Female	38^{+6}	11	right	hemivertebra T12	PPHN	congestion intra-alveolar and interstitial hemorrhage hyaline membranes thickened wall of arterioles thrombi stage: unable to determine
2	Male	37^{+6}	18	left	-	PPHN	(fibroblast isolation) congestion intra-alveolar hemorrhage hyaline membranes, mild septal fibrosis thickened wall of arterioles extramedullary hematopoesis local bronchopneumonia stage: saccular
3	Female	39^{+6}	12	left	-	PPHN	congestion intra-alveolar hemorrhage thickened wall of arterioles slight extramedullary hematopoesis stage: saccular
4	Female	36^{+5}	19	left	pneumothorax, small ependymal hemorrhage	PPHN	congestion hemorrhage hyaline membranes, septal fibrosis thickened wall of arterioles extramedullary hematopoesis atelectasis type II pneumocyte hyperplasia stage: unable to determine

Pt	Gender	GA	Death at	Side	Assoc Anomalies/ Morbidity	Clinical cause of death	Additional findings at lung biopsy
5	Female	38	14	left	sick euthyroid syndrome	PPHN	congestion intra-alveolar hemorrhage thickened alveolar septa thickened wall of arterioles extramedullary hematopoesis atelectasis type II pneumocyte hyperplasia stage: saccular
6	Male	37	23	left	-	PPHN + MOF	extensive hemorrhage thickened alveolar septa thickened wall of arterioles thrombi centroacinary unfolding with many atelectasis type II pneumocyte hyperplasia stage: saccular-alveolar
7	Female	38	12	right	-	Right-sided heart failure due to PPHN	extensive intra-alveolar hemorrhage slight fibrosis thickened wall of arterioles atelectasis type II pneumocyte hyperplasia stage: saccular
8	Female	38^{+1}	29	left	-	PPHN	congestion extensive hemorrhage start of hyaline membranes, septal fibrosis thickened wall of arterioles extensive type II pneumocyte hyperplasia stage: saccular

Pt	Gender	GA	Death at	Side	Assoc Anomalies/ Morbidity	Clinical cause of death	Additional findings at lung biopsy
9	Male	39^{+3}	12	left	intracerebral hemorrhage	PPHN	congestion extensive intra-alveolar hemorrhage thickened wall of arterioles extramedullary hematopoesis thrombi partial atelectasis stage: unable to determine
10	Male	39	16	left	prenatal cerebral infarction	PPHN	thickened alveolar septa thickened wall of arterioles centroacinary unfolding stage: alveolar
11	Female	41^{+1}	20	left	pneumothorax	PPHN	intra-alveolar hemorrhage thickened alveolar septa thickened wall of arterioles partial atelectasis type II pneumocyte hyperplasia stage: unable to determine
12*	Female	36^{+6}	1	left	severe left ventricle heart hypoplasia, mitral atresia, large ASD, double outlet right ventricle	PPHN	congestion extensive hemorrhage extensive hyaline membranes thickened wall of arterioles thrombi centroacinar unfolding stage: saccular
13*	Male	36^{+2}	2	left	Cornelia de Lange syndrome, hypospadias	PPHN	minimal hemorrhage thickened wall of arterioles extensive atelectasis amniotic fluid and meconium aspiration stage: alveolar

Pt	Gender	GA	Death at	Side	Assoc Anomalies/ Morbidity	Clinical cause of death	Additional findings at lung biopsy
14	Female	39^{+2}	16	left	-	PPHN	intra-alveolar hemorrhage hyaline membranes thickened wall of arterioles arterial thrombus atelectasis type II pneumocyte hyperplasia stage: saccular
15	Female	38^{+3}	21	left	pneumothorax	PPHN	congestion hemorrhages slight hyaline membranes, interstitial fibrosis thickened wall of arterioles atelectasis stage: unable to determine

* denotes this patient did not receive ECMO treatment.
Pt = patient anonymous code GA = gestational age at birth (in weeks $^{+\text{ days}}$) Death at = time of death in postnatal days Side = side of congenital diaphragmatic hernia Assoc an = associated anomalies PPHN = persistent pulmonary hypertension of the neonate MOF = multi-organ failure

However, significant changes in the cause of death were not identified. In one full autopsy report no vessel abnormalities were observed while the clinical cause of death was PPHN. Additional findings that were clinically relevant but were not considered the cause of death were findings of pneumonia and hemorrhage. This emphasizes the importance of full autopsies and postmortem biopsies [1, 33, 34]. Knowing the cause of death is also important for general knowledge of diseases and their complications to improve the quality of care and to enhance the accuracy of death statistics. In addition, full autopsies are necessary to monitor new clinical investigative techniques for accuracy and side effects and new treatment modalities for efficacy and complications [3, 35]. And, last but not least, autopsy results help the parents grieve, understand what happened, and whether the diagnosis was accurate and the treatment given appropriate. For instance, when the treatment team decides to withdraw further treatment, additional diagnostic insight from a lung biopsy could help parents cope with this decision. Simultaneously, results from an autopsy may provide information about obstetric or genetic risk of recurrence, and can therefore be important for future pregnancies or family members [1, 2, 5, 11-14, 35-39]. However, we are aware that not all benefits associated with full autopsies are present in postmortem biopsies through a mini-thoracotomy. Evaluating a small part of, in this instance, the lung might not be representative for the entire organ, even though most biopsy characteristics in our study did resemble full autopsy results of the lungs [30, 40]. In addition, validation of diagnostic tools and monitoring treatments are not feasible when we are not able to evaluate the entire body.

Even though we agree that a postmortem lung biopsy through a mini-thoracotomy could never replace a full autopsy [40], there are a few advantages to a postmortem biopsy through a mini-thoracotomy over full autopsy. Postmortem tissue availability is primarily necessary for diagnostic purposes, however can be useful for translational research as well. A scientific advantage of the biopsies is minimal delay between death and obtaining the tissue, which improves tissue quality [41]. Van Maldegem et al. demonstrated that RNA quality decreased when time till fixation of the tissue increased [42]. We demonstrated the potential to isolate fibroblasts for cell culture experiments from lung tissue of a CDH patient obtained from a postmortem lung biopsy through a mini-thoracotomy. This provides the unique opportunity for pathogenetic research to translate results from *in vitro* animal studies to the human situation. Nowadays, cells such as lung fibroblasts can be stored for a prolonged time, so cells derived from biopsies as well as the tissues itself could, with proper consent, be stored to create a biobank for future research [43]. Our biobank of lung biopsy samples has proved to be an invaluable resourch for many translational studies by our group as demonstrated in literature [20, 21, 44-46]. Examples of successful use of the biobank are Unger et al., who demonstrated that the expression of sonic hedgehog

is downregulated in pulmonary hypoplasia, and Rajatapiti et al., who demonstrated that the expression of glucocorticoid, retinoid and thyroid hormone receptors is not altered in lungs of patients with CDH [20, 21]. These studies both contributed to our current knowledge on CDH. The biobank allows us to collect specimens from more patients and use these in the future when new investigative techniques, such as the recent whole genome sequencing, become available or new results from animal studies warrant translation to the human situation. This is especially important in diseases such as CDH in which survival has improved (and therefore the opportunity to obtain material from deceased patients has become rare) but there is still a need for elucidating the exact pathogenetic mechanisms. This procedure might be applicable to a broader spectrum of pediatric diseases that result in death, and does not have to be restricted to newborns.

CONCLUSIONS

Given the low consent rate for full autopsy, postmortem biopsies through a mini-thoracotomy provide a valuable alternative to obtain human tissue samples for diagnostic and research purposes. The value of postmortem organ biopsies through a mini-thoracotomy for a broader spectrum of diseases that result in pediatric death should be evaluated in future studies.

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7

The Von Hippel-Lindau Pathway in Diaphragmatic Defects

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ABSTRACT

Background The Von Hippel-Lindau (VHL) pathway is involved in the morphogenesis of many organs amongst which kidneys, testes and lungs. In congenital diaphragmatic hernia (CDH) both the diaphragm and lungs are affected. Recent data suggest that these defects are caused by a similar insult. The exact pathogenesis of CDH has not been elucidated so far. The retinoic acid (RA) pathway has been implicated in CDH. Several transcription factor (regulators) are present in both lungs and diaphragm, and are located on chromosomes commonly deleted in CDH patients. This encourages the theory of a similar insult. Hypoplastic CDH lungs display abnormalities in the VHL pathway. The VHL pathway has not been investigated in the diaphragms of CDH patients, neither during normal diaphragm development. The objective of this study was to determine the expression pattern of VHL pathway proteins in healthy diaphragms and the diaphragm of patients with CDH and diaphragmatic eventration (CDE).

Methods After obtaining consent from parents, diaphragms were isolated during autopsies of deceased children with CDH (4), CDE (3) and children without congenital abnormalities (7). No chromosomal anomalies were present. In addition, diaphragm biopsies were collected from nine other CDH patients during operative repair. Standard histology and immunohistochemical staining for platelet endothelial cell adhesion molecule (PECAM), VHL, hypoxia-inducible factor-1α (HIF1α), vascular endothelial growth factor (VEGF), and endothelial nitric oxide synthase (eNOS) were performed. Quantitative real-time RT-PCR for eNOS was performed as well.

Results Protein expression of components of the VHL pathway in the interstitium, muscle, and vessel wall was similar between CDH, CDE and healthy diaphragms. Neither did we observe any differences at the gene expression level of eNOS between CDH and healthy diaphragms.

Conclusions The VHL proteins are present in healthy diaphragms while a disruption related to the VHL pathway appears unlikely as the common cause of lung and diaphragm anomalies in CDH patients.

INTRODUCTION

The Von Hippel-Lindau (VHL) pathway is a pathway involved in embryogenesis, and in formation of many organs such as kidneys, testes, and the central nervous system [1, 2]. The enzyme endothelial nitric oxide synthase (eNOS), a downstream target of the VHL pathway, converts l-arginine to nitric oxide (NO) (Figure 1) [3]. In development, NO is mainly involved in angiogenesis. The diaphragm is a crucial organ for breathing. To our knowledge, the VHL pathway has not been studied in diaphragms so far.

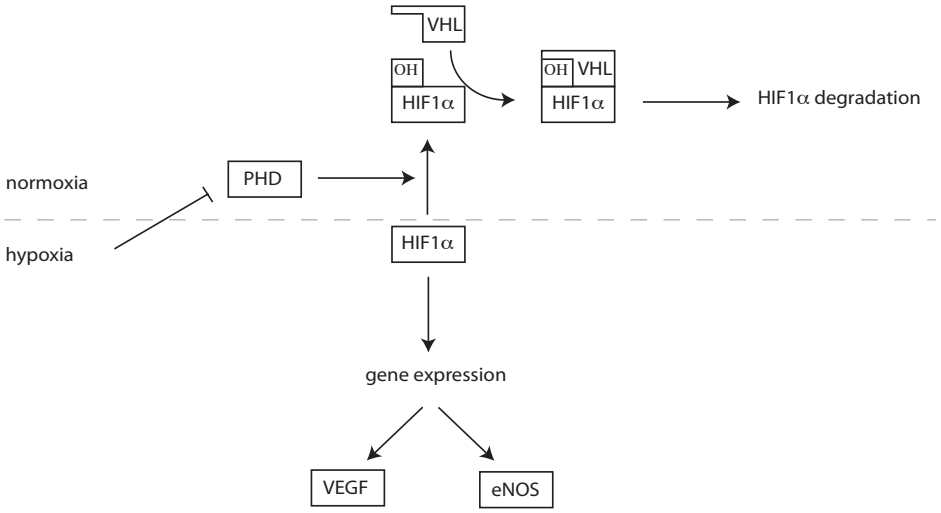

Figure 1. Schematic of the VHL pathway. In normoxic conditions prolyl hydroxylase (PHD) causes hydroxylation of hypoxia-inducible factor-1α (HIF1α) to allow Von Hippel-Lindau (VHL) protein to bind to HIF1α. This results in the degradation of HIF1α. In hypoxic conditions, PHD is inhibited, which allows HIF1α to induce gene expression of angiogenic factors such as vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS). The enzyme eNOS converts l-arginine to nitric oxide (NO). NO is involved in angiogenesis and vessel function.

Diaphragmatic defects such as congenital diaphragmatic hernia (CDH) or congenital diaphragmatic eventration (CDE) can severely impair oxygenation and can be incompatible with life. CDE, which is 7% (*versus* 93% CDH) of real diaphragmatic defects, has been proposed as a 'milder' form of CDH in which the diaphragm is affected by loss of muscle tissue without causing a 'hole in the diaphragm' [4]. Interestingly, pulmonary hypoplasia, heart and skeletal abnormalities are associated with both defects, which strengthens this assumption [5-11]. CDE has a lower mortality rate (1 – 8% *versus* ≥ 20% depending on case selection) and is less complicated to repair than CDH.

CDH consists of two major defects: a diaphragmatic defect and lung anomalies. For decades, lung anomalies were thought to be solely attributable to the abdominal organs 'compressing' the lungs. However, in 2000 our group demonstrated that the observed lung anomalies in the rat nitrofen model are due to an intrinsic defect in the lungs as well, the so-called dual-hit hypothesis [12]. Therefore, it has become likely that these defects occur through the same pathogenetic pathway. In addition, more recently, several transcription factor (regulators) of the retinoic acid (RA) pathway, located on chromosomes commonly deleted in CDH patients, are present in both organs [13-15]. During lung morphogenesis NO is important in the formation of vessels and after birth for pulmonary gas exchange as it acts as a prominent vasodilator. In human CDH lungs our group demonstrated earlier that the VHL pathway is disrupted [16-19]. We speculate this pathway might be disrupted in CDH diaphragms as well, as a common denominator between the lung and diaphragm anomalies in diaphragmatic defects. To test this hypothesis we investigated the expression of components of the VHL pathway in healthy diaphragms and, subsequently, in diaphragms from patients with CDH and CDE.

PATIENTS & METHODS

Human diaphragms

Informed consent was obtained from parents of children that succumbed from CDH (4), CDE (3) and other causes except congenital abnormalities in their neonatal period (controls) (7). Subsequently, diaphragms were isolated during autopsy. In CDH patients the median gestational age (GA) at birth or abortion was 40^{+1} ($28 - 42^{+1}$) weeks $^{+\text{days}}$; median survival time was 4 days (< 24 hours – 2 weeks). One CDH patient had undergone a prenatal tracheal occlusion procedure and was postnatally treated with extra-corporeal membrane oxygenation (ECMO), and died at the age of 14 days due to therapy-resistant pulmonary hypertension. The median GA of CDE patients was 21^{+1} ($17^{+3} - 21^{+3}$) weeks; these were all terminations of pregnancy. The median GA of control patients was 32^{+1} ($23^{+6} - 38^{+4}$) weeks. Most control patients had died intrauterine or immediately after birth.

Diaphragm tissue was also collected from living patients during operative hernia repair. Separate informed consent was obtained from parents. During the operation a biopsy from the diaphragm was collected (5) and tissue from the hernia sac, if present, was removed in four other patients (4). The median gestational age at birth was 39^{+1} ($35^{+5} - 40^{+3}$) weeks. Three patients were treated with ECMO before the hernia-repair. Tissues (except for biopsies) were processed for paraffin embedding according to the standard pathology protocol. Biopsies were frozen in liquid nitrogen. Sections of 5µm were stained with hematoxylin and eosin (H&E) staining for histology.

Genetic studies

Karyotyping was performed in 17 of 20 patients according to standard analysis methods. DNA for genomic analysis was extracted from peripheral blood or fibroblast cultures by the puregene DNA purification kit (Gentra Systems, Minneapolis (MN)). Genomic DNA of ten CDH patients was hybridized to a high-resolution Illumina cyto-SNP bead chips version 12.2 (Illumina, San Diego (CA)). Filtering, normalization and data analysis of each array was done using the Nexus® software programme (version five, Biodiscovery, El Segundo (CA)) as previously described [20]. To review functionality of each putative copy number variation (CNV), occurrence frequencies in qualified normal cohorts of CHOP [http://www.chop.edu/], DGV [projects.tcag.ca/variation/] and Decipher databases [https://decipher.sanger.ac.uk/] were checked. Since these populations display various ethnic backgrounds, comparison to an in-house local reference set of 470 normal individuals was executed also. Confirmation of each putative candidate with real-time RT-PCR and/or fluorescent in situ hybridization (FISH) was executed in the proband and his / her parents according to local standard protocols as recently described [21].

Immunohistochemistry

We used antibodies against members of the VHL pathway to determine whether they are differently expressed between the three groups. The VHL protein regulates the production of nitric oxide (NO) through hypoxia-inducible factor-1α (HIF1α) inhibition. When HIF1α is not degraded, it induces the production of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS). eNOS converts l-arginine into NO [22]. We investigated VHL, HIF1α, VEGF, and eNOS. We used platelet endothelial cell adhesion molecule (PECAM) as a positive control for vessels.

Following heat-induced antigen retrieval in Tris-EDTA pH 9.0 buffer, slides were treated with 3% (v/v) hydrogenperoxide in phosphate buffered saline to block endogenous peroxidase activity. We added blocking solution of 10% normal goat serum and 1% bovine serum album in PBS for one hour at room temperature. Subsequently, we incubated slides overnight with primary antibodies at 4°C. Slides were developed using Envision kit according to the manufacturer's instructions, and counterstained with hematoxylin. Slides were mounted with perfex (Histolab, Gothenburg (Sweden)). Primary antibodies used were undiluted mouse anti-VHL protein (Genetex, Irvine (CA)), mouse anti-HIF1α (1:200; Abcam, Cambridge (United Kingdom)), rabbit anti-VEGF (1:100; Abcam, Cambridge (United Kingdom)), anti-eNOS (1:100; BD Biosciences Pharmigen, Breda (the Netherlands)), and anti-PECAM (1:50; Dako, Heverlee (Belgium)).

Quantification

We investigated all diaphragms for the presence of staining of the VHL pathway antibodies in the muscle, interstitium and vessels of the diaphragm (if present), and scored blinded as strong staining (+++), average staining (++), weak staining (+), absence of staining (-).

Real-time RT-PCR

To validate our immunohistochemical results, we performed real-time RT-PCR in five patients (three CDH *versus* two controls) to compare eNOS expression at the RNA level. We used iScript cDNA synthesis kit (Bio-rad, Hercules (CA)) to reversely transcribe 2μg of total RNA sample to cDNA. Per well we used 0.6μl of each cDNA product, which was mixed with the KAPA-SYBR fast mastermix (KapaBiosystems, Woburn (MA)). Subsequently, for the quantitative RT-PCR analysis we used the ABI7300 Real-time PCR system. We performed each reaction in triplicate, designed as described by Boehm et al., with a region of the RPS18 human gene serving as a control [23]. We designed the primers from the unique cDNA sequences of the eNOS gene (forward ACTGAGATCGGCACGAGGAA; reverse GTCCATGCAGACAGCCA-CAT) with Primer Express software v2.0 (Applied Biosystems).

RESULTS

Genetic studies

All 17 patients displayed a normal 46.XX or 46.XY karyotype. Of the 11 patients in which genomic analysis was performed two demonstrated a known CDH related CNV (22q11 duplication and 15q25 deletion). No deletions or duplications were observed involving the VHL and related genes as prolyl hydroxylase (PHD)3, HIF1α, VEGFA and eNOS.

Human diaphragm morphology

H&E staining demonstrated that all eventration diaphragms did not have any muscularization present in the specimen (Figure 2C). CDH postmortem tissue and biopsy tissue did not appear different from control diaphragm tissue (Figure 2B *vs* 2A). Hernia sac tissue removed during hernia surgery did not have any muscularization either; the morphology was similar to CDE tissue (Figure 2D). Vessels did appear to be further apart and smaller in the CDE and hernia sac tissue than in postmortem diaphragmatic tissue from CDH patients and controls (Figure 2C and 2D *vs* 2B and 2A, respectively). However, due to the limited number of patients, we were not able to quantify this.

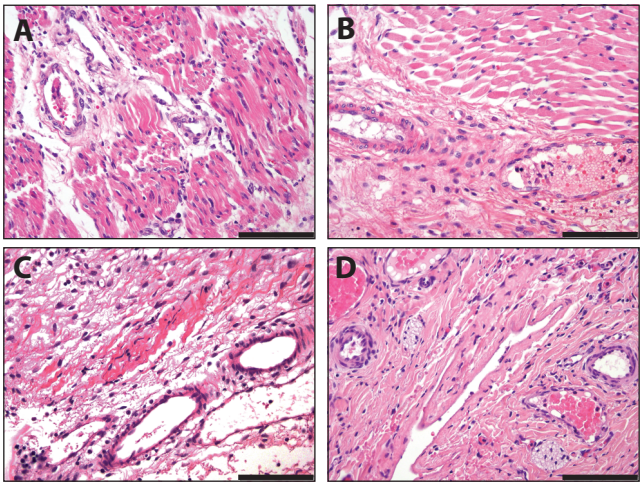

Figure 2. Diaphragm morphology. An H&E staining was performed for morphological analysis of diaphragms of control (A), CDH (B), CDE (C), and hernia sac (D). The scale bar represents 100μm

Human diaphragm VHL pathway

PECAM, VHL, VEGF, HIF1a and eNOS staining were present in control diaphragms, which means the VHL pathway is part of the vessel formation, and maybe vessel function, in healthy diaphragms. Background staining was present in muscle fibers when present in the sample, staining in interstitum was either weakly present or absent. eNOS staining was demonstrated in Figure 3, and is representative for the protein expression pattern of the other stainings. These five markers were similar in control

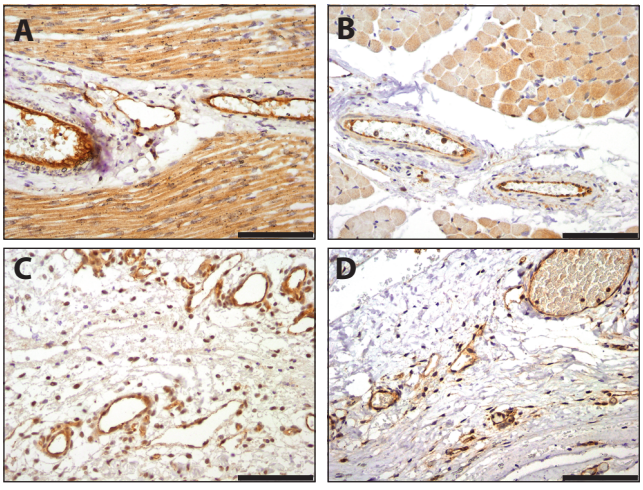

Figure 3. eNOS staining of diaphragms. Staining was performed on diaphragms of control (A), CDH (B), CDE (C), and hernia sac (D). No differences were quantified in intensity of the staining. The scale bar represents 100μm.

and CDH diaphragms (Figure 3A *vs* 3B, respectively). This was also similar in hernia sac tissue (Figure 3D). No effect of gestational age on staining pattern was observed as all antibodies were present in diaphragm tissue from 17^{+3} weeks onwards.

In CDE tissue PECAM, VEGF, and HIF1a staining were similar to controls and CDH tissue (data not shown). eNOS appeared slightly weaker in CDE tissue compared to the other groups. VHL had a weak intensity in CDE tissue compared to all other groups (data not shown). An overview of the intensity of staining in vessels is shown in Table 1.

Table 1. Semi-quantified data of protein expression

	PECAM	VHL	VEGF	HIF1a	eNOS
Control	+++	++	+++	+	+++
CDH	+++	++	+++	+	+++
Hernia sac	+++	++	+++	+	+++
Eventration	+++	+	+++	+	++

An overview of the semiquantified data from immunohistochemical analyses. No apparent differences were observed between groups.

Real-time RT-PCR

No differences were observed in eNOS expression between control and CDH. Differences between the two groups fell within the intra-group variability. Due to the relative low numbers of patient material we did not perform a statistical analysis (Figure 4).

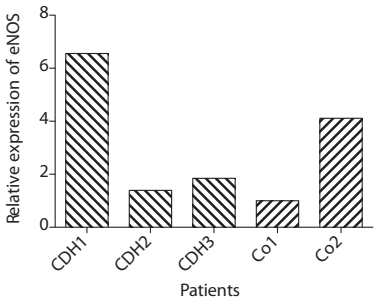

Figure 4. Relative expression of eNOS in CDH and controls. Real-time RT-PCR of eNOS was performed on diaphragms of three CDH and two control patients. No differences were observed between groups.

DISCUSSION

In this study, we investigated components of the VHL pathway in diaphragms. We demonstrated that these are present during prenatal and perinatal healthy diaphragm development. Subsequently, we investigated these proteins in diaphragms of patients with CDH and CDE. We observed that these proteins were present in diaphragms of CDH and CDE patients. However, no differences in expression were found compared to healthy diaphragms. Therefore, it is unlikely that this is the pathogenetic pathway that results in both the diaphragmatic defect and pulmonary hypoplasia seen in CDH patients.

To our knowledge this is the first study to investigate the VHL pathway in human (neonatal) diaphragms. All tested markers of the VHL pathway were present in diaphragms of control patients suggesting a role of the VHL pathway in normal vessel development and function of the diaphragm.

The second part of this study was prompted by the idea that one insult causes both the hypoplastic lungs and diaphragmatic defect, supported by studies that demonstrated a common expression of transcription factors in the developing lung and diaphragm mainly related to the RA pathway such as COUP-TFII [13-15]. Previously, our group demonstrated disruptions in the VHL pathway in CDH lungs [16-18, 24]. Both in the nitrofen CDH model and in human CDH disturbances in the RA pathway have been demonstrated [25, 26]. RA has been demonstrated to increase NO production by eNOS phosphorylation through activation of an RA receptor (RAR)-mediated pathway in endothelial cells [27, 28]. As eNOS is the downstream target of the VHL pathway, we were interested in the VHL pathway in diaphragms of CDH cases. The intensity of the immunostaining, although at best semi-quantitative, of the various VHL pathway associated proteins did, however, not differ amongst the investigated groups. In addition, real-time RT-PCR revealed gene expression of eNOS not to be altered. Therefore it is unlikely that a disturbance in the VHL pathway is the mutual link between CDH lung and diaphragm abnormalities.

The nitrofen mouse model is a well-established CDH rodent model to elucidate pathogenetic aspects of diaphragmatic defects and the associated pulmonary hypoplasia [26]. In CD1 mice, nitrofen induces pulmonary hypoplasia in 100% and diaphragmatic hernias in approximately 30% of mice (unpublished results and Iritani [29]). As stated previously, disruptions in the RA pathway have been observed in the nitrofen model. As RA has been demonstrated to increase NO production by eNOS phosphorylation, nitrofen causing an inhibition of RA could lead to a

downregulation of eNOS and as a result NO [27, 28]. This might disrupt vessel formation, and therefore diaphragm formation. We observed a lower incidence of CDH after nitrofen treatment of Tie2eNOS CD1 mice compared to control CD1 mice (0% *versus* 21%, p-value < 0.01) (unpublished results). In these mice, endothelial nitric oxide (eNOS) is overexpressed in endothelial cells using the endothelial cell specific promoter Tie2. This might indicate that nitrofen acts on the VHL pathway resulting in a lack of eNOS. This pathogenetic pathway would explain why mice overexpressing eNOS are resistant to the impact of nitrofen on the diaphragm, and therefore do not develop diaphragmatic defects.

We did not find any differences in protein expression in the residual diaphragmatic tissue of CDH cases. This is however residual tissue, and might therefore be unaffected tissue. The CDE diaphragms and the remaining part of the CDH diaphragms are however the best available tissues to study the defect. A limitation of this study is that we are evidently never able to investigate the actual 'missing tissue' in CDH patients. The CDE diaphragms we investigated originated from patients with a severe form of eventration, which was diagnosed prenatally. In all three patients pregnancy was terminated due to its severe phenotype. Therefore the gestational age of these patients was lower compared to other groups.

As described in the introduction, a defect in the VHL pathway could have been a potential candidate for several reasons. One major reason was that our group previously demonstrated human CDH lung vessel defects in the VHL pathway [16-18, 24]. We must however not forget that the abnormalities in lung vessels might have been caused by other mechanisms than the intrinsic defect. Vessels are subject to pulmonary hypertension and to the iatrogenic damage from intense treatments. Whether the defects of the VHL pathway are cause or effect, remains to be elucidated.

CONCLUSIONS

The VHL signaling pathway is present in the developing diaphragm and likely plays a role in vessel development and function of the diaphragm. In addition, this pathway does not seem to be affected in diaphragms of CDH and CDE patients. Therefore, it is unlikely that this is the common pathogenetic pathway linked to RA signaling that results in both major abnormalities as observed in CDH patients.

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8

General Discussion & Future Perspectives

GENERAL DISCUSSION

Congenital diaphragmatic hernia (CDH) consists of two major anomalies: a diaphragmatic defect and pulmonary anomalies consisting of a respiratory and vascular component. Despite extensive research, the pathogenesis and etiology of CDH and the pulmonary anomalies are still largely unknown. As children with CDH primarily suffer from the associated pulmonary anomalies, this dissertation mainly focused on deciphering the pathogenesis of pulmonary hypoplasia.

Lungs consist of two major tissue layers: epithelium and mesenchyme. Interactions between these tissue layers (epithelial-mesenchymal interactions) are crucial for proper embryonic lung development [1-7]. Several developmental entities such as proliferation, apoptosis, and differentiation, are essential to form a healthy lung [3, 8-11]. Apoptosis, for instance, is necessary during late lung development for thinning of the mesenchymal cell layer to improve gas exchange and as a result the oxygenation of blood in the vessels [12-15]. For these processes to occur correctly, interaction between epithelial and mesenchymal cells (fibroblasts) is a prerequisite. Cells in close proximity communicate via paracrine signals (soluble factors, matrix or cell contact) [16, 17]. To study the particular interactions between these two pulmonary cell types, we isolated, recombined, and cultured these cells together in different ways; and created 'cell recombinants'. Previously, our group demonstrated that fetal lung epithelial cells recombined with fetal lung fibroblasts reorganize in alveolar-like structures *in vitro* and that fibroblasts direct epithelial morphogenesis [18].

Understanding the pathogenesis of pulmonary hypoplasia is warranted to develop novel prenatal therapeutic interventions to prevent or minimize its severe morbidity. We utilized the recombinant model to investigate the pathogenesis of nitrofen-induced pulmonary hypoplasia. Obviously, the best tissue to investigate the pathogenesis of pulmonary hypoplasia would have been fresh lung tissue from a living patient instead of an animal model. However, obtaining a lung biopsy from a living fetus or newborn with CDH for research is unethical. The burden of such a biopsy in a very ill child is too high in terms of pain, risk of infection and bleeding, and loss of the already small amount of lung tissue (pulmonary hypoplasia). However, several animal models are available to study CDH (as discussed in **Chapter 2**). The nitrofen model is best suited to investigate the pathogenesis of pulmonary hypoplasia. Previously our group utilized this nitrofen model to demonstrate that CDH-associated pulmonary hypoplasia is a result of two hits: an intrinsic problem in the hypoplastic lungs itself before development of the diaphragmatic defect, and interference with fetal breathing movements and competition for space of the lungs due to herniation of abdominal organs through the diaphragmatic defect [19, 20]. In the

present study, we were interested in the primary cause of the pulmonary hypoplasia, and therefore we focused on the intrinsic lung defect of the nitrofen model (**Chapter 3**). Which of the two major tissue layers (epithelial or fibroblast / mesenchyme) is defective in the lung (and what epithelial-mesenchymal interactions are responsible for the pulmonary morphological characteristics of this disease) has never been addressed in detail. Such knowledge is required to design tissue-specific treatment modalities that can exclusively target the defective tissue layer in pulmonary hypoplasia, and thereby modulate or potentially prevent pulmonary hypoplasia in CDH. To determine which lung tissue layer is defective in CDH, we combined the above mentioned nitrofen rodent and *in vitro* recombinant model, and created an *in vitro* model for pulmonary hypoplasia. This cell recombinant model enabled us to investigate epithelial-mesenchymal interactions in nitrofen-induced pulmonary hypoplasia into more detail. These recombination studies demonstrated, for the first time, that the fibroblast (mesenchymal) layer is the defective tissue layer in hypoplastic lungs due to a decreased ability to undergo apoptosis and maintain overall proliferation. More importantly, this defect was intrinsic to the fibroblasts and this fibroblast tissue layer defect was not influenced by epithelial-mesenchymal interactions. As mentioned earlier, this newly gained knowledge is necessary to eventually develop fibroblast-targeted treatment strategies. Later on, we will discuss how this affects future research and focus on treatment modalities.

Diminished apoptosis of fibroblasts could explain the thickened fibroblast tissue layer seen in nitrofen lungs. Diminished apoptosis could also explain the fewer alveoli observed due to this increase in fibroblasts. Furthermore, inhibition of apoptosis of the fibroblast layer has been reported to reduce epithelial branching, which could also contribute to decreased formation of alveoli [15]. Healthy epithelial cells were unable to rescue this defect, implying that the diminished apoptosis was intrinsic to the nitrofen-exposed fibroblasts. In addition, nitrofen-exposed epithelial cells did not induce the observed defect in control fibroblasts, again indicating that the observed defect is inherent to the nitrofen-exposed fibroblasts.

In order to decipher the exact pathogenesis of developmental diseases, it is necessary to understand the physiology of normal development. We used the established recombination method in **Chapter 4** to investigate the influence of developmental stage on epithelial-mesenchymal interactions in developing lungs, and discovered that apoptosis of the mesenchyme during late prenatal lung development, is orchestrated by the surrounding epithelial cells, and is consequently not intrinsic to the mesenchyme itself. However, in our cell recombinant model we observed that apoptosis of nitrofen-treated fibroblasts was diminished and could not be rescued by a healthy epithelial opposing layer (**Chapter 3**). These seemingly controversial

results can be explained by the following: apoptosis of the fibroblasts is initiated by the surrounding epithelial cells however in nitrofen-treated fibroblasts the apoptotic process cannot be maintained. The exact processes responsible for these observations remain to be elucidated. One possible explanation could be interference in fibroblast growth factor-2 (FGF-2), which plays an important role in apoptosis. Later on, we describe an experimental setup to decipher the influence of FGF-2 on this process.

In contrast to earlier observations in apoptosis in our cultures, proliferation was intrinsic to the cells and therefore not triggered by surrounding cells (**Chapter 4**). In nitrofen lungs the decrease in proliferation was equally notable in epithelial cells and fibroblasts (**Chapter 3**). The decrease in proliferating epithelial cells likely explains the observed fewer number of alveoli and airways in nitrofen lungs. The 'fibroblast defect' must have influenced the proliferation of epithelial cells through epithelial-mesenchymal interactions, as no decrease was observed when healthy fibroblasts were used. Apparently, fibroblasts can influence proliferation of epithelial cells in nitrofen lungs in such a way that an arrest of the cell cycle occurs, while the initiation of proliferation remains intrinsic to the epithelial cells, as demonstrated in **Chapter 4**. The proliferative arrest however, does not explain the overall increase in fibroblasts. One possibility is that the reduction in apoptosis of fibroblasts outbalances the decrease in proliferation. A lack of retinoic acid (RA) has been demonstrated to cause an arrest in the transition from the G_1- to S-phase of the cell cycle [21]. A link to the RA pathway has been described in both the nitrofen model and human CDH patients. Nitrofen has been demonstrated to interfere with the RA pathway in several ways, in particular by blocking RALDH2, and consequently interferes with transcription factor (regulator)s such as GATA4, CoupTFII, etcetera [22, 23]. The so-called RA hypothesis was recently supported by human studies, which showed that in a case-control design human newborns with CDH had significant lower retinol and retinol binding protein (RBP) levels in umbilical cord blood [24]. No differences in serum levels were observed in the mothers. Whether low serum retinol and RBP were present during the early phase of lung development remains to be seen, as placental function is different at that time and free diffusion of blood between mother and child occurs. Nevertheless, it would be interesting to investigate the effect of supplementing RA to the recombinant cultures containing nitrofen fibroblasts to observe whether this would annul the cell cycle arrest. To confirm that those results are the effect of RA on the fibroblasts, and not epithelial cells, cultures with RA-pretreated fibroblasts should be performed additionally. Another possibility would be to use isolated pulmonary cells from a double-knockout model of the RA receptor described by Mendelsohn et al. to investigate whether this creates a similar morphology as the recombinants containing nitrofen fibroblasts [25].

Taken together, the diminished proliferative capacity probably reflects in the smaller size of the lungs (pulmonary hypoplasia), while the diminished apoptosis of the fibroblasts causes the thickening of the fibroblast tissue layer in nitrofen-treated lungs.

Our *in vitro* recombination experiments of nitrofen-treated and control epithelial and fibroblast lung cells suggest a defect in the fibroblast cell layer in the nitrofen model of pulmonary hypoplasia in CDH. The utilized *in vitro* cell recombinant model has its limitations. Endothelial cells were not added to the culture, and therefore the influence of vascularization and pulsations could not be studied. Fetal breathing movements, which have been demonstrated essential for proper lung development, were absent. The model also lacked the physiological ratio of lung cells. However, to study the pure interactions between epithelial cells and fibroblasts, we deemed this to be an appropriate model. In future experiments it could be interesting to implement endothelial cells in cultures as well as to observe their influence on *in vitro* morphogenesis and nitrofen-treated fibroblasts. Also, to provide more spacial accuracy cultures could be plated on 3D scaffolds to give cells the possibility to grow in all directions, and to mimic fetal breathing movements cell recombinants could be grown on stretch plates. Human hypoplastic CDH lungs contain thickened alveolar walls, an increase in interstitial tissue, reduced alveolar air spaces and a reduced gas-exchange surface area [26-29]. The defect in the fibroblast layer as we described above, could be a good explanation for these observations. A decrease in apoptosis of fibroblasts prevents the thinning of the interstitial fibroblast layer normally seen during later lung development [12, 30]. Identification of the timing and, eventually, targeted modulation of the defect in the fibroblasts during lung development warrants more research. However, the origin of the increase in fibroblasts is unknown, and would be the logical next step to address in order to elucidate the pathogenesis of pulmonary hypoplasia in CDH. Recently, researchers discovered that pulmonary fibrosis is partially due to a process called epithelial-mesenchymal transition (EMT) in both the bleomycin mouse model and idiopathic pulmonary fibrosis patients [31, 32]. EMT is the transformation of an epithelial cell into a mesenchymal (fibroblast) cell. EMT, and its opposite process mesenchymal-epithelial transition (MET), have been described in many organs (eg. the kidney and the lung), both as a normal part of (embryonic) development as well as a pathological process [33-37]. We hypothesized that EMT might explain the increase in fibroblasts observed in the previous cell recombinant experiments, but also in hypoplastic lungs in the nitrofen model and in human CDH. Another explanation within the concept of transitional processes would be a loss of physiological MET; this could explain the thickened fibroblast layer as well. However, even though it seems likely that EMT and MET are physiological processes in the lungs, this remains to be determined. In our pilot study in **Chapter 5** we demonstrated that nitrofen-treated lungs contain mesenchymal

cells from an epithelial descent. This suggests that EMT is a process that occurs in nitrofen-treated lungs *in vivo*, and may contribute to the CDH associated pulmonary hypoplasia. If EMT is the cause of the thickened fibroblast layer, therapeutic options to further pursue would be selectively blocking EMT-inducing factors such as transforming growth factor-beta.

A progressive obstacle in the field of research of CDH-associated pulmonary hypoplasia is the lack of human lung tissues available for research. As mentioned before, it is unethical to obtain a lung tissue biopsy, not merely due to the existing pulmonary hypoplasia. However, human tissue for translational research is necessary to validate the results from fundamental research in animal models to the clinical setting in human patients. While CDH animal models have generated new hypotheses for human CDH and its associated anomalies, tissue availability has decreased dramatically to test these hypotheses [38-41]. The accrual of fresh tissue to perform sensitive investigative techniques (ie. cell cultures, RNA extraction, etc) is even more difficult due to the postmortem delay, which affects tissue quality. In **Chapter 6** we described a new approach that was implemented in 2001 in the Sophia Children's Hospital in Rotterdam to increase the accrual of human CDH lung tissue: a postmortem lung biopsy through a mini-thoracotomy. Parents who refused to consent for a full autopsy were offered the opportunity of a minimally invasive lung biopsy for pathological evaluation (diagnostics) of the lung and translational research purposes. We managed to quadruple the amount of postmortem tissues obtained from 11% (autopsy) to 45% (autopsy and biopsy combined). In addition, we demonstrated that it is possible to isolate fibroblasts from these tissues. This is an interesting application to create human CDH biobanks to collect tissue and isolated cells to test future hypotheses and prospective investigative techniques (see 'fibroblasts in the biobank'). Due to its severe phenotype, parents with a prenatally diagnosed child have the option to terminate the pregnancy. Especially in the presence of chromosomal anomalies termination of pregnancy takes place on a regular base. Also in case of a predicted low chance of survival (lung area to head circumference-ratio (LHR < 1.0) and liver present in the thoracic cavity) termination of pregnancy is regularly considered in the Netherlands. Tissues from these prenatally terminated children are very valuable for research, as they are untreated and therefore little iatrogenic damage is present. In addition, cord blood is a valuable source of progenitor cells. Progenitor cells are cells that have the ability to differentiate into different cell types. They are an interesting source for the repair of damaged tissues as has recently been shown for other organs like the heart [42]. Isolated progenitor cells could be tested for their regenerative potential in hypoplastic lungs.

It is likely that the two major anomalies of CDH (the diaphragmatic and pulmonary defect) occur through a shared pathogenetic pathway: the 'two defects, one origin'-theory. Previously, the pulmonary defect was demonstrated to be present in the lungs before closure of the diaphragm, and consequently, not only a result of herniation of the abdominal viscera through the diaphragmatic hernia [20]. More recently, data that encourages this theory is that the chromosome regions that are commonly deleted in CDH patients are located in both lungs and diaphragm [43-45]. Examples of the RA pathway related genes are COUP-TFII and FOG2. In human CDH lungs, expression of proteins of the Von Hippel-Lindau (VHL) pathway is disrupted [46-49]. As vascularization is important for proper embryogenesis and therefore likely in diaphragm formation as well, a disruption in the VHL pathway could be the mutual cause of both major defects in CDH patients. In **Chapter 7** we performed experiments to investigate the expression of proteins in the VHL pathway in the remaining diaphragms of patients with CDH. We did not observe any differences compared to control human diaphragms. However, the pitfall of performing research on the residual diaphragm in CDH is that this is the *residual* tissue. Residual tissue might mean that it is unaffected tissue. Whether we should actually study diaphragm tissue from CDH patients is discussed below.

FUTURE PERSPECTIVES

Two central points of attention for future research should be considered: 1) searching for the intrinsic defect of the fibroblasts, and 2) exploring new (preventative) treatment modalities, both pre- and postnatal.

Fibroblasts: focus of future research

This dissertation focused on deciphering the malfunctioning tissue layer in nitrofen-induced pulmonary hypoplasia in CDH. We demonstrated that fibroblasts are the malfunctioning tissue layer, and therefore, these cells should be the focus of future research. The first step of future research should be to confirm that the fibroblast tissue layer is indeed the malfunctioning tissue layer in human hypoplastic CDH lungs. In addition, we should verify that these fibroblasts contain diminished apoptosis and a proliferative arrest. The performed recombinant experiments with healthy and hypoplastic isolated tissue layers have not been feasible in human CDH so far. However, in **Chapter 6** we did demonstrate the application of isolating fibroblasts from the postmortem biopsies of approximately two hours old to create an opportunity to study these defective fibroblasts in cell culture. If we would be able to do the same for epithelial cells, and store these in the biobank, human cell recombination experiments should be possible in the future. Previous attempts to isolate human epithelial

cells failed; therefore it is necessary to optimize these techniques. We should bear in mind that the interpretation of such experiments with human lung tissue should be done with caution, as it is difficult to distinguish whether the observed differences are due to the underlying pathogenesis or caused by the intense treatment these children undergo, for instance prenatal tracheal occlusion (PLUG). The best tissue source for unbiased results would be tissues from terminated pregnancies of children with isolated CDH, although the level of autolysis remains a matter of concern.

Fibroblasts: deciphering the defect

Isolation of human fibroblasts is not easily established and also a labour intensive process. It requires a delicate infrastructure and informed consent of the parents for obvious reasons. However, it is worth the effort to make it common practice to do so. Fibroblasts from CDH lungs are a unique material, especially as it seems increasingly likely that these contain the defect we are searching for. With fibroblast biobanking we could collect and analyze fibroblasts from several CDH patients simultaneously. More importantly, these materials will be available for novel future investigative techniques. Once we have established that human fibroblasts are indeed defective, the next step would be to search for the cause of this malfunctioning. Recently, advanced techniques for molecular profiling have become available such as genome-wide association studies. These compare the DNA of patients with healthy individuals to find abnormalities in the DNA sequence such as mutations. However, over the last decades no specific genes have been revealed that explain the phenotype of the group of isolated CDH patients. It seems likely that the abnormality does not lie within the DNA sequence itself. Hence, the next step in deciphering the intrinsic defect of human fibroblasts should be mRNA expression arrays. These arrays could demonstrate which mRNA pathways are altered in CDH fibroblasts compared to healthy fibroblasts. This information aids us in finding the affected pathway(s). MicroRNAs are important regulators of the expression of mRNA. MicroRNAs can bind to mRNA resulting in inhibition of the mRNA, and therefore determine protein expression. Once we know which mRNAs are abnormal in the fibroblasts, we can develop mRNA-specific treatment options, such as administrating microRNAs (to stop protein expression if abundant) or their antagonists (to increase the amount of particular proteins).

Fibroblasts and the retinoic acid signaling pathway

Another argument that supports the 'two defects, one origin'-theory is the common expression of transcription factor (regulator)s such as COUP-TFII, GATA-binding protein 4 (GATA4), and Friend of GATA2 (FOG2). Both are expressed in the developing lung and diaphragm, and are located on chromosome regions commonly deleted in individuals with CDH [43-45]. An interesting link is the connection to the RA signaling

pathway. These factors are all downstream targets of this pathway. Both human and nitrofen-induced CDH and pulmonary hypoplasia have been connected to perturbations in the RA signaling pathway for many years [50-53]. More recently this connection was supported by a study that showed that human newborns with CDH had significantly lower retinol and retinol binding protein levels in umbilical cord blood [24]. An intriguing insight into the late G_1-phase arrest we demonstrated in the nitrofen-treated fibroblasts is the link to RA. It has been shown that RA protects alveolar epithelial cells from a late G_1-phase arrest induced by hyperoxia [21]. Cells exposed to oxygen did form less cyclin E-Cdk complexes but these complexes regained normal values when oxygen-exposed cells were pretreated with RA. Interestingly, we observed a late G_1-phase arrest in the recombinants containing nitrofen-treated fibroblasts. Several treatment options have been aimed at increasing proliferation in CDH-associated pulmonary hypoplasia but none has specifically targeted a G_1-phase proliferative arrest [54, 55]. The application of prenatal tracheal occlusion was able to increase lung growth, branching and the gas exchange surface at least in nitrofen-treated lungs and larger animals such as sheep [54, 56-59]. It would be interesting to see whether this specifically targeted the G1-to-S-phase arrest, and influenced cyclin-dependent kinase inhibitors.

Several FGFs are known to play an important role in lung development [60, 61]. For instance FGF-2 plays a role in lung cell apoptosis, while FGF-1 is an example of a growth factor with proliferative qualities [62, 63]. In addition, in the nitrofen model both FGF-7 and FGF-10, and FGFRL-1 were down regulated, while recently LopezJimenez et al. demonstrated a CDH patient with a 4p16.3 deletion that included the FGFRL-1 region [64-66]. As we now know that fibroblasts are the malfunctioning tissue layer in the nitrofen model, it would be interesting to investigate FGFs and their influence. One approach would be to grow healthy fetal rat lungs *in vitro* together with nitrofen and FGFs to observe whether an abundance of FGF is able to save the hypoplasia nitrofen induces. Another option would be to administer FGFs *in vivo* to the fetus during nitrofen treatment. Finally, it would be interesting to investigate the effect of blocking FGFs *in vitro* and investigate the influence in healthy lungs. Especially the influence of adding FGF-2 and FGF-1, which play a role in apoptosis and proliferation, respectively, on the development of nitrofen lungs would be of interest. Previously, our group was able to grow organotypic cultures of human fetal lungs [67]. If we would be able to do this for CDH lungs as well, we could investigate the influence of FGFs on hypoplastic lungs. No major differences in timeframe of the termination of pregnancy exist (between 16 and 22 weeks in healthy fetuses *versus* 16 and 24 weeks in CDH fetuses), but there are some other concerns to this approach. First, there is a difference in delay before lungs can be isolated. Fetuses from an abortion clinic are readily available and cultures can start with little delay. However, deceased CDH

fetuses may stay in the womb for a couple of days till induced delivery takes place, which causes delay to culture. In addition, healthy lungs might be easier to culture compared to hypoplastic CDH lungs. Given the suggested importance of derailment of the RA pathway, RA would be an important factor for further investigation.

Towards therapeutics

It will be a while before we can implement our results in the clinical setting. First, possible treatments have to be tested in animal (model)s. Prenatal surgery was tested in animal models for many years before it was implemented in the clinical setting. Prenatal surgery has grown in the last decade, and is potentially relevant for a few congenital anomalies such as meningomyelocele and selected cases of lung cysts [68, 69]. Fetal surgeons have been able to improve their skills, and certain congenital anomalies are waiting to be added to the standard prenatal surgery list. As individual children with CDH suffer and succumb from their hypoplastic lungs, for these patients one logical treatment option would be lung transplantation. Lung transplantation in newborns is rarely performed as it has many associated problems such as the risk of rejection, not to mention the shortage of donor organs and technical difficulties in the newborn [70, 71]. In addition, prenatal transplantation of an entire lung has never been performed but would be very challenging and risky, if even, feasible. Recently, the area of tissue engineering has been explored. Lungs are removed from the thorax and acellularized, and used as an *in vitro* scaffold to grow cells and create a new 'lung'. Petersen et al. demonstrated that transplantation of such a recellularized scaffold participated in gas exchange within two hours and up to seven days in adult rats [72, 73]. This is a very new and promising approach to replace diseased lungs without the need for a donor and the associated risks of allogeneic transplantation. Previously, tissue engineered trachea was successfully transplanted in a patient with bronchomalacia. The trachea was functioning normally at four months and, despite not using any immunosuppressive drugs, no anti-donor antibodies were formed, which demonstrates that such approaches are feasible [74]. Interestingly, as we demonstrated that the nitrofen fibroblasts are the defective cell layer, which acts through cell-cell interactions, transplanting the acellularized scaffold of nitrofen-treated lungs might be able to solve this problem. Even before actually transplanting the recellularized scaffold, it would be interesting to see whether growing healthy lung cells onto a nitrofen-scaffold would 'rescue' pulmonary hypoplasia. As the interstitium is acellularized and only the scaffold remains, diseased nitrofen fibroblasts would not be able to result in defective growth of the lung. This approach has only been demonstrated for an adult full-grown lung, and not for a developing lung. It is a precarious approach but it would be interesting to see whether it has the potential to grow and be used in a developing lung as well.

Instead of transplanting an entire lung, single cells can be transplanted as well. Progenitor cells can be extracted from cord blood of the newborn. These days we are even able to create induced pluripotent stem cells (iPS cells) from skin fibroblasts [75]. To test the influence of pluripotent cells on lung growth, we could create fluorescent-labeled iPS cells and administer them to nitrofen-treated rodent embryos prenatally. The best method of administration with direct delivery of the cells to the damaged lungs would be to endoscopically inject the iPS cells in the trachea followed by tracheal occlusion. Another method would be microinjections in the right ventricle. We should however take into account that approximately 10% of the blood volume circulates through the lungs, and 90% will leave the heart through the ductus arteriosus and foramen ovale straight into the general circulation, which makes this method less desirable. However, other options could be examined as well such as administration through amniotic fluid injections, maternal circulation, or in the umbilical vein. Subsequently, lungs would be isolated and investigated for morphology and localization of the labeled iPS cells. In addition, isolated lung cells can be sorted by fluorescence-activated cell sorting (FACS) to determine their differentiation pattern. Another option would be to inject labeled pluripotent cells in the trachea of nitrofen explants. Using live imaging of cells (with confocal microscopy) it is possible to follow cells for a certain amount of time. Cells are photographed at regular time intervals over several hours and their behavior can be followed individually.

Fibroblasts in nitrofen-treated lungs and diaphragm: same intrinsic defect?

The pulmonary hypoplasia and diaphragmatic hernia are likely to have a similar origin. Chromosome regions that are commonly deleted in CDH patients such as COUP-TFII and FOG2 are located in both lungs and diaphragm. In addition, nitrofen blocks RALDH2 *in vitro* cell cultures. RALDH2 was demonstrated to be present in both the pleuroperitoneal fold of the rat diaphragm and lungs [76-79]. Based on the localization of the defect of the primordial diaphragms in several CDH animal models, Clugston et al. previously hypothesized that it is the non-muscular component that is defective [80]. In addition, we hereby, for the first time, demonstrate that the fibroblast tissue layer is also the defective tissue layer in nitrofen-induced pulmonary hypoplasia. These results combined encourage the theory that fibroblasts are the primary affected cells caused by the same insult in the nitrofen model for CDH.

Research in CDH diaphragms and/or CDH lungs?

Even though it is very likely that both anomalies contain a defective fibroblast tissue layer, the question arises whether we should indeed study both human tissues. The major issue with diaphragmatic material from patients with an isolated congenital diaphragmatic hernia (both postmortem or through a biopsy obtained during hernia repair), is that this diaphragm material is the *residual* 'healthy' tissue, and might

therefore not contain any defect at all. So far, the only evidence that the residual human diaphragm contains abnormalities was that the residual diaphragm tissue surrounding the hernia was thickened in CDH patients as well as in several CDH animal models [80]. Even though we believe that the diaphragmatic defect and pulmonary hypoplasia share the same original insult, research might have to focus on the pulmonary hypoplasia to uncover the shared origin of the fibroblast defect.

CONCLUSIONS

The aim of this dissertation was to improve our knowledge into the pathogenesis of pulmonary hypoplasia in CDH, to eventually aid in finding ways to modulate the natural course in the prenatally diagnosed child with CDH. The most important finding we uncovered was that fibroblasts are the actual malfunctioning tissue layer in the nitrofen lung, and orchestrate the characteristic morphology we observe in nitrofen lungs. Therefore, fibroblasts should be the primary focus of future research. The next step in our quest should be to elucidate what causes the intrinsic defect of the fibroblasts. This knowledge is necessary to develop new (prenatal) preventative or therapeutic strategies. These strategies should focus on correcting the defective fibroblast tissue layer, so that lungs of the future prenatally diagnosed child with CDH can develop (near) normal, and this future child does not suffer from the current severe morbidity and risk of dying in the newborn period even under conditions of maximal supportive care.

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9

Summary & Samenvatting
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ENGLISH SUMMARY

Congenital diaphragmatic hernia or CDH is a developmental defect of the diaphragm that allows abdominal organs, such as intestines and liver, to herniate into the thoracic cavity and compromise lung development. CDH has a prevalence of one in 2000 – 3000 newborns. Children with a CDH suffer from a substantial amount of morbidity and mortality due to the associated abnormal pulmonary development. This results in two clinical problems: pulmonary hypoplasia and persistent pulmonary hypertension of the neonate (PPHN). New treatment modalities, such as extracorporeal membrane oxygenation (ECMO), are designed for treating the sequelae of CDH, pulmonary hypoplasia and PPHN, but do not contribute to the prevention of these conditions. The pathogenesis and etiology of CDH and its associated pulmonary anomalies are still largely unknown despite all research efforts over the past years. A basic understanding of these anomalies is fundamental in our quest for new answers how to protect these children from the sequelae of this anomaly. Consequently, the aim of this dissertation is to improve our understanding of the pathogenesis of pulmonary hypoplasia in CDH, to eventually aid in finding ways to modulate the natural course in a prenatally diagnosed child (**Chapter 1**).

The best tissue to investigate the pathogenesis of pulmonary hypoplasia would be a lung biopsy from a living fetus or newborn with CDH. The burden of such a procedure would be unethical due to the high associated risks and loss of the already small amount of hypoplastic lung tissue. Fortunately, several animal models are available to study CDH, including the nitrofen rodent model, a surgical lamb or rabbit model and multiple genetic mouse models. In **Chapter 2** we discuss the strengths and limitations of these three models, and their relevance to the human situation. Surgical models are preferred for validating new interventions in CDH. Genetic mouse models are necessary to study the influence of genes on lung and diaphragm development. The nitrofen model is best suited to investigate the pathogenesis of pulmonary hypoplasia. Although none of the animal models have been perfect in mimicking human CDH and its associated anomalies, they all contributed to improve our understanding of the underlying pathogenesis and pathology of this disease.

Previously, the associated pulmonary hypoplasia was interpreted as a result of the 'compression' by herniated abdominal organs of the developing lung. A decade ago, pulmonary hypoplasia was found to be present before closure of the diaphragm, which prompted the postulation/foundation of the dual-hit hypothesis: pulmonary hypoplasia was not solely caused by the herniated organs but also the results of an intrinsic defect in the lungs itself. Subsequently, research focused on the intrinsic de-

fect. In **Chapter 3** we utilized an *in vitro* cell recombinant model (a model appropriate to investigate epithelial-mesenchymal interactions) for pulmonary hypoplasia to demonstrate that the fibroblast tissue layer (or mesenchyme) is the primary affected tissue layer in nitrofen-induced pulmonary hypoplasia. The fibroblast tissue layer has a decreased ability to undergo apoptosis and maintain overall proliferation. This knowledge is necessary to eventually develop fibroblast-targeted treatment strategies, to prevent severe pulmonary hypoplasia in children with CDH.

In order to decipher the exact pathogenesis of developmental diseases, it is necessary to understand the physiology of normal, healthy development. In **Chapter 4** we utilized the *in vitro* cell recombinant model to study the influence of developmental stage on epithelial-mesenchymal interactions during normal fetal rat lung development. Apoptosis of the fibroblast tissue layer, a physiological phenomenon during late prenatal lung development, appears to be orchestrated by the surrounding epithelial cells and is consequently not intrinsic to the mesenchyme itself.

In **Chapter 5** we present data from our pilot study of epithelial-mesenchymal transition (EMT) in nitrofen-treated lungs. In nitrofen-treated lungs we established previously that the fibroblasts are the primary affected tissue layer, which disturbs the normal epithelial-mesenchymal interactions necessary for proper lung development. The origin of these cells however is unknown. Recently, a new theory involving cell transformations arose to explain both normal morphogenesis and the development of diseases such as fibrosis and malignancies. EMT is the transformation of an epithelial cell into a mesenchymal cell (fibroblast). One explanation for the origin of the defective fibroblasts could be this process. Our preliminary results demonstrated that nitrofen-treated lungs contain mesenchymal cells from an epithelial descent. This suggests that EMT is a process that occurs in nitrofen-treated lungs *in vivo*, and may contribute to the CDH associated pulmonary hypoplasia.

Translational research is necessary to validate the results of fundamental research in the clinical setting. While CDH animal models have generated new hypotheses of human CDH and its associated anomalies, tissue availability has decreased dramatically to test these hypotheses. Both the improved survival and decrease in permission to perform full autopsy can be held responsible for this development. In 2001 a new protocol was instituted in our hospital to increase the accrual of CDH lung tissue via a quick and minimal invasive method: postmortem lung biopsy through a mini-thoracotomy. In **Chapter 6** we described that this new procedure to obtain human CDH lung tissue for both diagnostic and translational research quadrupled the amount. In addition, we were able to isolate CDH fibroblasts from one of these biopsies. Human CDH fibroblasts are very precious and necessary, as it seems

increasingly likely that these cells contain the defect we are after. These isolated fibroblasts are the key to eventually develop and implement fibroblast-targeted treatment modalities.

It is very likely that the two major defects of CDH, the diaphragmatic hernia and pulmonary anomalies, share the same original insult. This is confirmed by the presence of disruptions in similar developmental pathways of both organs such as the retinoic acid signaling pathway. During normal organogenesis, vessel development is a prerequisite. The Von Hippel-Lindau (VHL) pathway is important for angiogenesis and vessel function. Previously, disruptions in the VHL pathway were observed in human CDH lungs. In **Chapter 7** we investigated the VHL pathway in diaphragmatic tissues from healthy controls, patients with CDH and patients with congenital diaphragmatic eventration (CDE). We demonstrated that the VHL signaling pathway is present during diaphragm development at early stages of gestation, and likely plays a role in vessel development and function of the diaphragm. The VHL pathway does not appear affected in diaphragms of CDH and CDE patients. Therefore, it is unlikely that this is the common pathogenetic pathway linked to retinoic acid signaling that results in both major abnormalities as observed in CDH patients.

The general discussion in **Chapter 8** describes the interpretation of the studies reported in this dissertation. In addition, it provides suggestions for future research to continue the quest for answers to eventually develop prenatal tissue-targeted treatment modalities

NEDERLANDSE SAMENVATTING

Congenitale hernia diafragmatica of CDH is een defect in de ontwikkeling van het middenrif dat organen van de buikholte, zoals darmen en lever, de kans geeft om ruimte in te nemen binnen de borstkas en zo de ontwikkeling van de longen te beperken. CDH komt voor bij één op de 2000 – 3000 pasgeborenen. Pasgeborenen met CDH lijden onder ernstige restverschijnselen en sterfte door de abnormale long ontwikkeling die hiermee gepaard gaat. Dit resulteert in twee klinische problemen: een vorm van abnormale long ontwikkeling (pulmonale hypoplasie) en abnormale long vaat ontwikkeling (persisterende pulmonale hypertensie van de neonaat (PPHN)). Nieuwe therapeutische mogelijkheden, zoals extracorporele membraan oxygenatie (ECMO of hart-longmachine), zijn ontwikkeld om de gevolgen van CDH, pulmonale hypoplasie en PPHN, te behandelen, maar dragen niet bij aan het voorkómen hiervan. De ontstaanswijze (pathogenese en etiologie) van CDH en zijn aanverwante long afwijkingen zijn nog grotendeels onbekend ondanks het vele onderzoek van de afgelopen jaren. Basale kennis van deze aandoeningen is noodzakelijk in onze strijd naar antwoorden hoe deze kinderen beschermd kunnen worden tegen deze gevolgen. Het doel van dit proefschrift is om de kennis van de pathogenese van pulmonale hypoplasie in CDH te verbeteren, om uiteindelijk het natuurlijke beloop van een prenataal gediagnosticeerd kind ten goede te kunnen beïnvloeden (**Hoofdstuk 1**).

Het beste weefsel voor onderzoek naar de ontstaanswijze van pulmonale hypoplasie zou een long biopt zijn van een levende foetus of pasgeborene met CDH. Zo'n procedure is echter niet ethisch verantwoord door het hoge risico dat het met zich meebrengt naast het verlies van het al minimaal aanwezige (hypoplastische) long weefsel. Gelukkig bestaan er meerdere diermodellen om CDH te onderzoeken, zoals het nitrofen model, het chirurgische model, en een aantal genetische modellen. In **Hoofdstuk 2** bespreken we de sterke en zwakke punten van deze drie modellen, en hun relatie tot de humane situatie. Het chirurgische model verdient de voorkeur om nieuwe interventies te testen. Genetische modellen zijn noodzakelijk om de invloed van genen op de ontwikkeling van de long en het diafragma te analyseren. Het nitrofen model is het meest geschikte model om de pathogenese te bestuderen. Alhoewel geen enkel model hét perfecte model is, zijn ze belangrijk om nieuwe inzichten te verkrijgen ten aanzien van de pathogenese en pathologie van de aandoening.

In het verleden werd pulmonale hypoplasie geïnterpreteerd als het gevolg van 'compressie' van de ontwikkelende long door de buik organen. Ruim tien jaar geleden werd ontdekt dat pulmonale hypoplasie aanwezig is vóórdat de fysiologische slui-

ting van het middenrif plaatsvindt. Dit vormde de basis van de 'dual-hit' hypothese: pulmonale hypoplasie wordt niet puur door 'compressie' van de binnendringende buik organen veroorzaakt maar de long is zelf afwijkend. Zodoende verschoof de focus van het onderzoek naar dit intrinsieke long defect. In **Hoofdstuk 3** toonden we aan, middels gebruik te maken van een *in vitro* cel recombinatie model (een model geschikt om interacties tussen twee weefsellagen (epitheel en fibroblasten) te onderzoeken), dat de fibroblasten (of het mesenchym) de weefsellaag is die primair is aangedaan in nitrofen-geïnduceerde pulmonale hypoplasie. Tevens liet dit model zien dat de defecte fibroblasten een verminderd vermogen hebben om geplande celdood (apoptose) te ondergaan en cel vermeerdering (proliferatie) te behouden. Dit is noodzakelijke kennis om uiteindelijk fibroblast-specifieke therapeutische strategieën te ontwikkelen ter voorkoming van ernstige pulmonale hypoplasie bij pasgeborenen met CDH.

Om de exacte pathogenese van ziektes die tijdens de ontwikkeling ontstaan te achterhalen, is het nodig om de fysiologie van normale, gezonde ontwikkeling te begrijpen. Middels het *in vitro* cel recombinatie model onderzochten we in **Hoofdstuk 4** de invloed van de ontwikkelingsfase op epitheliale-mesenchymale interacties tijdens normale foetale rat long ontwikkeling. Apoptose van de fibroblasten, een fysiologisch fenomeen aan het eind van de prenatale long ontwikkeling, wordt gereguleerd door de omliggende epitheelcellen, en is dus niet intrinsiek aan de fibroblasten zelf.

In **Hoofdstuk 5** presenteerden we onze pilot studie naar epitheliale-mesenchymale transitie (EMT) in nitrofen-behandelde longen. In nitrofen longen zagen we eerder dat de fibroblasten de primair afwijkende weefsellaag zijn, die de normale epitheliale-mesenchymale interacties, noodzakelijk voor normale long ontwikkeling, verstoren. De oorsprong van deze cellen is echter onbekend. Recent is er een nieuwe theorie ontstaan rondom cel transformaties die zowel normale orgaan ontwikkeling als de ontstaanswijze van ziektes zoals fibrose en maligniteiten verklaart. EMT is het transformeren van een epitheelcel naar een mesenchymale cel (fibroblast). De oorsprong van de fibroblasten kan zo verklaard worden. Onze eerste resultaten laten zien dat nitrofen-behandelde longen mesenchymale cellen bevatten van een epitheliale afkomst. EMT zou plaats kunnen vinden in nitrofen-behandelde longen *in vivo*, en dus bij kunnen dragen aan de pulmonale hypoplasie bij kinderen met CDH.

Translationeel onderzoek is nodig om de resultaten van basaal wetenschappelijk onderzoek te vertalen naar de kliniek. Terwijl diermodellen nieuwe hypotheses over CDH bij de mens en de aanverwante afwijkingen teweeg hebben gebracht, is de hoeveelheid humaan weefsel om deze hypotheses op te testen aanzienlijk gedaald.

Zowel de verbeterde overlevingskans als de afname in toestemming voor obductie zijn verantwoordelijk voor deze ontwikkeling. In 2001 is er een nieuw protocol in ons ziekenhuis geïntroduceerd om een toename van het verkrijgen van weefsel te bewerkstelligen via een snelle en minimaal invasieve procedure: een postmortaal long biopt middels een mini-thoracotomie. In **Hoofdstuk 6** beschreven we dat deze nieuwe procedure de hoeveelheid weefsel voor zowel diagnostiek als translationeel onderzoek verviervoudigde. Daarnaast waren we in staat om CDH fibroblasten uit één van de long biopten te isoleren. Humane CDH fibroblasten zijn erg waardevol en noodzakelijk nu het in toenemende mate duidelijk wordt dat deze cellen het defect bevatten waar we naar op zoek zijn. Deze geïsoleerde fibroblasten zijn de sleutel om uiteindelijk fibroblast-specifieke therapeutische mogelijkheden te onderzoeken en toe te passen.

Het is zeer waarschijnlijk dat de voornaamste defecten van CDH, het gat in het middenrif en de longafwijkingen, een zelfde oorsprong delen. Dit wordt bevestigd door de aanwezigheid van verstoringen in overeenkomstige pathways zoals de vitamine A cascade tijdens de ontwikkeling van beide organen. Vaatontwikkeling (angiogenese) is een vereiste tijdens normale orgaan ontwikkeling. De Von Hippel-Lindau (VHL) pathway is belangrijk voor angiogenese en vaatfunctie. In eerder onderzoek zijn verstoringen in de VHL pathway gezien. In **Hoofdstuk 7** bestudeerden we de VHL pathway in middenrif weefsel van gezonde controle patiënten, patiënten met CDH, en patiënten met congenitale eventeratie van het diafragma (CDE). De VHL cascade was aanwezig tijdens de ontwikkeling van het diafragma en vroeg in de zwangerschap, waardoor het aannemelijk is dat de VHL cascade een rol speelt in de ontwikkeling en functie van het diafragma. De VHL pathway blijkt niet afwijkend te zijn in diafragmata van CDH en CDE patiënten. Daardoor is het onwaarschijnlijk dat dit de gemeenschappelijke pathway is, verbonden door vitamine A, van de beide afwijkingen in CDH patiënten.

De algemene discussie in **Hoofdstuk 8** beschrijft de interpretatie van de studies beschreven in dit proefschrift. Daarnaast geeft het suggesties voor toekomstige onderzoeksprojecten in de zoektocht naar weefsel-specifieke therapeutische mogelijkheden.

ABBREVIATIONS

ARDS	acute respiratory distress syndrome
αSMA	alpha-smooth muscle actin
BASC	bronchoalveolar stem cell
BSA	bovine serum albumine
CCSP	clara cell secretory protein
CDE	congenital diaphragmatic eventration
CDH	congenital diaphragmatic hernia
Cdk	cyclin-dependent kinase
COUP-TFII	chicken ovalbumin upstream promotor-transcription factor 2
CRABP1	cellular retinoic acid binding protein
Cyp	cytochrome p450
DAB	3,3′-Diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
Disp1	Dispatched homolog 1
E	embryonic day
E_C	control epithelial cells
E_N	nitrofen epithelial cells
E-Cadh	E-Cadherin
ECMO	extracorporeal memebrane oxygenation
EdU	5-ethynyl-2′-deoxyuridine
EMT	epithelial-mesenchymal transition
Endo	endodermal cells
eNOS	endothelial NO synthase
Epi	epithelial cells
EXIT	ex-utero intrapartum treatment
F_C	control fibroblasts
F_N	nitrofen fibroblasts
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FETO	fetoscopic endoluminal tracheal occlusion
FGF	fibroblast growth factor
FGFRL1	FGF receptor L1
Fib	fibroblasts
FITC	fluorescein isothiocyanate
FOG2	friend of GATA2
GA	gestational age
GATA	GATA-binding protein
GFP	green fluorescent protein

GNZ	GFP and LacZ
H&E	hematoxylin and eosin
HBSS	Hanks' Balanced Salt Solution
HFO	high frequency oscillation
HIF1α	hypoxia-inducible factor 1-alpha
IF	immunofluorescence
iPS cell	induced pluripotent stem cell
IRDS	infant respiratory distress syndrome
LacZ	beta-galactosidase fusion protein
LHLBR	lung hypoplasia based on lung weight : body weight-ratio
LHR	lung area to head circumference-ratio
LHW	lung hypoplasia based on lung weight *versus* GA normal values
LRAT	lecithin : retinol acyltransferase
Mes	mesenchymal cells
MET	mesenchymal-epithelial transition
MOF	multi-organ failure
NGS	normal goat serum
nitrofen	2,4-dichlorophenyl-*p*-nitrophenyl ether
NO	nitric oxide
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PECAM	platelet endothelial cell adhesion molecule
PGE2	prostaglandin E2
pH3	phosphohistone H3
PHD	prolyl hydroxylase
PLUG	'plug the lung until it grows'
PPF	pleuroperitoneal fold
PPHN	persistent pulmonary hypertension of the neonate
PROM	premature rupture of membranes
pro-SFTPC	pro-surfactant protein-C
RA	retinoic acid
RALDH	retinal dehydrogenase
RAR	RA receptor
RARE	RA response element
RBP	retinol binding protein
RE	retinyl ester
RXR	retinoid X receptor

Shh	sonic hedgehog
STRA6	membrane receptor for retinol
T3	triiodothyronine
T4	thyroxine
TGFβ	transforming growth factor beta
TH	thyroid hormone
THT	TH transporters
TOTAL-trial	TO to accelerate lung growth-trial
TR	thyroid hormone receptor
TRE	thyroid response element
TRH	thyrotropin-releasing hormone
TITF-1	thyroid transcription factor-1
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick-End labeling
VEGF	vascular endothelial growth factor
VHL	Von Hippel-Lindau
Vim	vimentin
VSD	ventricular septal defect
Wt1	Wilm's tumor 1

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Beste (bijna-professor) Richard, ik zal nooit onze skype sessie vergeten waarin jij afgeleid werd door Pippa (het zusje van..). Buiten deze skype sessie, liepen de gesprekken en discussies via skype bijzonder goed. Bedankt voor je enthousiasme, je immer positieve instelling, gevoel voor humor, en het pushen voor 'out of the box' denken. Ik ben er enorm trots op dat ik jouw eerste promovendus mag zijn!

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om je oratie mee te kunnen maken. Komende zomer maar weer een keertje fietsen? (wanneer ik een periode veel getraind heb, en jij 'ff een rustig rondje' wil doen).

Beste Robbert, bedankt voor het welkom in je lab en je hulp bij alle praktische zaken – jij stond altijd klaar om te helpen en mee te denken!

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Dear members of the Post lab, thank you all so much for your support those years, even after I had left you were still willing to help me with anything. Can't thank you enough! Emily, my bestest lab friend, still miss our early morning coffee dates; thanks for all the little (and long) chats we had! Steve, thanks for your sense of humor, still have the sketch on my fridge! Jeroen, ik ben blij dat je tot inzicht bent gekomen dat de Mac het wint van de PC ;) Maciej, our lab grandpa, thanks for your everlasting enthousiasm. André, Irene, Jinxia, Angie, Sanita, Palma, Anna, Zhen, Daochen, Stephane, David, Hayley, and Pascal, thanks for all those years! Lab members of the Rottier lab: thanks for your help and interest in my projects. Can't say that I will miss the Wednesday morning meetings but will definitely miss having you around! Daarnaast wil ik ook de lab mensen van de moleculaire diagnostiek en de pathologie bedanken en dan met name voor de borrels! Annemarie Illsley, ontzettend bedankt voor al je hulp, zeker in de laatste fase!

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Lieve paranimfen! Kirsten, we kennen mekaar inmiddels al tien jaar, waarvan we zo'n beetje de helft van die tijd op grote afstand (Toronto, Singapore, Zürich) van elkaar leefden. Toch hoop ik dat we eind dit jaar wat langer in hetzelfde land zullen wonen, want ik mis je! Annemieke, we hebben mekaar leren kennen in de sneeuw op de heuvels van Blue Mountain maar de echte vriendschap begon in Rotterdam toen we beiden 'opnieuw' moesten beginnen. Ik bewonder je enthousiasme en positieve insteek voor elke situatie. Meiden, jullie zijn fantastisch, en ik ben er trots op dat jullie naast mij zullen staan tijdens mijn verdediging!

Catherine, jarenlang spraken we af in 'exotische' landen, nu toch ook wel erg leuk om weer in hetzelfde land (misschien snel zelfde stad?) te zijn! Sharon, regelmatig verlang ik terug naar Aruba – wanneer gaan we weer? Fabiënne, club- en dispuutsgenoot, bedankt voor al je support, met name je opvang toen ik net weer terug was in Nederland – ik zal nooit vergeten hoe jij naast je megadrukke baan tijd maakte om doordeweeks me op te komen zoeken in Dordt! Daphne, bedankt voor de vele Doppio-dates om alle promotie issues te bespreken. Inger, hopelijk snel samen een keer tennissen! Ella, van de zomer maar eens serieus die 'fietsclub' opzetten? Joyce, ik sta versteld van jouw management van de combinatie werk, man, dochtertje, kindje-op-komst en onderzoek – mocht het Noorwegen worden dan komen we daar wel uit! Danielle, van collega naar vriendin – bedankt voor de vele koffie meetings; en gezellig dat we nu ook het TULIPS Curriculum samen doen, op naar dat Professor-schap hè ;) Karin, bedankt voor de talloze borrels! MC, mekaar leren kennen in Toronto, en nu allebei in Rotterdam promoveren in dezelfde week – als ik het me goed herinner heb we van mekaar nog overnachtingen in TO tegoed; een idee voor 2013? Lalini, bedankt voor het altijd klaar staan met een kopje thee om samen te kunnen schelden op 'wat er nu weer mis ging' – ik ben blij dat we zoveel konden delen! Heel veel succes met je promotie in juni en je opleiding tot dokter. Mariska, jij kwam me zelfs twee keer opzoeken – ongelooflijk dat wij bij de eerste overwinning op de Spelen van Nederland met goud in de sneeuw hadden kunnen zijn, mits onze kaartjes niet gecanceld waren.. Gezellig om met jou op avontuur te gaan!

Cara crazy Monica, grazie per la tua amicizia. Spero di viaggiare a Milano piu spesso in futuro. Mi manchi..

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Lieve oud-huisgenoten, Linda, Tom, Martijn, Edwin en Marcel, met jullie heb ik verhalen om nooit te vergeten, van 'kijk een boot!' tot slapen in een zitzak. Ik hoop dat we onze borrels erin houden!

My 'international group of girls' – Yafit, Anastasia, Larissa, Andrea, and Tumay – so glad I had you around in Toronto! Thanks for all those parties and weekends away. Looking forward to all the future places where we'll meet, for starters sun, sea, beaches and a wedding in Barbados!

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- liefs, Rhiannon

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PHD PORTFOLIO

Summary of PhD training

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Erasmus MC:	Department of Pediatric Surgery
Hospital for Sick Children:	Department of Physiology & Experimental Medicine
PhD period:	2007 – 2012
Supervisors:	Prof.dr. D. Tibboel
	Prof.dr. M. Post
	Dr. R. Keijzer

General Courses

-	Scientific Writingin English for Publication	2010 – 2011
-	Photoshop & Illustrator Course	2010
-	Research Ethics' Introductory Tutorial TCPS	2008
-	Biology, Chemical and Radiation Safety Course	2007
-	Lab Animal Services General Orientation	2007
-	Lab Animal Services Animal Handling	2007
-	Lab Animal Services Injectable Anaesthesia	2007
-	Lab Animal Services Gaseous Anaesthesia	2007
-	Lab Animal Services Survival Surgery	2007

Presentations

-	Pediatric Academic Societies (PAS)-meeting [oral]	2011
-	Symposium Experimental Surgical Research SEOHS [oral]	2010
-	American Transplantation Congress (ATC) [oral]	2010
-	Transplant Rounds, Hospital for Sick Children [oral]	2010
-	P.E.M. retreat, Hospital for Sick Children [poster]	2010
-	Research Institute retreat, Hospital for Sick Children [poster]	2009
-	Research Institute retreat, Hospital for Sick Children [poster]	2008
-	American Thoracic Society (ATS) conference [poster discussion]	2008
-	Research Institute retreat, Hospital for Sick Children [poster]	2007
-	Summer Programme, Hospital for Sick Children [poster]	2007

Seminars & Workshops

-	TULIPS PhD Clinician-Scientist Programme	2011 – 2012
-	Lab Meetings Cell Biology	2010 – 2012
-	CCHCSP Canadian Clinician-Scientist Programme	2008 – 2010
-	Lab Meetings Lung Biology	2007 – 2010

- Seminars P.E.M. Programme 2007 – 2010
- Seminars University of Toronto 2007 – 2010
- ATS Course Stem Cells in Lung Development 2007

Master of Science in Clinical Epidemiology 2003 – 2005

Erasmus University Rotterdam, Rotterdam, the Netherlands

- Emerging Infectious Diseases
- Genetic Epidemiology
- Causal Modelling
- History of Epidemiologic Ideas
- Maternal and Child Health
- Advanced Topics in Clinical Trials
- Seminars in Clinical Research
- Introduction to Data-analysis
- Regression Analysis
- Survival Analysis
- Introduction to Clinical Research
- Intervention Research and Clinical Trials
- Diagnostic Research
- Advances in Prognostic Research
- Decision Making in Medicine
- Pharmaco-epidemiology
- Erasmus Course Study Design
- Principles of Research in Medicine and Epidemiology
- Clinical Decision Analysis
- Methods of Clinical Research
- Methods of Health Services Research
- Prevention Research
- Topics in Evidence Based Medicine

University Medical Center Utrecht, Utrecht, the Netherlands

- Advanced Diagnostic Research

Bloomberg School of Public Health, Johns Hopkins University, Baltimore, USA

- Intermediate Epidemiology
- Epidemiology in Evidence-Based Decisions
- Social Epidemiology

Cambridge University, Cambridge, United Kingdom

- Cardiovascular Module
- Diabetes Module

CURRICULUM VITAE AUCTORIS

Rhiannon was born on December 2nd of 1981 in Dordrecht, the Netherlands. She attended Thuredrecht College in Dordrecht from 1994 to 2000. She finished one year of Kinesiology at the VU University Amsterdam before she started medical school at the Erasmus University Rotterdam in 2001.

Her research career started in 2003 when she participated in a study at the Pediatric Oncology department about end-of-life decisions in children dying from cancer. Soon thereafter she was invited to apply to the talent programme of the Netherlands Institute for Health Sciences to obtain a MSc degree in Clinical Epidemiology. During this programme she spent time at Cambridge University and the Bloomberg Institute at Johns Hopkins Hospital. She graduated from the programme in 2005 with an article about decision models to determine serious bacterial infections in febrile children at the Emergency Department at the Sophia Children's Hospital.

In 2007 she obtained her medical degree and started her PhD training at the Hospital for Sick Children in Toronto (ON), Canada, at the Lung Development programme at the Department of Physiology and Experimental Medicine. Under supervision of Dr. Richard Keijzer and Prof Dr. Martin Post she investigated pulmonary hypoplasia in congenital diaphragmatic hernia (CDH) in the nitrofen rat model. Simultaneously, she performed a clinical study on hemophagocytic lymphohistiocytosis in children with a liver transplant at the General Surgery Department of the Hospital for Sick Children together with Dr Annie Fecteau and Dr Sheila Weitzman.

After three years of basic research she returned to Rotterdam to investigate human CDH lung samples at the department of Pediatric Surgery (Prof Dr Dick Tibboel) and Pathology (Prof Dr Ronald R. de Krijger) and worked in the laboratory of Dr Robbert J. Rottier. Concurrently, she started a two-year clinician-scientist programme to optimize her skills to continue research in her future clinical career.

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