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The Pulmonary Mesenchymal Tissue Layer Is Defective in an *in Vitro* Recombinant Model of Nitrofen-Induced Lung Hypoplasia

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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 treatments, improved comprehension of the pathogenesis of lung hypoplasia is warranted. We developed an *in vitro* cell recombinant model to mimic pulmonary hypoplasia and specifically to investigate epithelial-mesenchymal interactions and to decipher which tissue layer is primarily defective in nitrofen-induced CDH-associated lung hypoplasia. 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. Recombinants containing F_N fibroblasts had a thickened fibroblast tissue layer and there were fewer organized alveolar-like epithelial structures compared with those in control (F_C)(E_C) recombinants. These F_N recombinants exhibited a decrease in terminal deoxynucleotidyl transferase dUTP nick end labeling 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. In conclusion, 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. (*Am J Pathol* 2012, 180:48–60; DOI: 10.1016/j.ajpath.2011.09.032)

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, lung hypoplasia is a major associated anomaly. Characteristics of human lung 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 pre-acinar vessels, and adventitial thickening.⁸ It has been demonstrated that the lung, in itself, is defective, and that lung hypoplasia is not caused by compression of the lungs by herniated abdominal organs alone.⁹ However, as to which of the two major tissue layers (epithelial or fibro-

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blast/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 lung hypoplasia, and thereby modulate or potentially prevent lung hypoplasia in CDH.

Several animal models have been used to study CDH and/or the associated lung hypoplasia such as the nitrofen rodent model, a surgical lamb or rabbit model, and multiple genetic mouse models. The nitrofen model is the preferred model to study the pathogenetic aspects of CDH. For the past three decades, the nitrofen model has been used extensively to investigate the anomalies associated with CDH. Originally, nitrofen was used as an herbicide, and while toxicology screens on adult rats did not reveal any apparent toxicological effects, the administration to pregnant dams during midgestation caused developmental anomalies in the lungs, diaphragm, heart, thymus, parathyroid glands, and skeleton of the offspring.^{10–12} Numerous groups including our own demonstrated that nitrofen-induced diaphragmatic hernias 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.^{13–16} 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.^{17–19} Previously we used this nitrofen model to demonstrate that CDH-associated lung 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.⁹ Thus, lung hypoplasia is already present before development of the diaphragmatic defect, but will worsen as a result of interference with fetal breathing movements and compression by the abdominal organs. In the present

study, we were interested in the primary cause of the lung hypoplasia, and merely focused on “the first hit,” the intrinsic lung defect of the nitrofen model. Previous studies by others and us have demonstrated abnormal patterns in proliferation, apoptosis, and cell differentiation in hypoplastic nitrofen-lungs, but the defective tissue layer has not been identified.^{9,20}

Forty years ago it was reported that interactions between the different tissue layers are crucial for proper embryonic lung development.²¹ Since then, many studies have contributed to the analysis of molecular determinants of lung growth.^{22,23} 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.²⁴ In addition, distal lung embryonic mesenchyme has been shown to induce expression of distal epithelial markers in proximal (tracheal) lung epithelial cells.²⁵ 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 lung 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.²⁴ Because access to human (hypoplastic) lungs to perform such experiments is at best very limited, we used 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

Figure 1. Schematic representation of the formation of the homotypic and heterotypic recombinants. Black represents control cells (C), and gray indicates 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, whereas model 3 (F_C)(E_N) and model 4 (F_N)(E_C) are heterotypic recombinants.

lung 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 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.

Materials and Methods

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, Quebec, Canada).

Nitrofen Treatment

Congenital diaphragmatic hernia and lung 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, Canada). 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 lung 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 (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). 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.²⁶ After overnight culture, cells were washed, trypsinized and collected by centrifugation. Cells were counted and recombined (3.0×10^6 epithelial cells with 3.0×10^6 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 1 hour, and transferred onto inserts (Millipore, Etobicoke, ON, Canada). The recombined cells were cultured according to Deimling et al.²⁴ After 5 days of culture, recombinants were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated and em-

bedded in paraplast, and 5- μ m sections were cut. To ensure that isolated cells did not differ between the groups before recombination with respect to differentiation markers and number of apoptotic cells, freshly isolated cells were grown on coverslips for immunocytochemical analysis.

Hematoxylin and Eosin Staining

Sections were rehydrated and stained with hematoxylin (Sigma, Oakville, ON, Canada). 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) in 95% ethanol, dehydrated, and mounted with 70% permount (Fisher, Pittsburgh, PA) in xylene.

Immunofluorescence

Immunofluorescence (IF) analysis was performed as described previously.²⁴ Briefly, tissue sections were rehydrated and antigen retrieval was performed in 10 mmol/L pH 6.0 sodium citrate 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 1 hour. Subsequently, the primary antibody in blocking solution was added and sections were incubated overnight at 4°C. Following three washes with PBS containing 0.05% (v/v) Tween-20 (PBST), slides were incubated with a secondary antibody in blocking solution for 1 hour. The slides were then washed and the samples were mounted with DAPI hard mounting medium (Vector, Burlington, ON, Canada). Primary antibodies were: 1:500 rabbit anti-cytokeratin (Dako, Mississauga, ON, Canada), 1:50 mouse anti-vimentin (Dako), 1:400 mouse anti- α -smooth muscle actin (α SMA; 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; Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 rabbit anti-platelet endothelial cell adhesion molecule (PECAM; Santa Cruz Biotechnology). Secondary antibodies (dilution of 1:200) were fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Calbiochem, San Diego, CA) for vimentin and α SMA, rhodamine-labeled anti-mouse IgG (Invitrogen, Eugene, OR) for vimentin, rhodamine-labeled anti-rabbit IgG (Invitrogen) 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) hydrogen peroxide in methanol to block endogenous peroxidase activity. Blocking solution, containing avidin (Vector), was added for 1 hour according to the manufacturer's instructions. Primary antibody in blocking solution containing biotin (Vector) was added and slides were incubated overnight at 4°C. The following day a biotinyl-

ated secondary antibody was added for 1 hour in blocking solution. Subsequently, ABC-complex (Vector) was added for 30 minutes. Slides were developed using ImmPACT 3,3'-Diaminobenzidine (DAB; Vector), 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 Technology, Danvers, MA), 1:2000 rabbit anti-ki67 (Dako), 1:1000 mouse-anti-cyclin D3 (Abcam), 1:1000 rabbit anti-cyclin E (Abcam), 1:400 rabbit anti-phosphohistone H3 (pH3) (Upstate, Atlanta, GA), and 1:100 rabbit anti-thyroid transcription factor-1 (TTF-1) (NeoMarkers) in blocking solution. Biotinylated anti-mouse IgG (for cyclin D3; Vector) and anti-rabbit IgG (for cleaved caspase-3, ki67, cyclin E, pH3, and TTF-1; Calbiochem) were used as secondary antibodies in a dilution of 1:200. Whole lungs of E19 rat embryos were used as positive controls for immunohistochemistry.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-assay (Roche, Toronto, ON, Canada) 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 1 hour at 37°C. Slides were washed three times with PBS and counterstained with DAPI mounting medium.

5-Ethynyl-2'-deoxyuridine Uptake

5-Ethynyl-2'-deoxyuridine (EdU)-incorporation assay was carried out according to the manufacturer's instructions (Invitrogen). Briefly, recombinants were incubated for 2 hours with 10 $\mu\text{mol/L}$ EdU component A on day 5 of culture before fixation. Sections were incubated with 0.5% (v/v) Triton X-100 for 20 minutes to make the sample permeable. EdU detection was performed by incubation with a freshly prepared reaction cocktail of fluorescein isothiocyanate-labeled 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 made permeable with 0.2% Triton-X100 (Sigma Life Science, St Louis, MO) in 1% BSA for 5 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 struc-

tures were counted. Simultaneously, we determined cell origin (epithelial cell or fibroblast). Volocity4 software (Quorum Technologies Inc., Guelph, ON, Canada) 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 radioimmunoprecipitation assay buffer and sonicated. Western blot analysis was performed as previously described.²⁷ 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). Anti-rabbit and anti-mouse secondary antibodies (Vector) 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 $\pm$ SEM. For statistical analyses we used an analysis of variance 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* < 0.025.

Results

Organogenesis

In all four types of recombinants (Figure 1), cells spontaneously organized in alveolar-like structures (Figure 2, A–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 2, F and H versus E and 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).

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 (see Supplemental Figure S2 at <http://ajp.amjpathol.org>).

Figure 2. Organogenesis in homotypic and heterotypic recombinants. Alveolar-like structures developed in all four types of recombinants spontaneously (**A–D**). IF staining of the recombinants for cytokeratin (epithelial cells; red) and vimentin (fibroblasts; green) that were 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 compared with F_C -containing recombinants (**E**, **G**). Scale bar = 50 μ m.

Apoptosis

To determine whether 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 4, B, D, F, and H versus A, C, E, and G). Freshly isolated F_N and F_C cultures were analyzed for TUNEL-positive cells, but no differences between the cultures were observed before recombination (see Supplemental Figure S2K and L at <http://ajp.amjpathol.org>). E19 nitrofen-treated rat lungs also demonstrated less apoptosis compared with E19 control rat lungs (see Supplemental Figure S3 at <http://ajp.amjpathol.org>).

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 5, E–H) and cyclin E (results not shown) were similar in all four types of recombinants. However, EdU-incorporation assays demonstrated less cells in the S-phase of the cell cycle in (F_N)(E_N) recombinants compared with (F_C)(E_C) recombinants (Figure 5, J versus I). Ki-67 (Figure 5, A–D) and pH3 (Figure 5, K–N) immunohistochemistry corroborated the finding of less proliferating/dividing cells in F_N -containing recombinants versus F_C -containing recombinants (Figure 5, B, D, L, and N versus 5, A, C, K, and 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 p57^{Kip2} in nitrofen-treated fibroblasts compared to control fibroblasts (Figure 7). These inhibitors are known to inhibit the transition from the G_1 -to S-phase of the cell cycle. The other Cip/Kip family member of Cdk inhibitors, p21^{Waf1/Cip1}, was not detect-

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 Volocity4 software (Quorum Technologies Inc). The epithelial-to-fibroblast (E/F) ratio (**A**) and fraction (**B**) of fibroblasts in the recombinants were determined. The E/F ratio was significantly decreased whereas the number of fibroblasts increased in (F_N)(E_N) recombinants compared with 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. * $P < 0.025$. The bars represent SEM.

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) revealed less TUNEL-positive fibroblasts of both F_N containing recombinants in comparison to F_C containing recombinants (**B, D** versus **A, C**). Cleaved caspase-3 (brown) immunoreactivity was less in both F_N containing recombinants in comparison to F_C-containing recombinants (**F, H** versus **E, G**) as well. Scale bar = 50 μ m.

able 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 (see Supplemental Figure S3 at <http://ajp.amjpathol.org>).

Cell Differentiation

To determine whether the recombinants demonstrated differences in cell differentiation besides the reduced proliferation and apoptosis *in vitro*, we performed IF for α SMA (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 for endothelial cells). α SMA IF staining was similar in myofibroblasts in all four types of recombinants (Figure 8, A–D). In addition, IF staining for TITF-1 (results not shown), pro-SFTPC (Figure 8, E–H), or 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 (see Supplemental Figure S2, E–H at <http://ajp.amjpathol.org>).

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 *in vitro* pulmonary cell recombinant model that resembles human CDH-associated lung hypoplasia. This cell recombinant model enabled us to investigate epithelial-mesenchymal interactions in nitrofen-induced lung hypoplasia in more detail and resulted in the following observations. Recombinants containing *in vivo* nitrofen-treated fibroblasts (F_N) exhibited decreased apoptosis

and a late G₁ cell cycle arrest. Therefore, we postulate that fibroblasts are the defective cell layer in CDH-associated hypoplastic lungs.

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.^{5–7,16,28–30} Hence, we believe the pulmonary cell recombinant model is an adequate *in vitro* model for lung 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 cross talk between the two tissue layers. After establishing a functional model for homotypic recombinants (recombining lung epithelial cells and fibroblasts from the same treatment group), we created heterotypic recombinants (recombining lung epithelial cells and fibroblasts from opposing treatment 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.³¹ We hereby, for the first time, provide evidence that the fibroblast tissue layer is also the defective tissue layer in nitrofen-induced lung hypoplasia. More importantly, this defect was intrinsic to the fibroblasts and not influenced by epithelial-mesenchymal interactions.

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**) compared with 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) revealed a decrease in cells in the S-phase in $(F_N)(E_N)$ recombinants (**J**) compared with $(F_C)(E_C)$ recombinants (**I**). PH3 (brown) staining indicated less cells undergoing mitosis in F_N -containing recombinants (**L, N**) compared with F_C -containing recombinants (**K, M**). Scale bar = 50 μ m.

Apoptosis is one of the developmental entities essential to the formation of a healthy lung.³² Immunohistochemical (TUNEL and cleaved caspase-3) analyses demonstrated less apoptosis in F_N -containing recombinants compared with 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.^{32,33} 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.³³ 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 explants⁹ nor in whole lungs after nitrofen treatment *in vivo*.^{20,34} 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.^{34,35} 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.³² 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^{9,36} 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.

Figure 6. Decrease in mitosis in affected recombinants. To quantify the mitotic index of pH3 positive cells in Figure 5, they 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 compared with both (F_C)(E_C) and (F_C)(E_N) recombinants. **P* < 0.0001.

In the present study, we found a late arrest in the G₁-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 G₁ arrest and are unable to continue the proliferation cycle. The exact mechanism of this G₁ arrest remains to be elucidated. Similar to our observation with E19 lungs, previous studies have reported decreased proliferation in nitrofen-treated rat lungs.^{9,20} Jesudason et al²⁰ reported reduced lung cell proliferation 24 hours before diaphragm closure (E15.5) in nitrofen-treated rats, whereas we found a diminished fibroblast proliferation in nitrofen-exposed lung explants.⁹ In addition to lung, Clugston et al³⁴ demonstrated a decrease in cellular proliferation of the pleuropertitoneal fold (diaphragm) in nitrofen-treated rat embryos. None of these studies addressed the specific phase of the cell cycle involved and the present study is the first one to report in the G₁-to S-phase transition in nitrofen-induced pulmonary hypoplasia. Quantitative analyses of pH3-positive cells demonstrated a more than twofold 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 G₁-to S-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 G₁-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 Andersen⁴⁰ and has been reviewed by others.^{18,19} RA treatment has demonstrated to reduce the number of hernias and to induce lung growth in nitrofen-treated embryos.^{29,41,42} It has been shown that RA protects alveolar epithelial cells from a late G₁-phase arrest induced by hyperoxia.⁴³ 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 G₁-phase arrest in the F_N-containing recombinants. Several treatment options have been aimed at increasing proliferation in CDH-associated lung hypoplasia, but none has specifically targeted a G₁-phase proliferative arrest.^{39,44} An overview of the literature is shown in Table 1.

Four investigated cell differentiation markers (TITF-1, CCSP, αSMA, and pro-SFTPC) were present and not differently distributed among isolated cells and the recombinants. PECAM was absent in freshly isolated cells before recombination and in the recombinants, which indicates no endothelial cells were isolated during primary culture. The standard primary culture techniques we used 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.⁴⁵ 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.^{46,47} Furthermore, the hypoplastic CDH human lung contains a decrease in interstitial and bronchial smooth muscle cells.⁴³ In previous studies, differences between normal lungs and nitrofen-treated lungs have been reported for these differentiation markers.^{9,28,30,38,48–50} 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.^{36,51} 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

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 p27^{Kip1} and p57^{Kip2} proteins.

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) revealed 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**). Scale bar = 50 μm.

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. Based on our findings, we postulate that lung fibroblasts are the primary malfunctioning layer in the nitrofen model (Figure 9). Previously, others and our group demonstrated that hypoplastic lungs of both experimentally-induced CDH and human CDH possess a thickened mesenchyme.^{7,9,16,20} Until now there has been no good explanation for this observation. Moreover, no studies have investigated whether this thickening is due to defects in the mesenchyme, epithelium, or the interaction between both tissue layers. Forty years ago interactions between the different tissue layers were shown to be crucial for proper embryonic lung development.²¹ Epithelial-mesenchymal interactions are important throughout lung development. Therefore, the thickened mesenchymal layer that is observed in hypoplastic lungs is not

necessarily caused by an intrinsic defect in this layer. To our knowledge, we are the first to demonstrate that fibroblasts from nitrofen-induced hypoplastic lungs are identified to be intrinsically defective, and more importantly, that this is not regulated by interactions with the epithelial tissue layer. Because cell markers were similar in nitrofen-treated and control cells just after isolation of the cells and before 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 revealed a defective fibroblast (mesenchymal) compartment of the pleuroperitoneal fold as the underlying mechanism resulting in diaphragmatic defects.^{31,52,53} The fact that we observed similar structures in F_C-containing recombinants can be explained by the postulated hypothesis, because there are no malfunctioning F_N cells in these recombinants present that makes them appear like normal lungs. The abnormal structures of F_N-containing re-

Table 1. Overview of the Literature E19 Nitrofen-Treated Rat Lungs

	Reference	E19 nitrofen-treated rat lungs
Apoptosis		
Cleaved Caspase-3	Jesudason ²⁰	No difference (however: E13.5–E16.5 rat)
TUNEL	This study	Decrease
Proliferation		
Ki67	This study	Decrease
Cyclin D	This study	No difference
EdU (BrdU)	Jesudason ²⁰	Decrease (however: E15.5)
Phosphohistone H3	Kling ³⁵	Decrease (however: E14 explants <i>in vitro</i> exposure to nitrofen)
Cell differentiation		
Surfactant proteins	Van Tuyt ³⁷	No difference
	Guilbert ³⁰	
αSMA	Coleman ³⁸	Increase mesenchyme (however: E17 mice)
TITF-1	Chinoy ²⁸	No difference (however: E19 mice)
	Sugimoto ³⁹	Increase (however: E21 rat)

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

combinants 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. The $(F_C)(E_C)$ 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 or cause this defect, respectively, implying 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 (lung hypoplasia), whereas 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.

Our study is associated with some limitations. First of all, we are dealing with an animal model for lung 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. However, the exact working mechanism of nitrofen on embryonic development has just recently become more clear as nitrofen was found to inhibit RALDH2, an enzyme involved in the RA pathway.⁵⁴ In addition, our group recently demonstrated lower levels of retinol and retinol binding protein in human umbilical cord blood of infants with CDH.¹⁹ This suggests a possible epigenetic role of RA metabolism in the pathogenesis of human CDH. However, despite its shortcomings, the nitrofen model is the preferred model to investigate pathogenetic aspects of (lung hypoplasia in) CDH because it

has very similar characteristics to human CDH.^{13,16} 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 lung hypoplasia by mechanic interference although the lungs are normal, the relatively late creation of the defect during lung development, and the almost completed alveolarization in sheep whereas in humans only 20% of adult alveolarization is achieved at that timepoint. Genetic mouse models have also been used to study lung 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 factors (regulators), 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.^{53,55,56} However, no single gene mutation has been identified to date and, because the CDH phenotype is so variable, it is unlikely that it is caused by a single gene mutation and may be 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 previous reports.^{17,18} 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 factors (regulators) (GATA4, CoupTFII, etc).^{17,18} The so-called RA hypothesis was recently supported by our human studies, which found that human newborns with CDH had significant lower retinol and retinol binding protein levels in umbilical cord blood.¹⁹

A second limitation of our recombination model is that it is an *in vitro* cell culture model based on an experimental approach. Even though the hernia and lung 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.²⁴ 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, because our goal was to investigate the interactions between different tissue layers during (ab)normal lung development, this *in*

vitro model was deemed appropriate. 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.

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 lung hypoplasia. Characteristics of human lung 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.^{57,58} 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.^{5-7,28} Our findings do not support an immature state of CDH lungs, as reported by others.^{59,60} The idea that the CDH hypoplastic lung is immature remains controversial because there have been no studies reporting primary surfactant deficiencies in human and nitrofen-induced CDH lungs,^{37,61} nor a decreased pool size of surfactant.⁶² Oligohydramnios due to obstructive uropathy, another cause of human lung hypoplasia, has different morphological features and may not be associated with the pathogenesis for lung hypoplasia in CDH.⁶³ 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 because 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.

Using 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.^{64,65} For instance, FGF2 plays a role in lung cell apoptosis whereas FGF1 is an example of a growth factor with proliferative qualities.^{66,67} In addition, in the nitrofen model both FGF7 and FGF10, and FGFR1 were down-regulated whereas LopezJimenez et al⁶⁸ and others^{69,70} recently demonstrated a CDH patient with a 4p16.3 deletion that included the FGFR1 region.⁶⁸⁻⁷⁰ The next step to elucidate the pathogenesis and work toward 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.²⁹

In conclusion, we show that the fibroblast tissue layer is malfunctioning in nitrofen-induced lung hypoplasia. Knowing the malfunctioning tissue layer will aid in developing 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 surgical- and nitrofen-induced diaphragmatic hernia models, tracheal occlusion induces fibroblast apoptosis and accelerates cell proliferation,^{41,71-74} which are the main defects according to our recombinant model. Thus, this treatment modality appears to have great potential.

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