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ORIGINAL RESEARCH

Fetal Tracheal Occlusion Correlates with Normalized YAP Expression and Alveolar Epithelial Differentiation in Congenital Diaphragmatic Hernia

Ophelia Aubert[1,2], Yuichiro Miyake[3], Gaurang M. Amonkar[1], Olivia M. Dinwoodie[1], Brian M. Varisco[4,5], Mario Marotta[6], Caiqi Zhao[1], Richard Wagner[2], Ya-Wen Chen[7,8], Alessandra Moscatello[7,8,10], Caterina Tiozzo[9], Xaralabos Varelas[11], Paul H. Lerou[1], Jose L. Peiro[12], Richard Keijzer[3], and Xingbin Ai[1]

[1]Division of Newborn Medicine, Department of Pediatrics, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; [2]Department of Pediatric Surgery, University Hospital Leipzig, Leipzig, Germany; [3]Division of Pediatric Surgery, Department of Surgery, Rady Faculty of Health Sciences, Max Rady College of Medicine, University of Manitoba and Children's Hospital Research Institute of Manitoba, Winnipeg, Manitoba, Canada; [4]College of Medicine, University of Arkansas for Medical Sciences, Little Rock, Arkansas; [5]Arkansas Children's Research Institute, Little Rock, Arkansas; [6]Bioengineering, Cell Therapy and Surgery in Congenital Malformations Laboratory, Vall d'Hebron Hospital Research Institute, Universitat Autonoma de Barcelona, Barcelona, Spain; [7]Department of Otolaryngology, [8]Department of Cell, Developmental, and Regenerative Biology, and [9]Division of Neonatology, Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, New York; [10]School of Medicine, New York Medical College, Valhalla, New York; [11]Department of Biochemistry and Cell Biology, Boston University School of Medicine, Boston, Massachusetts; and [12]Center for Fetal and Placental Research, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio

ORCID IDs: 0009-0007-4785-6462 (O.A.); 0000-0002-3035-2619 (R.W.); 0000-0003-2382-8444 (Y.-W.C.).

Abstract

Congenital diaphragmatic hernia (CDH) is characterized by incomplete closure of the diaphragm. Although the ensuing compression to the fetal lung causes lung hypoplasia, specific cellular phenotypes and developmental signaling defects in the alveolar epithelium in CDH are not fully understood. Employing lung samples from human CDH, a surgical lamb model, and a nitrofen rat model, we investigated whether lung compression impairs alveolar epithelial differentiation and Yes-associated protein (YAP)-mediated mechanosensing. We showed that CDH in humans and lambs caused defective alveolar epithelial differentiation manifested by more alveolar epithelial type II (ATII) cells, fewer ATI cells, and the emergence of cells coexpressing ATI and ATII markers. Associated with these alveolar epithelial defects, we found a decrease in the level and nuclear localization of YAP. Reduced YAP and abnormal distal lung development were evident as early as 21 weeks of gestation in human CDH. In addition, rat fetuses with CDH also showed diminished nuclear YAP and more abundant ATII cells. In contrast, the littermates without the hernia had no such alveolar phenotypes. Furthermore, fetal tracheal occlusion in the surgical lamb model of CDH fully normalized nuclear YAP and rescued alveolar epithelial defects in a gestational age-dependent manner. Taken together, our findings across species indicate that lung compression in CDH is sufficient to disrupt alveolar epithelial differentiation and impair YAP signaling. Tracheal occlusion can restore nuclear YAP and rescue the alveolar defects in CDH, depending on the timing and the duration of this prenatal surgical intervention.

Keywords: congenital diaphragmatic hernia; fetal tracheal occlusion; alveolar epithelial differentiation; YAP

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Correspondence and requests for reprints should be addressed to Xingbin Ai, Ph.D., Barlett Hall Extension, Room 414, Division of Newborn Medicine, Department of Pediatrics, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114. E-mail: xai@mgh.harvard.edu.

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Clinical Relevance

Congenital diaphragmatic hernia (CDH) is an unmet medical condition that causes significant morbidity and mortality in patients, mostly because of lung defects. Repair of the diaphragm *in utero* remains the only available treatment strategy so far. This study employed patient samples and animal models of CDH to investigate molecular pathways that can be normalized by the fetal surgical procedure in CDH.

Congenital diaphragmatic hernia (CDH) affects 1 out of 3,000 live births and is characterized by incomplete closure of the diaphragm, lung hypoplasia, and pulmonary hypertension (1). Over the past decades, outcomes of patients with CDH have improved, but worldwide mortality remains high (30–50%). Patients who survive the neonatal period often face significant long-term pulmonary morbidity (1, 2). Reasons for ameliorated odds of survival are twofold: better neonatal intensive care and the establishment of fetal tracheal occlusion (TO) as an indispensable prenatal treatment option (3). To date, TO represents the only available prenatal therapeutic intervention that has been shown to significantly improve survival in a cohort of patients with severe CDH (3).

The etiology of CDH is complex, and the clinical presentation is heterogeneous. Compression of the lung is a shared pathogenic event during fetal lung development in all patients with CDH. Patient-specific genetic factors and *in utero* exposure also contribute to pathogenesis of lung defects (4). Our recent study has indicated causative proinflammatory pathways in the conductive airway epithelial compartment in human CDH (5). However, despite the fact that alveolar epithelial cells are responsible for gas exchange and cover more than 99% of the internal lung surface area (6), our knowledge about alveolar defects in CDH stems predominantly from the nitrofen rodent model because of limited access to CDH patient lung samples (7–10). Nitrofen is a carcinogenic herbicide that causes tissue-nonspecific and only partially defined damage to fetuses. Oral gavage of nitrofen to rat dams at embryonic day 9.5 (E9.5) causes CDH in ∼60% of the fetuses, with the rest exhibiting pulmonary hypoplasia without hernia, which provides an opportunity to distinguish the role of compression in fetal lung development from other undesirable effects of nitrofen (7, 8). Of note, nitrofen has never been implicated in human CDH, and previous findings from the nitrogen model are often conflicting (11–14). As such, specific molecular phenotypes of alveolar epithelium in human CDH remain to be fully characterized. In addition, whether TO benefits alveologenesis is unclear.

Lung compression is known to disrupt mechanotransduction in the fetal lung (15). We thus hypothesize that lung compression secondary to the hernia may be a driver for impaired alveolar epithelial development by disrupting the activity of mechanosensitive signaling effectors. Yes-associated protein (YAP) is a central mechanotransducer that plays an essential role in branching morphogenesis and alveolar epithelium differentiation during embryonic lung development, and YAP dysregulation was previously implicated in CDH (15–22). The activity of YAP signaling is regulated by protein phosphorylation and subcellular distribution (17, 20). When localized to the nucleus, YAP is considered active and acts as a transcription factor, whereas cytoplasmic localization promotes degradation of YAP. In this study, employing lung samples of control subjects and patients with CDH at two different developmental stages, a surgical model of CDH with TO in lambs, and the nitrofen rat model with and without CDH, we performed detailed characterization of alveolar phenotypes in CDH, assessed the associated changes in the level and nuclear localization of YAP, and evaluated the benefit of TO to alveologenesis.

Methods

Human Fetal Lung Samples

Histological sections of full-term fetal human lungs were obtained from a tissue bank at the University of Manitoba (IRB HS15293) (23). CDH cases were identified by whole-text search of autopsy records dated between 1980 and 2017. Control samples were collected from stillborn cases whose main causes of death included vasculopathologies of the placenta, placental abruption, preeclampsia, or unknown cause. These archived lung samples were fixed in formalin and stored in paraffin blocks before collection of 5-μm tissue sections.

Fresh control and CDH lung samples at 21 weeks of gestation were collected by the Developmental Origins of Health and Disease Biorepository at the Icahn School of Medicine at Mount Sinai (IRB STUDY-22-00065). Consent for tissue donation was obtained after the patient had already made the decision of pregnancy termination and by a different clinical research coordinator from the physician who performed the procedure. All tissues were deidentified, and the only clinical information collected was the gestational age and the presence of any maternal or fetal diagnoses. Lung tissues were fixed in 4% formaldehyde in PBS overnight, paraffinized, and 5-μm sections were sectioned for histological analysis.

Surgical Model of CDH in Lamb

Surgical procedures to induce CDH and TO in fetal lambs were approved by the institutional animal care and use committee at Cincinnati Children's Hospital Medical Center Animals and the Vall d'Hebron Institute of Research Institutional Animal Care and Use Committee (no. 49/14 CEEA). Experiments were performed at the Vall d'Hebron Institute of Research between 2011 and 2013. The surgical procedure was previously described (24, 25). Briefly, timed pregnant ewes were anesthetized at Day 65 of gestation, and a midline laparotomy was performed to expose the uterus. A short hysterotomy exposed the head and left upper extremity of the fetus, and a left lateral thoracotomy was performed on the fetus. A left diaphragmatic hernia was created by perforating and disrupting the muscle with scissors, and the stomach and intestines were placed into the left thoracic cavity. Identical anesthesia and midline maternal laparotomy were used for the TO procedure at 85 days (early tracheal occlusion [ETO]) or 105 days (standard TO). The fetal head and neck were exposed by a small hysterotomy, and cervical dissection of the fetus exposed the trachea. Two titanium clips (LIGAMAX, Ethicon Endo-Surgery) were applied externally to occlude the tracheal lumen, and the hysterotomy and laparotomy were closed. Sham-operated lambs were non-CDH control animals. Between 135 and 145 days of gestation, the ewe was anesthetized, and, after cesarian section, fetal tissues were collected. The left lower lobe was fixed, paraffinized, and sectioned for histological analysis.

Nitrofen Rat Model of CDH

All animal work was approved by the institutional animal care and use committee at Massachusetts General Hospital (Institutional Animal Care and Use Committee no. 2022N000003; X.A., principal investigator). Timed pregnant Sprague-Dawley rats were purchased from Charles River Laboratories. Rat dams at E9.5 were orally gavaged with either nitrofen (100 mg in 1 ml olive oil; Cayman Chemical) or olive oil alone (vehicle) (8). Rat fetuses were harvested at E21.5.

Statistical Analyses

For details on statistical analyses, the numbers of samples, and the experimental repeat, *see* the corresponding figure legends and the Results section. For comparisons between two experimental groups, the Mann-Whitney *U* test was performed. For comparisons between more than two groups, the Kruskal-Wallis test followed by a *post hoc* corrected Dunn's test or one-way ANOVA followed by a *post hoc* corrected Tukey's test was performed when appropriate. All statistical tests were performed using GraphPad Prism 8. A calculated *P* value less than 0.05 is considered statistically significant. Additional methods (antibody staining, RNAscope, and imaging) are provided in the data supplement.

Results

Human Full-Term CDH Lungs Exhibit Impaired Alveolar Epithelial Differentiation

We characterized alveolar epithelial differentiation in human full-term, control, and CDH lungs by antibody staining for HOPX and SPC as markers of alveolar epithelial type I (ATI) and ATII cells, respectively. Lung samples ($n = 4$ for each group) were obtained from infants who died at the time of or immediately after birth, allowing tissue assessment without the confounding effect of mechanical ventilation. We found an increase in SPC^+ ATII cells ($10.5 \pm 1.3\%$ control, $35.6 \pm 5.4\%$ CDH; $P < 0.05$) and a decrease in $HOPX^+$ ATI cells in CDH lungs ($29.3 \pm 2.2\%$ control, $13.8 \pm 2.9\%$ CDH; $P < 0.05$) (Figures 1A and 1B). In addition, human CDH lungs had more abundant SPC^+HOPX^+ double-positive cells than control human lungs ($1.0 \pm 0.3\%$ control, $12.0 \pm 2.0\%$ CDH; $P < 0.05$) (Figures 1A and 1B), suggestive of an abnormal transitional state during alveolar development or delayed differentiation.

Epithelial Defects in Human CDH Can Be Evident as Early as 21 Weeks of Gestation

To investigate how early the epithelial phenotype occurs in human CDH, we procured left lobes from a CDH fetus with a left-sided diaphragmatic hernia at 21 weeks of gestation and two gestational stage-matched, non-CDH controls. Antibody staining for HOPX showed robust expression in the stalk of branching airways and excluded from the distal airway or surrounding mesenchyme, a pattern comparable between controls and this CDH lung (Figure 1C) (26). In control fetal lungs, SPC was restricted to the epithelium in distal airways (Figure 1C). In comparison, this CDH lung at 21 weeks of gestation showed a broader expression pattern of SPC than gestation age-matched control lungs (Figure 1C).

Lung compression is known to disrupt mechanotransduction in the fetal lung (15). Given a critical role of YAP in mechanosensory and alveolar epithelial development (16–21), we assessed the level and intracellular localization of YAP by antibody staining of human fetal lung samples. In contrast to nuclear YAP in a number of airway epithelial cells in control lungs, YAP was found almost exclusively in a perinuclear and cytoplasmic localization in this CDH lung (Figure 1D). Because YAP signaling plays a critical role in the proximal-to-distal patterning of airway epithelial cells during branching morphogenesis (20, 22), we stained 21-week, control, and CDH lungs for SOX2 and SOX9 and found a comparable expression pattern (*see* Figure E1 in the data supplement). Therefore, impaired YAP signaling in airway epithelial cells may occur as early as 21 weeks of gestation in human CDH and may not be sufficient to disrupt patterning of airway epithelial cells at this age of gestation.

Lung Compression in the Nitrofen Rat Model of CDH Impairs Nuclear YAP Localization and Disrupts Alveolar Epithelial Development

About 60% of the rat fetuses from nitrofen-exposed dams (Figure 2A) had diaphragmatic hernia (8). We thus compared rat fetuses with hernia and the littermates without hernia at E21.5 to evaluate alveolar epithelial defects caused specifically by lung compression. Fetuses from oil-exposed pregnant dams were included as normal controls. Among several antibodies tested for SPC and YAP staining in rat lung tissues, we only found two rabbit antibodies that generated specific signals. Therefore, we performed antibody staining for SPC and YAP using two adjacent paraffin sections (5 μm apart) and then colocalized the signals to determine ATII cell expression of YAP. We showed that YAP was expressed by ATII cells in rat lungs (Figure 2B). In control and no-hernia rat lungs, YAP was abundant and localized to the nucleus and cytoplasm (Figure 2C). However, CDH in rats caused decreases in overall YAP levels and the relative abundance of nuclear YAP^+ cells in alveoli ($44.3 \pm 5.5\%$ control, $39.0 \pm 4.1\%$ no hernia, $10.4 \pm 4.2\%$ CDH; $P < 0.0001$) (Figure 2D). In addition, YAP was mostly found in a perinuclear and cytoplasmic location in alveolar cells in the CDH group (Figure 2C). Loss of nuclear YAP was associated with a significant increase in SPC^+ ATII cells in CDH rat fetuses compared with control and no-hernia rat fetuses ($22.2 \pm 3.0\%$ control, $22.6 \pm 0.8\%$ no hernia, $37.3 \pm 3.9\%$ CDH; $P < 0.001$) (Figure 2E). Notably, unlike HOPX staining in human CDH lungs, we found no change in the abundance of $HOPX^+$ cells or double $HOPX^+SPC^+$ cells in rat CDH fetuses (Figure E2), which may be due to differences in the time course of alveolar development between species. Taken together, lung compression is associated with reduced nuclear YAP localization and an increase in the number of ATII cells in the rat model of CDH.

CDH in the Surgical Lamb Model Impairs Alveolar Epithelial Differentiation that Is Reversed by TO

We evaluated in a surgical lamb model of CDH whether compression of the fetal lung is sufficient to induce similar alveolar epithelium defects found in human CDH and the nitrofen rat model (Figures 1 and 2). A left-sided diaphragmatic hernia was introduced at Day 65 of gestation (~12th week of gestation in humans), and fetuses were delivered at term via cesarean section around Day 145 (Figure 3A) (25, 27). Antibody staining for SPC showed CDH lambs had more SPC^+ ATII cells than control animals ($n = 47.8 \pm 7.3$ control, $n = 144 \pm 36.9$ CDH; $P < 0.01$) (Figures 3B and 3C), similar to our findings in human and rat CDH (Figures 1 and 2). Because of poor cross-species reactivity of the HOPX antibody, we performed RNAscope to detect *HOPX* mRNA expression and coexpression

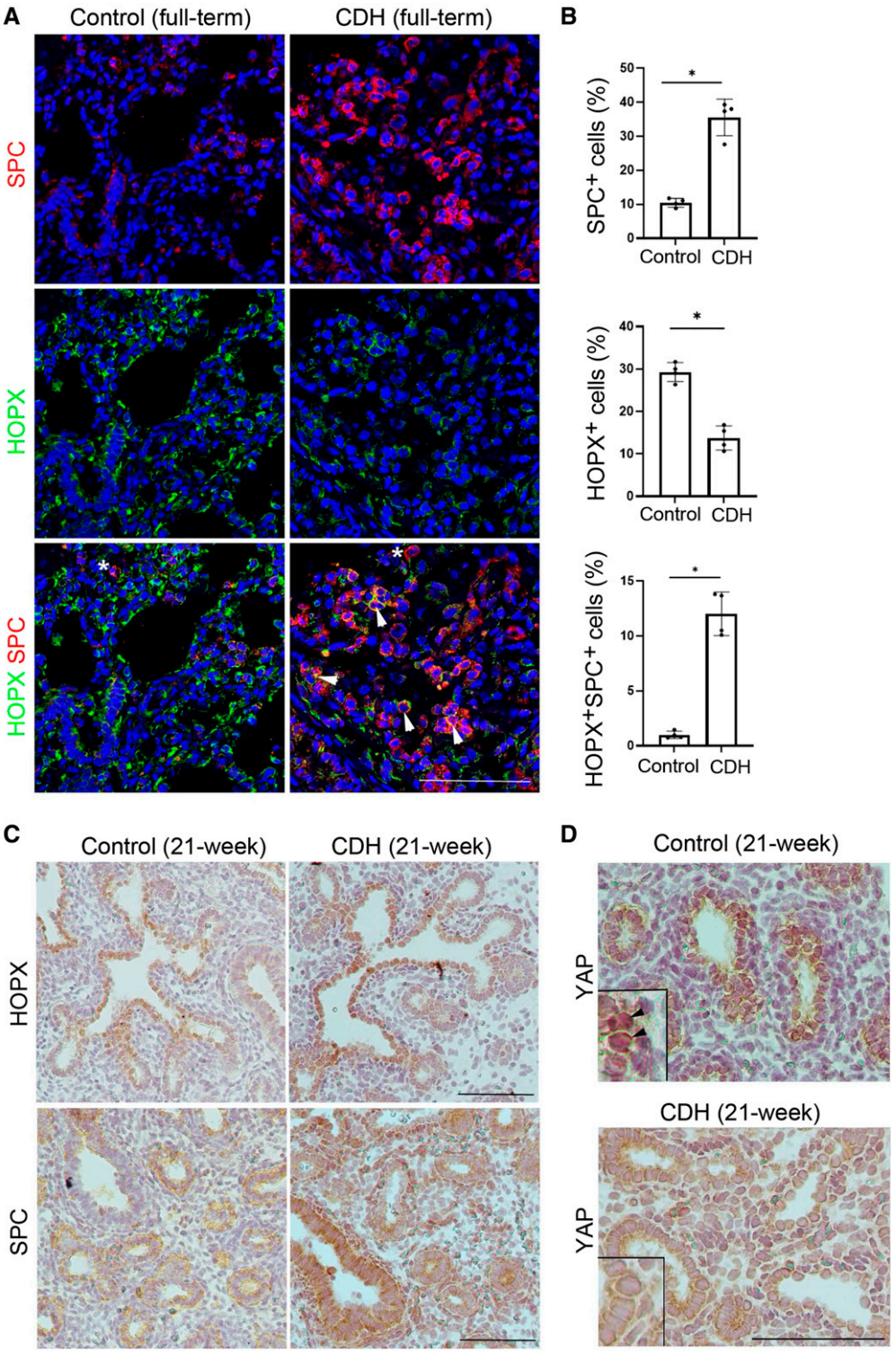

Figure 1. Human congenital diaphragmatic hernia (CDH) lungs exhibit defective alveolar differentiation associated with impaired nuclear Yes-associated protein (YAP). (*A*) Representative immunofluorescence images of antibody staining for HOPX and SPC in control and CDH full-term human lung sections. Arrowhead marks double-positive $HOPX^+SPC^+$ cells in CDH fetal lungs. Asterisk marks single-positive SPC^+ cells. Nuclei were counterstained by DAPI. (*B*) Quantification of relative abundance of $HOPX^+$ and SPC^+ single-positive and $HOPX^+SPC^+$ double-positive cells in control and CDH ($n = 4$ per group) human lung sections. More than 1,500 nuclei were counted for each marker. Each dot represents one patient sample. (*C* and *D*) Representative immunohistochemical images of antibody staining for HOPX, SPC, and YAP in one 21-week fetal CDH lung and two age-matched control lungs. Arrowheads mark nuclear YAP^+ cells. Statistical analyses were performed using the Mann-Whitney *U* test for comparisons between two groups. Significance is indicated as $^*P < 0.05$. Scale bars, 100 μm.

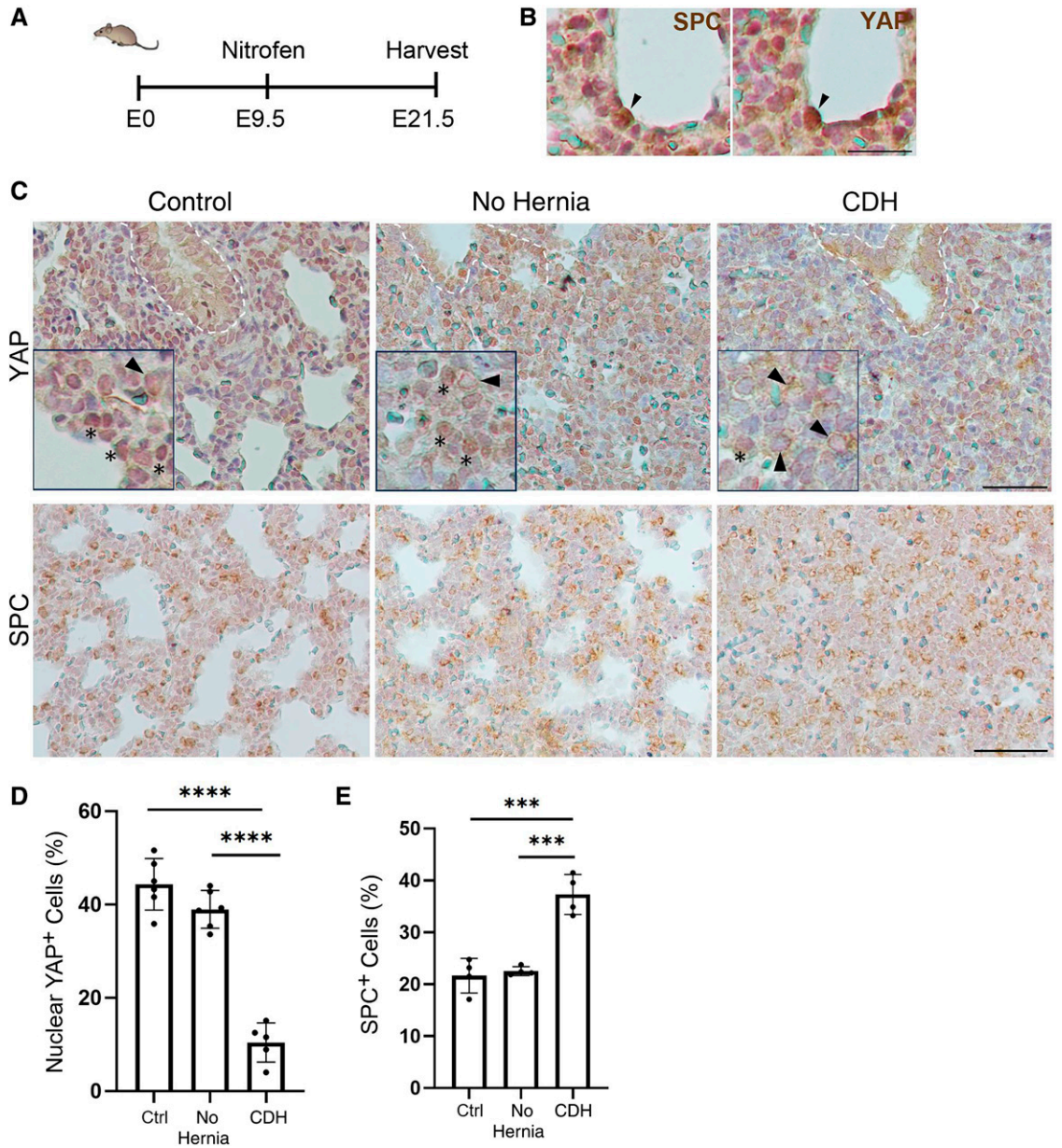

Figure 2. CDH in the nitrofen rat model causes reduced nuclear YAP and impaired alveolar epithelial differentiation. (*A*) Schematic of the nitrofen rat model. (*B*) Representative immunohistochemical images of antibody staining for SPC and YAP of two consecutive lung sections (5 μm apart) in the distal lung of embryonic day 21.5 (E21.5) control rat pups. Arrowheads mark double-positive SPC^+YAP^+ cells. Scale bars, 25 μm. (*C*) Representative immunohistochemical images of antibody staining for YAP and SPC in control, no-hernia, and CDH rat pups. Insets show enlarged areas with arrowheads marking perinuclear/cytoplasmic YAP localization and asterisks marking nuclear YAP^+ cells. Scale bars, 50 μm. (*D*) Quantification of the relative abundance of nuclear YAP^+ cells in fetal rat alveoli of each group. (*E*) Quantification of the relative abundance of SPC^+ cells in fetal rat alveoli of each group. At least three 20× images were counted for each rat pup. Each dot represents one sample. $n = 5$–6 samples per group. Bar graphs represent mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by a *post hoc* corrected Tukey's test for comparisons of multiple experimental groups. Significance is indicated as $^{***}P < 0.001$ and $^{****}P < 0.0001$.

with *SPC*. RNAscope revealed a significant decrease in the abundance of $HOPX^+$ ATI cells in CDH lambs compared with control animals (54.7 ± 5.6% control, 25 ± 9.7% CDH; $P < 0.001$) (Figures 3D and 3E). In addition, in contrast to control lamb lungs in which *HOPX* and *SPC* gene expression marked two different alveolar epithelial cell types, almost all *SPC*-expressing cells in CDH lambs were also positive for *HOPX* mRNA (Figure 3F). Furthermore, because TO counters lung compression in CDH, we tested in the surgical lamb model whether TO normalizes alveolar epithelial defects caused by lung compression. TO was achieved through clipping of the trachea at Gestation Day 105 (~27th week of gestation in humans). We found that TO fully rescued alveolar epithelial defects by restoring normal SPC and *HOPX* expression in CDH lambs (SPC^+ cells, $n = 48.0 \pm 6.1$;

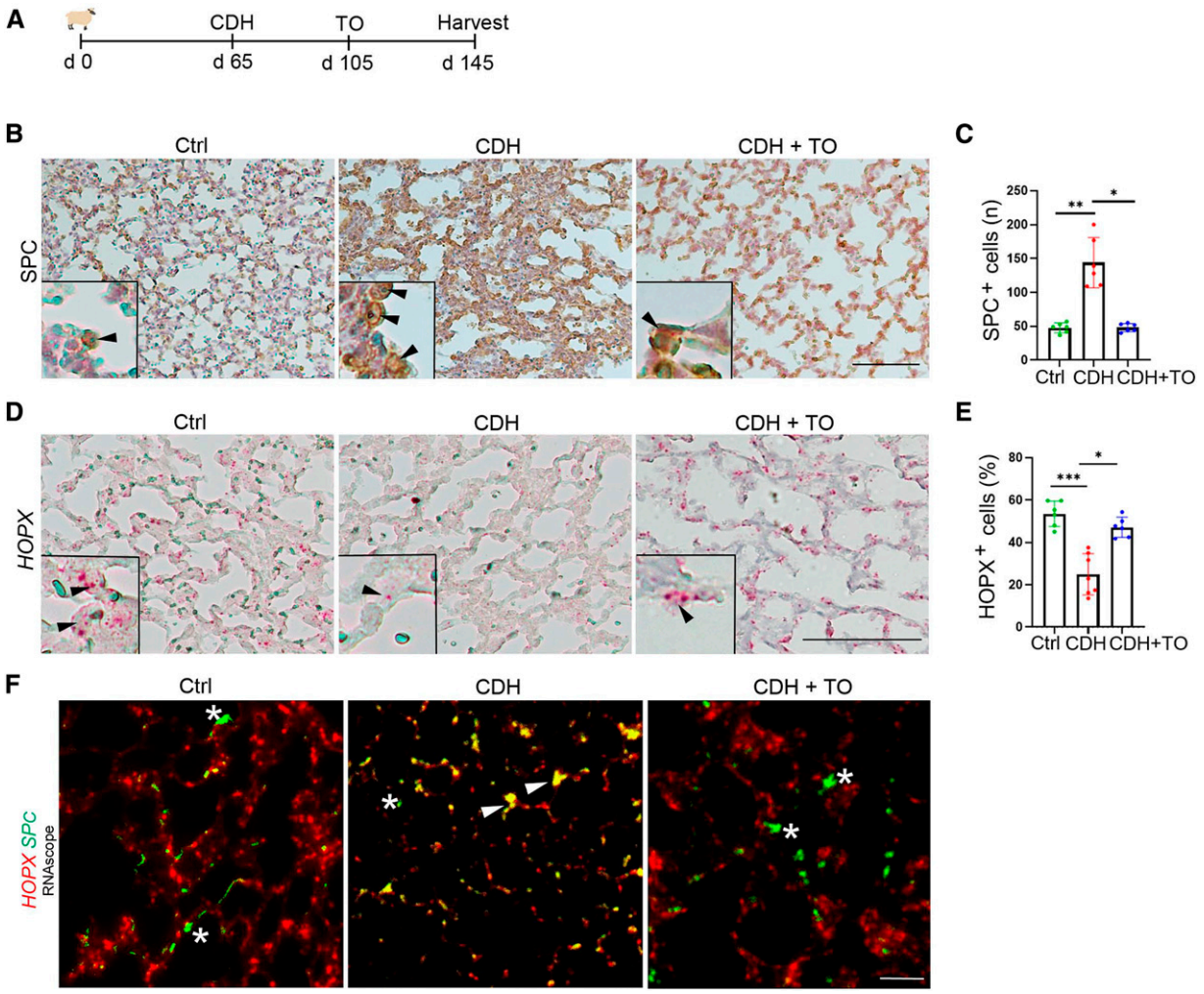

Figure 3. CDH causes defective alveolar epithelial differentiation that is restored by tracheal occlusion (TO) in the surgical CDH lamb model. (*A*) Schematic of the CDH+TO lamb model. Fetal lungs were harvested at Gestation Day 145 for assays. (*B*) Representative immunohistochemical images of antibody staining for SPC in distal lamb lungs of each group. (*C*) Quantification of the number of SPC^+ cell per 20× image. (*D*) Representative RNAscope images to detect *HOPX* gene expression in distal lungs of control, CDH, and CDH+TO lambs. Arrowheads mark $HOPX^+$ cells. (*E*) Quantification of the relative abundance of $HOPX^+$ cells in distal lamb lungs of each group. (*F*) Representative fluorescence images of RNAscope detection of *HOPX* (red) and *SPC* (green) gene expression in control, CDH, and CDH+TO lambs. Arrowhead marks $HOPX^+SPC^+$ double-positive cells in CDH lambs. Asterisk marks SPC^+ single-positive cells. For quantifications in *C* and *E*, three randomly chosen, nonoverlapping 20× images were counted for each lamb. Each dot represents one lamb. Bar graphs represent mean ± SEM. Statistical analyses were performed using the Kruskal-Wallis test followed by a *post hoc* corrected Dunn's test for comparisons of the three experimental groups. Significance is indicated as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Scale bars, 100 μm.

$HOPX^+$ cells, 47.2% ± 4.7% in CDH+TO group) (Figures 3B–3F). Morphometric measurements also showed that CDH lungs had reduced alveolar space and decreased collagen deposition in the perivascular area and septal tips, which were all normalized after TO (Figures E3A and E3B). Because the surgical model is devoid of patient-specific genetic factors and *in utero* exposure, these results indicate that lung compression alone can induce alveolar epithelial differentiation defects in CDH.

Lung Compression Disrupts Nuclear YAP Localization in the Alveolar Epithelium of CDH Lambs

We tested whether YAP signaling was dysregulated by lung compression in CDH lambs in association with abnormal alveolar epithelial phenotypes. To do so, we performed RNAscope to detect *HOPX* and *SPC* gene expression. After imaging, we washed the sections extensively with saline to remove the water-soluble chromogen for *SPC* RNAscope detection and then performed antibody staining for YAP. We showed that YAP was almost exclusively localized to SPC^+ ATII cells in control lamb lungs (Figure 4A). Furthermore, in control lambs, YAP was readily detectable in the nucleus and cytoplasm in ATII cells (Figures 4B and 4C). In contrast, in CDH lambs, the overall level of YAP and the relative abundance of nuclear YAP^+ alveolar epithelial cells were significantly reduced, and YAP was found mostly in a perinuclear and cytoplasmic localization (Figures 4B and 4C).

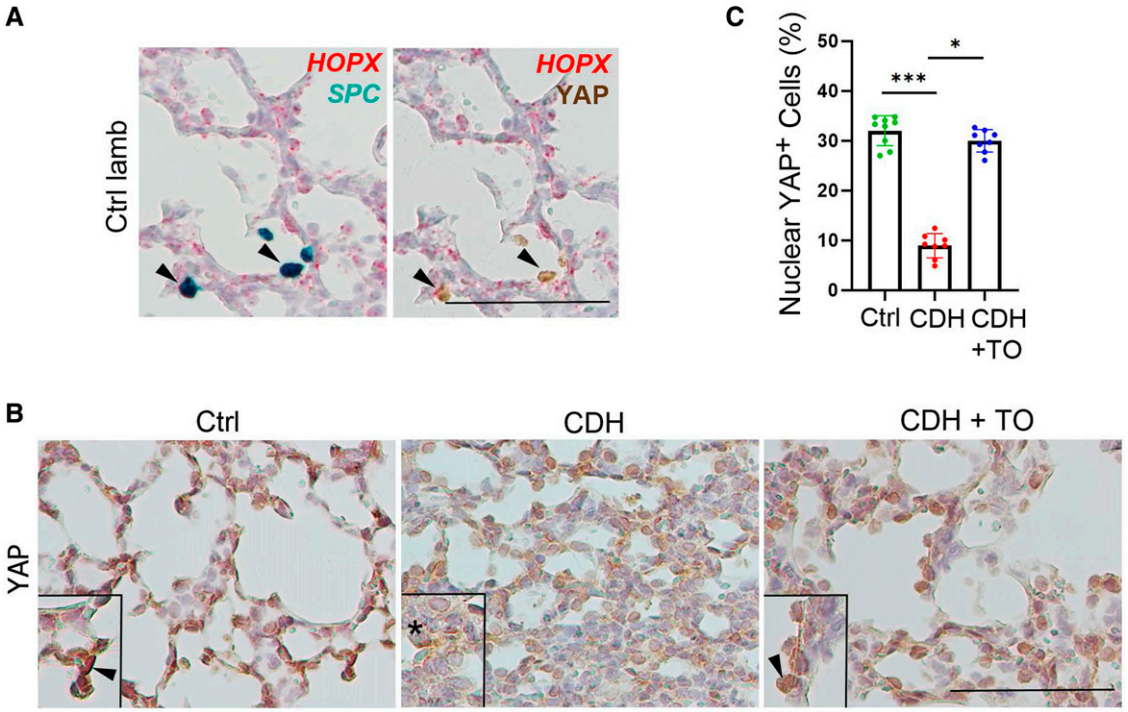

Figure 4. YAP is expressed in alveolar epithelial type II cells in lamb lungs and is decreased by lung compression in CDH. (*A*) Representative images of RNAscope to detect *HOPX* (red) and *SPC* (blue) gene expression and antibody staining for YAP in distal lamb lungs. RNAscope images were recorded before the lung sections were washed extensively with saline to remove the chromogenic substrate (water soluble) for *SPC* detection (in blue) followed by antibody staining for YAP. Arrowheads mark $SPC^{+}YAP^{+}$ double-positive cells. (*B*) Representative immunohistochemical images of antibody staining for YAP in distal lungs of control, CDH, and CDH+TO lambs. Arrowhead marks nuclear YAP^{+} cells, and asterisk marks cells with perinuclear YAP localization. (*C*) Quantification of the relative abundance of nuclear YAP^{+} cells from two or three nonoverlapping 20× images of each lamb lung; $n = 3$ lungs per group. Each dot represents quantification of one image. Bar graphs represent mean ± SEM. Statistical analyses were performed using the Kruskal-Wallis test followed by a *post hoc* corrected Dunn's test for comparisons of multiple experimental groups. Significance is indicated as $^{*}P < 0.05$ and $^{***}P < 0.001$. Scale bars, 100 μm.

TO in CDH lambs completely restored the level and nuclear localization of YAP (Figures 4B and 4C). Taken together, our results are consistent with previous findings in support of the role for YAP in ATII/ATI differentiation during embryonic alveologenesis (20, 22, 28) and suggest that YAP normalization may be functionally connected to the rescue of alveolar epithelial phenotypes in the CDH+TO group (Figure 3).

Early TO Disrupts YAP Signaling and Epithelial Differentiation in the Surgical CDH Lamb Model

Because human CDH can show abnormal epithelial phenotypes as early as 21 weeks of gestation (Figures 1C and 1D), we tested whether an earlier and prolonged duration of TO would be even more beneficial to lung development than the current surgical protocol (3). We employed an ETO model of CDH in lambs, where TO was performed at Day 85 of gestation (~21st week of gestation in humans) (Figure 5A). In contrast to TO that normalized fetal lung development in CDH lambs (Figures 3 and 4), ETO resulted in an almost complete suppression of nuclear YAP in the alveolar compartment, rendering technical difficulties of reliably detecting YAP by antibody staining (Figure 5B). In addition, lamb lungs with ETO showed disorganized expression of *HOPX* and *SPC* genes by RNAscope and had enlarged alveoli reminiscent of an emphysematous phenotype (Figures 5C and E3). A subset of *SPC*-expressing cells were also positive for *HOPX* transcripts, shown by a darker blue color in areas with an overlapping *HOPX* signal (in red) compared with the color of single *SPC* positivity (in blue) (Figure 5C). Double fluorescence RNAscope similarly found cells coexpressing *SPC* and *HOPX* genes in the distal lung of the ETO group (Figure E4). Importantly, the damaging effects of ETO extended beyond the distal lung, evidenced by poor differentiation of conducting airway epithelial cells, such as ciliated cells (27.2 ± 5.0% control, 7.0 ± 2.9% CDH+ETO; $P < 0.0001$) (Figure E5). Taken together, earlier and prolonged TO results in severe disruption of lung structure and epithelial cell differentiation.

Discussion

By systematically characterizing the alveolar epithelium in human CDH and two distinct CDH animal models, we report consistent changes in alveolar epithelial differentiation, which helps clarify conflicting results in previous studies stemming from differences in assays and models and the limitations inherent in analyzing human autopsy specimens (11–13, 29, 30). Contrary to the prevailing dual-hit hypothesis, which stipulates that first an intrinsic lung defect followed by secondary compression of the lung is causative of lung hypoplasia in CDH (7), our findings suggest that compression alone is sufficient to drive impaired epithelial

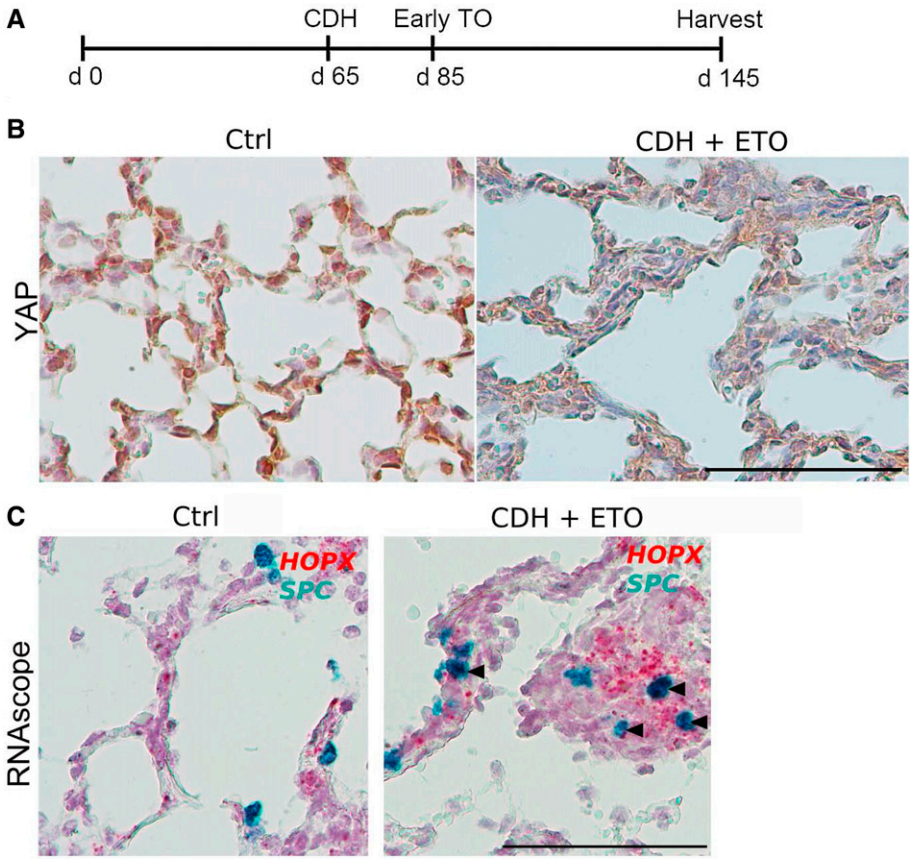

Figure 5. Early tracheal occlusion (ETO) disrupts YAP signaling and alveolar epithelium differentiation in surgical CDH lamb model. (*A*) Schematic of the CDH+ETO sheep model. (*B*) Representative immunohistochemical images of antibody staining for YAP in the distal lung of control and CDH+ETO ($n = 3$ per group). (*C*) Representative images of RNAscope detection of *HOPX* and *SPC* gene expression in CDH+ETO lambs ($n = 3$ per group). Arrowheads mark $HOPX^{+}SPC^{+}$ double-positive cells in CDH+ETO lungs. Scale bars, 100 μm.

differentiation. A parallel study in the conducting airway epithelium in CDH (data not shown) also indicates that compression-induced YAP defects can induce proinflammatory phenotypes in the conducting airway epithelium. Additional patient-specific, genetic, or *in utero* factors may explain the heterogeneity seen in a clinical setting (31–33). Furthermore, we identified that some of the phenotypical characteristics of CDH can be found as early as 21 weeks of gestation, extending our current understanding of how early the CDH phenotype is detectable in humans. This finding holds important clinical implications because it evidences that the CDH phenotype is at least partially present at the time of prenatal intervention, providing a rationale for the timing of the procedure. In addition, we found that CDH leads to nuclear YAP deficiency. On the basis of previously established roles of YAP in fetal alveologenesis (20, 28, 34), impaired nuclear YAP activities may serve as a potential mechanism for alveolar differentiation defects and thus a therapeutic target. Importantly, fetal TO fully normalizes epithelial differentiation as well as the level and nuclear localization YAP, demonstrating the benefit of the intervention.

Fetal breathing is impeded by CDH during pregnancy, and CDH leads to profound alterations of distal lung morphology, epithelial differentiation defects, and reduced gas exchange surface (25, 35–38). Consistent with our results, previous studies have shown an increase in SPC^{+} ATII cells in CDH and beneficial effects of TO on ATI cell differentiation (13, 39, 40). In contrast, others reported an almost complete suppression of SPC expression in the nitrofen rat model of CDH, which we cannot reconcile with our findings (11, 12). Mechanical forces exerted by breathing movements and mediated by YAP nuclear cytoplasmic localization have previously been shown to direct ATII/ATI differentiation (34). Lungs from oligohydramnios models, where fetal breathing is significantly impaired, also exhibit lung hypoplasia and an increase in ATII cells, further cementing the role of mechanical cues in alveolar differentiation (41, 42). Loss of nuclear YAP has also been shown to result in reduced ATI cell differentiation in an ATII-specific YAP/Taz knockout mouse model (28).

We show the emergence of a $HOPX^{+}SPC^{+}$ double-positive cell alveolar phenotype in CDH lungs of lambs and humans, suggestive of delayed or defective alveolar differentiation in CDH and that other mechanisms besides nuclear YAP deficiency likely play a role. An intermediate ATII/ATI cell state has recently been identified during alveolar regeneration and is implicated in idiopathic pulmonary fibrosis and acute respiratory distress syndrome (43–47). IL-1β–mediated inflammation was also found to promote

intermediate cell types and impede ATI cell differentiation (48). Transgenic activation of NF-κB in club cells has also been shown to disrupt alveolar development, supporting the idea of miscommunication between proinflammatory airway epithelium with alveolar progenitors during fetal lung development in CDH (49). Thus, the increase in ATII cells and the emergence of an intermediate alveolar cell phenotype seen in CDH may be explained by a combined effect of loss of YAP activity and chronic inflammation due to, for example, elevated nuclear NF-κB in the conducting airway, which we had previously demonstrated using patient-specific tracheal aspirate–derived basal stem cells from control subjects and patients with CDH (5).

Because of a lack of temporal characterization of human CDH alveolar phenotypes *in vivo* and *in vitro* using ATI/II culture models, it is challenging to determine whether impaired ATI/ATII differentiation at birth reflects a delayed or defective process. However, in a previous study, we have identified differentiation defects of basal progenitor cells in conducting airway epithelium in CDH (5) that are caused by compression-induced reduction in nuclear YAP activities (data not shown). Comparison of basal progenitors in CDH with the counterparts in full-term (36–40 wk) and preterm (24–28 wk) control subjects by transcriptomic profiling and differentiation assays has indicated that the defects in CDH basal progenitors are not associated with prematurity (5). As such, compression in CDH may also disrupt developmental pathways involved in distal airway patterning and differentiation causing defective rather than delayed alveologenesis, which warrants future investigation.

Limitations

We acknowledge limitations of our study. The lung defects in CDH at 21 weeks of gestation were from one lung sample. In addition, future work is warranted to elucidate how nuclear cytoplasmic YAP localization or previously identified proinflammatory pathways impact alveolar epithelial differentiation in CDH. Identification of direct target genes of YAP in alveolar progenitors will help address the possibility that impaired nuclear YAP may alter the expression of genes involved in differentiation of alveolar epithelial cells in CDH.

Conclusions

Neonatal intensive care and medical therapy for CDH has been optimized and plateaued for many patients. TO currently represents the only available prenatal treatment that can effectively improve survival in patients with severe CDH (3). The key to further improving outcomes may lie in optimizing TO protocols in terms of both timing and duration. The FETO (Fetoscopic Endoluminal Tracheal Occlusion) trial for moderate CDH showed no benefit, but the intervention was performed several weeks later compared with the clinical trial for severe CDH, because of institutional review board constraints related to the increased risk of premature membrane rupture (3, 50). Our findings show that phenotypical abnormalities can already be found at very early stages, suggesting that the intervention should be performed in a timely manner when cells still exhibit plasticity and the CDH phenotype is not fully developed (26). Furthermore, they could support the rationale for a clinical trial for moderate CDH at earlier time points, which would dramatically extend the patient population eligible for the intervention. On the other hand, results from the ETO model highlight that TO performed too early or with a longer duration is harmful to lung development. ETO resulted in severe disruption of lung structure and epithelial differentiation, which is consistent with early observations in the lamb model after tracheal ligation and corresponds to what we see clinically and histologically in patients with laryngeal or tracheal atresia in the spectrum of congenital high airway obstruction syndrome (51, 52). Future studies using fetal human TO samples could explore how TO can rescue the phenotype. This may be achieved by performing functional assays using our established tracheal aspirate basal stem cell *in vitro* system or in organoid models, alone or in combination with modulators of the YAP signaling pathway or antiinflammatory therapeutics. ■

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