Increased TGF-β: a drawback of tracheal occlusion in human and experimental congenital diaphragmatic hernia?

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ABSTRACT

Survivors of severe congenital diaphragmatic hernia (CDH) present significant respiratory morbidity despite lung growth induced by fetal tracheal occlusion (TO). We hypothesized that the underlying mechanisms would involve changes in lung extracellular matrix and dysregulated transforming growth factor (TGF)-β pathway, a key player in lung development and repair. Pulmonary expression of TGF-β signaling components, downstream effectors and extracellular matrix targets were evaluated in CDH neonates who died between birth and the first few weeks of life after prenatal conservative management or TO, and in rabbit pups that were prenatally randomized for surgical CDH and TO versus sham operation. Before tissue harvesting, lung tissue mechanics in rabbits was measured using the constant-phase model during the first 30 minutes of life. Human CDH and control fetal lungs were also collected from midterm onwards. Human and experimental CDH did not affect TGF-β/Smad2/3 expression and activity. In human and rabbit CDH lungs, TO upregulated TGF-β transcripts. Analysis of downstream pathways indicated increased Rho-associated kinases to the detriment of Smad2/3 activation. After TO, subtle accumulation of collagen and α-smooth muscle actin within alveolar walls was detected in rabbit pups and human CDH lungs with short-term mechanical ventilation. Despite TO-induced lung growth, mediocre lung tissue mechanics in the rabbit model was associated with increased transcription of extracellular matrix components. These results suggest that prenatal TO increases TGF-β/Rho kinase pathway, myofibroblast differentiation, and matrix deposition in neonatal rabbit and human CDH lungs. Whether this might influence postnatal development of sustainably ventilated lungs remains to be determined.

Keywords: fetal tracheal occlusion; congenital diaphragmatic hernia; lung hypoplasia; TGF-β signaling; rabbit model.
INTRODUCTION

Occurring in one out of 3,000 live births (56), congenital diaphragmatic hernia (CDH) is a life-threatening birth defect of the diaphragm that compromises the normal growth and maturation of fetal lungs. Pathological findings of subsequent lung hypoplasia include hypermuscularized arteries, diminished airspaces, alveolar wall thickening, and poor gas-exchange surface area (2, 48), which contribute to neonatal respiratory insufficiency and pulmonary hypertension. High mortality rates in spite of increased prenatal diagnosis and advances in neonatal care (14) led to the development of fetal tracheal occlusion (TO), a prenatal therapy designed to remedy lung hypoplasia. In nitrofen-induced CDH rats and surgically induced CDH sheep and rabbits, TO prevents the egress of lung fluid, stimulates lung tissue stretch and cell proliferation (40), and ameliorates lung growth and morphology (49, 66). However, prolonged TO induces an excess of alveolar type II epithelial cell transdifferentiation in CDH models (18, 66). Hence, the concept of temporary TO has been applied to avoid surfactant depletion (17). In the clinical setting, temporary fetoscopic endoluminal TO (FETO), a minimally invasive surgical technique intended to the most severely affected CDH fetuses (27), improved prenatal imaging findings (8) and neonatal survival rates (27, 50). Nevertheless, only sparse functional and morphometric findings have been reported (16, 24, 31).

Various growth factor signaling pathways seem to be involved in the pathogenesis of human CDH (6, 7, 44, 60) as well as in the lung response after experimental TO (6, 64). Transforming growth factor (TGF)-β is a key player in lung branching morphogenesis and alveolization (5). The three TGF-β isoforms are secreted as latent complexes bound to the extracellular matrix. Latent complexes release mature TGF-β upon proteolytic cleavage by matrix metalloproteinases (33), acidification (34), mechanical stretch (65), or conformational changes induced by thrombospondin-1 (5). Following binding of mature forms to TGF-β type
I and II receptors, signal transduction is initiated through Smad-dependent or independent routes that modify transcription of genes controlling extracellular matrix homeostasis and various cell functions (5). TGF-β has not been directly assessed in human CDH fetuses, while animal models have yielded contradictory findings (10, 36, 43,47). Besides, increased TGF-β transcripts (47, 64) have been reported after TO in intact and CDH sheep lungs, but none of these studies has intrinsically addressed signaling activity.

Besides its developmental role, TGF-β contributes to bronchopulmonary dysplasia (BPD) in premature infants, a chronic lung disease resulting from hyperoxia, barotrauma, and inflammation (37). Because mechanical ventilation and oxygen therapy are standard treatments for neonatal respiratory insufficiency, CDH survivors treated or not by FETO are at high risk of developing BPD (15, 59). Despite increased lung growth, temporary TO did not noticeably improve neonatal respiratory function in human and experimental CDH (17, 31). Since the underlying biological mechanisms are unknown, clarifying the status of TGF-β after TO might be clinically relevant. We hypothesized that lung overdistension induced by TO would enhance the TGF-β pathway prenatally and sensitize CDH lungs to postnatal injury. Herein, TGF-β signaling activity and expression of representative targets were evaluated after TO in human CDH neonates and in the rabbit CDH model. In the absence of previous literature, human CDH and control fetuses were preliminary analyzed from midterm onwards to test whether CDH was associated with disturbed TGF-β signaling.

MATERIALS AND METHODS

Human lung tissue

Between September 2009 and October 2012 lung specimens were collected after medical terminations of pregnancy or neonatal deaths. Medical abortions were performed according to Belgian and French legislations (laws of April 1990 and July 1994, respectively). The study
was approved by the local ethics committees of the participating centers (CHU Brugmann, Brussels, Belgium; Assistance Publique-Hôpitaux de Paris, Paris, France; Research Institute Polish Mother's Memorial Hospital, Lodz, Poland). Parents were informed about the research protocol and gave their signed consent for routine autopsy and/or postmortem lung biopsy by means of mini-thoracotomy. Depending on the tissue availability, non-macerated samples obtained within 24 hours of death were used for biological and/or histological studies. For CDH and FETO lungs, samples ipsilateral to the hernia were considered.

Patients' characteristics

The severity of CDH based on prenatal ultrasound was assessed by the observed to expected lung to head ratio (O/E LHR) (28). Eight fetuses had severe isolated CDH (O/E LHR less than 25%) and four fetuses had moderate CDH (O/E LHR between 26 and 45%) associated with other malformations. Pulmonary hypoplasia was defined by typical histological findings and reduced lung-to-body weight ratio (below 1.5% before 28 weeks of gestation, and below 1.2% thereafter) (3). CDH fetuses were compared to sixteen control fetuses with cerebral or skeletal anomalies without signs of thoracic compression (Table 1). Control lungs showed no microscopic features of lung hypoplasia and had lung-to-body weight ratios within the range of normality. Inclusion criteria, timing of FETO and patient management were as previously described (27). The lung response to FETO was appraised by increased O/E LHR and lung-to-body weight ratio. Two patients were born during the late saccular stage of lung development (10 and 30 days after FETO) and died soon after birth due to unsuccessful emergency FETO removal. Three other patients underwent six weeks of temporary FETO, were late preterm, and died after 6 to 42 days of intensive care. FETO patients were compared to six neonates with isolated left-sided CDH of comparable severity and expectant prenatal management, who died between postnatal days 0 and 10 from respiratory insufficiency and/or intractable pulmonary hypertension. In both groups, the postnatal management included
mechanical ventilation and permissive hypercapnia, early surgical hernia repair, vasopressor infusion, and/or inhaled nitric oxide therapy (Table 2). All newborns were mechanically ventilated from birth to death, except for the FETO patient with the longest survival, who was mechanically ventilated for a total of 31 days with short periods of non-invasive ventilation in between times. Complete pairwise matching could not be obtained, but groups were not statistically different for severity of lung hypoplasia before prenatal therapy, birth weight, and gestational age at birth (Table 2).

Animal model

Time-dated pregnant New Zealand white rabbits were obtained at 15 days of gestational age from Charles River Laboratories (Germany) with approval by the Ethics Committee for Animal Experimentation of the District Government of Upper Bavaria (Munich, Germany; number 55.2-1-54-2531-149-08). Animals were housed as reported (63). All experiments were conducted in agreement with the guide for the care and use of laboratory animals of the U.S. National Institutes of Health. Anesthesia and surgical procedures for the creation of CDH and TO have been detailed elsewhere (22, 62). Briefly, rabbit fetuses were randomly operated at day 23 (pseudoglandular stage of lung development) for partial diaphragm excision (DH) or thoracotomy (sham DH), and at day 28 (early saccular stage) for tracheal ligation (TO) or tracheal dissection without ligation (sham TO). Four groups were obtained (n = 8-10 per group): SHAM (fetuses with sham DH and sham TO, used as the control group), DH (fetuses with lung hypoplasia and sham TO), DH+TO (hypoplastic lungs treated with TO), and TO (fetuses with sham DH and TO, reflecting the effect of TO on intact lungs). After term delivery (day 31), lung tissue mechanics were assessed for 30 minutes using the forced oscillation technique as previously described (22, 26). After euthanasia, lungs were removed and weighed for the calculation of the lung-to-body weight ratio. Due to high fetal losses and the small size of neonatal rabbit lungs, left and right lungs from each pup were
differently allocated to postmortem analyses. We previously showed that biological and
histological features of lung hypoplasia were also present in the right lung, i.e. contralateral to
the hernia (62, 63). The right upper lobe was gently expanded with 4% paraformaldehyde
under vacuum and stored for 24 hours at 4°C in fixative solution. Following fixation, the lung
lobe was dehydrated in alcohol, embedded in paraffin, and sliced into 5-µm sections. Separate
sections were randomly used for hematoxylin and eosin staining, immunohistochemistry, and
immunofluorescence. The remaining right and left lungs were immediately shock frozen and
stored at –80°C for subsequent gene and protein assays.

Quantitative real-time PCR

Total RNA from rabbit and human lungs was extracted and quantified by absorbance (63). In
rabbit samples, integrity of extracted RNA was tested by gel electrophoresis (63). RNA
degradation was expected in human specimens because of the unavoidable postmortem
interval. RNA integrity was therefore verified by chip-analysis (Agilent Technologies,
Diegem, Belgium) using the calculation of a RNA Integrity Number (RIN). RIN values above
6 were considered as acceptable (21). Random hexamers for reverse transcription and small
PCR product sizes below 200 base pairs were also used to achieve reliable qPCR
measurements from autopsy specimens (21, 42). Before reverse transcription into cDNA (63),
genomic DNA contamination was excluded by quantitative real-time PCR using total RNA as
a template. SYBR Green quantitative real-time PCR was performed in triplicate as reported
(63) using previously validated primers (63) and newly designed sequences (Table 3).
Negative and positive controls were included to ensure qualitative measurements. Data
transformed following the efficiency-corrected model were normalized against the geometric
mean of validated reference genes (61, 62), which were identified by the genorm software
(ATP5B, RPLP0, and PGK1 in humans; SDHA and TOP1 in rabbits).
Western blot analysis

Protein extraction, gel electrophoresis, transfer and immunoblotting were performed using human and rabbit whole lung homogenates as previously reported (62). After the blocking step, membranes were blotted with one of the following primary antibodies: goat polyclonal anti-phospho-Smad2/3 (p-Smad2/3, Ser 423/425; 1:200; Santa Cruz, Heidelberg, Germany), rabbit monoclonal anti-Smad2/3 (1:100; Cell Signaling Technology, Leiden, The Netherlands), mouse monoclonal anti-ROCK1 (1:100; R&D Systems, Abingdon, UK), goat polyclonal anti-ROCK2 (1:100; R&D Systems), and mouse monoclonal anti-α-smooth muscle actin (α-SMA; 1:1000; Dako, Heverlee, Belgium). For detection of total Smad2/3 in rabbit lungs, a mouse monoclonal antibody was used to avoid masking the region of interest by recognition of rabbit heavy chain IgG (1:200; BD Biosciences, Erembodegem, Belgium).

Blocking buffer and antibody diluent were made of 5% non-fat dry milk in TBS/0.1% Tween-20. Non-fat dry milk was replaced by 5% fish serum (Seablock blocking buffer, Thermo Scientific) for p-Smad2/3 antibody. Membranes were stripped and reprobed with GAPDH (1:5000; Sigma-Aldrich, Bornem, Belgium) as loading control. Signals were quantified by densitometry using the NIH ImageJ gel analysis tool as previously reported (62). For rabbit analyses, four samples per group were included, which were run in two different gels processed in parallel. A standard sample made from lung lysates of three untouched rabbit pups were run in each gel to correct for technical variations.

Immunoassays

Using commercial kits (Quantikine human TGF-β1 and TGF-β2 kits, R&D Systems; human TGF-β3 kit, Aviscera Bioscience, Santa Clara, CA, USA), mature TGF-β peptides could not be detected in undiluted human lung homogenates. As sample concentration may induce loss of mature forms or activation of latent ones, only total (latent plus mature) TGF-β peptides were quantified after acid activation. Additionally, mature TGF-β1 was quantified in rabbit
lung homogenates (Gentaur, Brussels, Belgium). TGF-β levels were normalized to total protein content assessed by BCA assay (Thermo Scientific, Erembodegem, Belgium).

**Histochemical stainings**

As previously detailed (48, 66), mean terminal bronchial density and mean wall transection length were measured on hematoxylin-eosin stained rabbit lung sections. Mean terminal bronchial density reflects the number of terminal bronchioles, which is inversely related to the number of alveoli supplied by each bronchiole. Mean wall transection length is an index of the thickness of alveolar septa. Human lung specimens were not fixed under pressure. Sections were stained with Masson’s trichrome for the assessment of collagen deposition in airway septa (Sigma-Aldrich, Bornem, Belgium).

**Immunohistochemistry**

Avidin-biotin-peroxidase immunohistochemistry was performed exactly as previously described (62) with mouse monoclonal anti-α-smooth muscle actin (α-SMA; 1:100; Dako), mouse monoclonal anti-collagen I (1:500; abcam, Cambridge, UK), or mouse monoclonal anti-collagen III (1:500; abcam) antibodies for rabbit lung sections, and with mouse monoclonal anti-TGF-β1 (1:25; R&D Systems), goat polyclonal anti-TGF-β2 (1:20; R&D Systems), mouse monoclonal anti-TGF-β3 (1:25; R&D Systems), or mouse monoclonal anti-α-SMA (1:1000) antibodies for human lung sections. Primary antibodies were incubated overnight, except for the detection of α-SMA (49). Negative controls were performed by primary antibody omission. Slides were counterstained with hematoxylin before mounting. Color pictures were captured using a light microscope (Zeiss, Oberkochen, Germany) connected to a digital camera. Images were linearly adjusted for brightness.

**Immunofluorescence**

Indirect immunofluorescence detection of p-Smad2/3 was performed on rabbit lung sections. After deparaffinization and rehydration, heat-induced antigen retrieval with citrate buffer was
used, followed by permeabilization for 30 minutes with 0.1% Triton-X 100 in PBS and blocking of non-specific antigenic sites with undiluted fish serum (Thermo Scientific). Sections were then incubated with goat polyclonal anti-p-Smad2/3 (1:50 in 10% fish serum; Santa Cruz) overnight at 4°C. After washing, slides were treated with Alexa 594-conjugated donkey anti-goat antibody (1:300; Life Technologies, Ghent, Belgium) for 1 hour at room temperature. After rinsing, slides were counterstained with DAPI (Sigma-Aldrich) for nuclear staining before mounting. Human lung sections were processed using a simultaneous double-staining protocol for p-Smad2/3 and α-SMA. Adaptation of the aforementioned procedure consisted of simultaneous incubation of primary antibodies overnight (goat polyclonal anti-p-Smad2/3 and mouse monoclonal α-SMA; respectively 1:25 and 1:300 in 10% fish serum), followed by simultaneous treatment with Alexa 594-conjugated donkey anti-goat and Alexa 488-conjugated donkey anti-mouse (1:300; Life Technologies). Double immunofluorescence detection was not done on rabbit lung sections, because bright green autofluorescence was not successfully quenched. Negative controls were performed by primary antibody omission. Afterwards, the slides were analyzed using an Axioskop fluorescence microscope (Zeiss). Digital pictures were captured sequentially at x40 magnification ensuring the same settings for all sections across all study groups. Next, images were merged using Adobe Photoshop software. In all cases, brightness was similarly increased in the red channel, ensuring equal application across the entire image.

Statistical analysis

Data are reported as mean ± SE unless stated otherwise. Depending on normality and equality of variance, two-group comparisons were done using Student’s t-test or the Mann-Whitney U-test. In case of extremely low sample size, meaningful comparisons between means of normally distributed samples could be obtained using Student’s t-test, as previously validated (19). Multiple comparisons were performed with one-way ANOVA or the Kruskal-Wallis
test, followed by Bonferroni’s correction. Correlations were assessed with Pearson’s coefficient (r). Significance was considered at \( P \leq 0.05 \).

RESULTS

Undisturbed TGF-β/Smad2/3 pathway in human fetal lungs with CDH

CDH fetuses and controls with normally developing lungs were compared to test whether the TGF-β signaling pathway was impaired in CDH. Because decent RNA quality was found in fetal lung samples that did not represent all stages of lung development, experiments focused on the post-translational level only. From the canalicular until early alveolar stages, concentrations of the three TGF-β ligands assessed by sandwich ELISA did not vary in control and CDH lungs (Fig. 1A−C). Moreover, CDH did not obviously change TGF-β immunolocalization. In both groups, TGF-β isoforms were expressed in the conducting airway epithelium (Fig.1D, upper row). TGF-β1 and TGF-β3 immunoreactivity predominated in vascular smooth muscle, while TGF-β2 was immunolocalized in arterial adventitia (Fig. 1D, lower row). Connective tissue septa were also immunoreactive for TGF-β2 (Fig. 1E, lower row). Immunolabeling of terminal airways was detected during the canalicular stage for TGF-β2, but persisted until the late saccular stage for TGF-β1 and TGF-β3 (Fig. 1E, upper row). In both groups protein abundance of p-Smad2/3, indicative of signaling activity (5), increased steadily from the early saccular stage, while total Smad2/3 remained stable (Fig. 2A; semi-quantified in Fig. 2B&C). In the CDH group, this resulted in a progressive elevation of the ratio of p-Smad2/3 over total Smad2/3. A similar trend was observed in controls, but failed to reach statistical significance (Fig. 2D). Localization of p-Smad2/3 immunoreactivity was similar in non-parenchymal structures of control and CDH lungs (Fig. 2E, upper and intermediate rows). Because the interstitium of terminal airways was diffusely labeled for p-Smad2/3 (Fig. 2E, lower row), simultaneous immunodetection of α-SMA helped to
distinguish myofibroblasts, a population of α-SMA-positive interstitial cells producing
tropoelastin and crucial for alveolar septation, from lipofibroblasts, a population of α-SMA-
negative cells involved in surfactant synthesis (12). In control and CDH lungs, p-Smad2/3
predominated in α-SMA-negative interstitial cells (Fig. 2E, lower row). Round-shaped
myofibroblasts concentrating in septal protrusions were labeled (Fig. 2E, arrows), while
elongated myofibroblasts lining the primary septa were not (Fig. 2E, arrowheads).

Improved airway morphology and increased lung weight after TO

In agreement with previous reports (48, 66), the surgical creation of diaphragmatic hernia in
fetal rabbits mimicked features of lung hypoplasia, while fetal TO restored lung tissue
morphology (Fig. 3A). However, the present study was based on a 2x2 factorial design in
which lung morphometry has yet to be evaluated. Especially, SHAM fetuses used as controls
were obtained after two consecutive operations. This might decrease the basal degree of lung
expansion due to altered chest compliance and oligohydramnios, and thus induce lung
hypoplasia. Nevertheless, by comparison with SHAM fetuses, DH fetuses still showed
reduced lung-to-body weight ratio (Fig. 3B) and increased mean terminal bronchial density
(Fig. 3C) as well as septal wall thickness (Fig. 3D). As expected (62), TO increased the lung-
to-body weight ratio of DH lungs (Fig. 3B), while morphometric parameters normalized (Fig.
3C&D). Finally, intact lungs subject to TO displayed elevated lung-to-body weight ratio with
preserved airway morphometry. Accordingly, FETO increased the lung-to-body weight ratio
as compared with untreated human CDH lungs (Fig. 3E). Moreover, FETO caused airspace
enlargement even after a short period of occlusion (Fig. 3F). However, expanded lungs
remained hypoplastic with dense parenchyma, poorly formed alveolar septa, and remodeled
vessels. Yet, this could not be confirmed by morphometric analyses, since the absence of
fixation under pressure prevented from rigorous morphometric measurements. These data
collectively indicated the suitability of rabbit and human lung samples for the subsequent evaluation of TGF-β signaling pathway in the setting of stretch-induced lung growth after TO.

**Increased lung expression of TGF-β and ROCK pathways after FETO**

Pulmonary expression and activity of TGF-β signaling components were assessed in the four groups of ventilated rabbit pups. Consistent with elevated TGF-β transcription and signal activity after in vitro mechanical stretch (65), experimental TO increased TGF-β isoform transcripts (Fig. 4A) and mature TGF-β1 peptide levels (Fig. 4B). However, mature TGF-β2 and TGF-β3 levels could not be accurately measured because of a lack of specific ELISA detection kits for the rabbit species. According to human CDH, expression of TGF-β isoforms was not altered in DH lungs as compared with SHAM lungs. Finally, since TGF-β receptor genes were not modulated in either group (Fig. 4A), they were not further evaluated at the post-translational level. As an integrative approach, whole tissue experiments were performed using lungs from CDH newborns with expectant prenatal management or FETO. Analyses included only lungs that were ventilated for a relatively short period (i.e. less than 72 hours after birth). This aimed at reducing confounding factors related to the duration of mechanical ventilation, which may alter TGF-β expression per se (57). Consistent with the rabbit model, FETO upregulated TGF-β1 and TGF-β2 mRNA levels (Fig. 4C). However, total TGF-β peptide levels using ELISA were not significantly changed (Fig. 4D). Because whole lung analyses might not take into account possible variations in tissue distribution, immunohistochemistry was intended to localize TGF-β isoforms in the different lung compartments. Since the chosen antibodies failed to detect corresponding antigens in rabbit lung tissue in spite of various pretreatment regimens, qualitative analysis of immunostained human lungs was informative. In case of short duration of respiratory support, parenchymal immunoreactivity of TGF-β was globally superimposable in FETO and untreated CDH lungs (Fig. 4E). Yet, alveolar macrophages immunostained for the three TGF-β isoforms were
obviously detected in lungs from FETO patients who rapidly deceased after birth (Fig. 4E, insets). In agreement with features of bronchopulmonary dysplasia in premature infants (57), sustained mechanical ventilation in FETO patients was associated with large infiltrates of alveolar macrophages immunostained for TGF-β isoforms and enhanced interstitial immunoreactivity of TGF-β1 (Fig. 4E). Otherwise, non-parenchymal structures were similarly labeled in untreated CDH and FETO lungs (Fig. 4F).

Unexpectedly, increased TGF-β expression after experimental TO did not activate the canonical Smad2/3 signaling (Fig. 5A; semi-quantified in Fig. 5B). Likewise, alveolar septa were similarly labeled for p-Smad2/3 in all rabbit groups. Predominant cytoplasmic localization after TO indicated that p-Smad2/3 probably did not reach target genes (Fig. 5E).

In case of short duration of respiratory assistance, FETO lowered p-Smad2/3 protein expression as compared with untreated CDH (Fig. 5C; semi-quantified in Fig. 5D). This was consistent with the apparent decrease in parenchymal immunoreactivity after FETO, irrespective of the duration of mechanical ventilation (Fig. 5F&G). Finally, non-parenchymal structures were similarly labeled in untreated CDH and FETO lungs (Fig. 5H). A possible role for non-Smad signaling pathways activated by TGF-β was considered to understand the paradox between increased TGF-β and decreased p-Smad2/3 after FETO. The small GTPase RhoA and its major downstream effector Rho-associated kinase (ROCK), which regulate the actin cytoskeleton, participate in mechanotransduction-mediated differentiation of myofibroblasts (13) and alveolar type II epithelial cells (23) during lung development. Because the ROCK pathway has been involved in cytoskeletal rearrangements induced by TGF-β in vitro (29), expression of ROCK1 and ROCK2 isoforms was assessed using immunoblotting. ROCK1 protein expression was higher in TO-exposed rabbit lungs, whereas ROCK2 was unchanged (Fig. 6A; semi-quantified in Fig. 6B). As compared with untreated human CDH lungs, ROCK1 and ROCK2 protein abundance significantly increased in FETO
lungs that underwent a short period of invasive ventilation (Fig. 6C; semi-quantified in Fig. 6D). In addition ROCK1 was negatively correlated with p-Smad2/3 (Fig. 6E).

Increased extracellular matrix synthesis and myofibroblast differentiation after TO

Because the aforementioned results were compatible with enhanced TGF-β signaling after stretching of fetal lungs by TO, pulmonary expression of typical TGF-β downstream targets was evaluated in the rabbit model at transcriptional and post-translational levels. Extracellular matrix synthesis, which increases in vitro under mechanical load (65), is a hallmark of TGF-β activation (46). Herein, fibronectin as well as collagens I and III were preferentially evaluated since they co-localize with TGF-β during lung morphogenesis (25). Genes encoding these molecules were upregulated in TO-exposed rabbit lungs but similarly expressed in DH and SHAM lungs (Fig. 7A). Likewise, collagen immunoreactivity apparently increased in alveolar septa after experimental TO (Fig. 7C), while Masson’s trichrome stain showed subtle deposits of subepithelial collagen fibers in FETO lungs that were shortly ventilated after birth (Fig. 7D, arrows). According to the development of neonatal lung fibrosis after prolonged respiratory support (37), interstitial collagen was particularly abundant in FETO lungs that were sustainably ventilated (Fig. 7D, arrows). Beside its role in extracellular matrix synthesis, TGF-β induces the transdifferentiation of alveolar epithelial type II to type I cells in vitro (67). Therefore, the expression of epithelial-specific markers was assessed in the rabbit model. Surfactant protein transcripts were downregulated in TO-exposed rabbit lungs, while caveolin-1 increased, suggesting a shift towards the alveolar type I cell phenotype (Fig. 7B). Lower caveolin-1 levels were found in DH lungs, which may indicate delayed type I cell maturation. Epithelial markers were not evaluated in human lungs since prenatal steroids were administered in some patients from untreated CDH and FETO groups (Table 2). Extracellular matrix remodeling and changes in surfactant synthesis can be functionally evaluated using lung hysteresivity, a variable coupling dissipative and elastic behaviors measured by the
Forced oscillation technique (22). Using the rabbit model, hysteresivity was lower in the DH+TO group as compared to SHAM (Fig. 7E). Negative associations between hysteresivity and transcripts of fibronectin and collagens suggested that lung tissue heterogeneity owed to changes in the extracellular matrix network (Fig. 7F–G).

Because myofibroblasts are an important source of matrix components during late lung development (32), expression of α-SMA was assessed in rabbit lungs at transcriptional and post-translational levels. Enhanced myofibroblast phenotype after TO was suggested by gene (Fig. 8A) and protein (Fig. 8B; semi-quantified in Fig. 8D) analysis. On the other hand, DH and SHAM rabbit lungs shared comparable expression levels of α-SMA. Transcripts of α-SMA as well as matrix molecules produced by myofibroblasts correlated with mature TGF-β1 levels (Fig. 8E–H). In case of short-term mechanical ventilation, human CDH lungs treated with FETO displayed an apparent increase, yet not significant, in α-SMA protein abundance (Fig. 8C; semi-quantified in Fig. 8D). According to increased septal immunoreactivity of α-SMA after experimental TO (Fig. 8I), elongated myofibroblasts accumulated along terminal airways after FETO by comparison with untreated CDH lungs with similar duration of respiratory assistance. In agreement with increased α-SMA deposits in bronchopulmonary dysplasia (57), abundant clusters of septal myofibroblasts were found in human lungs subject to sustained mechanical ventilation (Fig. 8J).

**DISCUSSION**

In the present study, the TGF-β/Smad2/3 pathway was not altered in human and experimental CDH. Prenatal TO upregulated the TGF-β and ROCK pathways to the detriment of Smad2/3 activation in neonatal rabbit CDH lungs with increased extracellular matrix and alveolar type II cell transdifferentiation, and in human CDH lungs with subtle interstitial deposits of collagen and myofibroblasts after short-term mechanical ventilation. Therefore, these results
strongly suggest prenatal induction of TGF-β signaling by fetal therapy. Features of BPD were obvious in FETO patients who underwent sustained respiratory support. However, extremely low sample size and disparity in duration of ventilatory assistance cannot confirm whether BPD was boosted after prenatal sensitization of fetal lungs by FETO or postnatal ventilator-induced lung injury.

The present study provides an outstanding opportunity to understand CDH pathogenesis and FETO mechanisms. However, small sample sizes, postmortem delays and limited tissue analyses are inevitable constraints of studies involving fetal and neonatal specimens. In particular, the rarity of CDH and FETO-treated patients prevented from a complete pairwise matching regarding postnatal management. The suitability of controls may also be questioned, since terminations of pregnancy were performed for major birth defects in which TGF-β might have been implicated. Finally, autopsy-derived data cannot be extrapolated to survivors in whom TGF-β activity is unknown. Nevertheless, disturbed respiratory function in FETO survivors presupposes abnormal lung architecture (15). Notwithstanding these limitations, the reliability of human findings is reinforced by resemblances with a validated animal model. Because mechanical ventilation was similarly performed in rabbit pups regardless of group affiliation, increased tissue stretch or inflammatory processes due to mechanical ventilation may not explain potential differences between groups. However, the descriptive nature of the current study prevents from establishing robust cause-and-effect relationships between increased TGF-β signaling and extracellular matrix remodeling,

Lung alveolization requires tropoelastin and collagen synthesis by myofibroblasts, which drive secondary septation of saccules and septal elongation (12, 32). Consistent with previous findings (1), the TGF-β/Smad2/3 signaling takes part to this process as indicated by Smad2/3 activation in terminal airways of normal human fetal lungs. However, lung hypoplasia related to human CDH did not involve deficient TGF-β/Smad2/3 signaling in the present study. The
scarcity of previous reports limits comparisons. Reduced expression of TGF-β signaling components was found in a newborn with pulmonary acinar aplasia (11). Also, four infants carrying mutations in latent TGF-β binding protein-4 gene developed multiorgan impairment of elastin assembly with airway underdevelopment and diaphragmatic defects (58). Finally, decreased TGF-β2 immunoreactivity was recently reported during alveolar formation in three CDH newborns (45). However, the intensity of chromogen staining does not necessarily correlate linearly with antigen concentration (55). In the present study, this shortcoming was cautiously bypassed by immunoassays and immunoblottings to complement immunohistochemical data, which yielded consistent results across well-preserved lung samples that were not subject to mechanical ventilation or prenatal steroids. Nevertheless, the present study lacks CDH specimens from the pseudoglandular stage and we cannot therefore preclude a role of TGF-β in airway branching inhibition. Yet, this is unlikely given the persistence of defective branching morphogenesis in nitrofen lungs ex vivo after dampened TGF-β type II receptor (36).

In animal models, the role of TGF-β in CDH pathogenesis is equivocal. In nitrofen-induced CDH lungs TGF-β1 transcription was variable (10, 43), while ovine CDH lungs displayed unchanged TGF-β1 and TGF-β2 gene and protein levels (47). Accordingly, the rabbit CDH model did not disturb TGF-β machinery from transcription to signal transduction. Discrepant results may arise from different animal models, various lung developmental stages at birth, and choice of laboratory methods. Surgical CDH, which results from late insult to developing lungs, is fundamentally different from nitrofen-induced lung hypoplasia, which develops earlier even in the absence of CDH. Moreover, alveolization begins prenatally and extends postnatally in rabbits and humans, is completed in utero in sheep, and begins after birth in rodents. Finally, previous studies have used conventional end-point or competitive PCR, which are currently not the most sensitive and specific methods for gene expression analysis.
Rapid cell proliferation following prenatal TO has been linked to increased growth factor expression (40). However, consistent with intact sheep lungs (64), increases in TGF-β transcripts and peptides did not coincide with the TO-induced proliferation peak in rabbit lungs (18). What therefore are the pulmonary consequences of TGF-β activation after TO? In accordance with previous studies (see Table 4 for literature overview), fetal rabbits after TO displayed transcriptional changes compatible with accelerated alveolar type II cell transdifferentiation. Among factors regulating this process, transcription thyroid factor-1 decreased after TO in nitrofen-induced CDH lungs (9), but increased after TGF-β blockade in lung epithelial cells (38). Consistent with in vitro data (67), these findings infer a role for TGF-β in TO-mediated epithelial transdifferentiation. The timing and duration of TO influence epithelial phenotype (17, 18), raising the concept of temporary TO. Nevertheless, we were unable to study surfactant status after FETO because of administration of prenatal steroids. Coinciding with alveolization, experimental TO modified extracellular matrix homeostasis (see literature overview in Table 4), as denoted by increased elastogenesis (6, 30, 62), myogenic markers (51), and reshaping molecules like tissue inhibitor of matrix metalloproteinase-1 (62) and connective tissue growth factor (39, 53). However, despite improved lung morphology and growth, interstitial changes might be inappropriate for immediate neonatal lung mechanics, as denoted by low hysteresivity in the present study, altered lung compliance after reversible TO in CDH sheep despite normal surfactant status (17), and negligible improvement of neonatal respiratory function after FETO (31). Correlations between changes in TGF-β expression, downstream targets and hysteresivity in the current study do not imply causality, but potentially suggest that upregulated TGF-β after TO might favor extracellular matrix remodeling. This assumption is supported by the observation of pulmonary fibrosis rather than hypoplasia in fetal monkeys subject to TGF-β1 overexpression during the saccular stage (54).
Rhythmic variations in intraluminal fluid volume during the fetal respiratory cycle participate in lung development. As such, cyclic distension of fetal trachea restores optimal alveolization of ovine CDH lungs (41). Inversely, pulmonary vascular resistance and blood flow, which improved after brief TO, were blunted after prolonged static TO (4). Therefore, matrical changes after sustained TO might be attributed to permanent loss of cyclical stretch. Considering the present findings and literature reports (Table 4), hypothetical molecular mechanisms may be proposed. Mechanical stretch after TO induces TGF-β isoform transcription (47, 64) and activates matrix metalloproteinases (20) and thrombospondin-1 (52), which favor mature TGF-β release (33, 47). Besides, ROCK-induced cytoskeleton reorganization is rapidly initiated after TO in the terminal airways (13). Consequently, both signalings contribute to accelerate alveolization owing to rapid upregulation of genes encoding myogenic factors, tropoelastin, and collagens. Recently, higher expression levels of microRNA-200 family members in the tracheal fluid of human CDH fetuses undergoing FETO were interpreted as part of a negative feedback loop on microRNA-200 expression due to stretch-induced increase in TGF-β expression (45). Consistent with this assumption and the inhibition of Smad2/3 activity in vitro by microRNA-200b (45), the present study demonstrates elevated TGF-β transcripts and decreased p-Smad2/3 levels in FETO lungs. Likewise, increased TGF-β expression and absence of Smad2/3 activation were described after experimental TO in rabbits. Because Smad2/3 activity increases as gestation proceeds in normal and hypoplastic human lungs, TO appears to destabilize the crosstalk between Smad2/3 and ROCK pathways induced by TGF-β. This might explain, at least in part, the subtle changes in lung tissue mechanics and extracellular matrix deposition observed at birth in rabbit pups. Half of CDH survivors present BPD (59), resulting from ventilator-induced injury, lung hypoplasia, and pulmonary hypertension. Despite increased lung growth, respiratory
morbidity after FETO is comparable to that of severe CDH with conservative management (15). Dysregulation of myofibroblast differentiation and maintenance is involved in fibrotic lung diseases (37). Myofibroblast deposition in ventilated FETO lungs is indeed coherent with “old” BPD, which developed before the era of modern neonatology in moderately preterm infants subject to high airway pressures and oxygen concentrations (57). Infiltration of inflammatory cells expressing TGF-β in long-term ventilated FETO lungs was consistent with findings in premature infants with chronic lung disease (37, 57). ROCK signaling has not been described in human BPD, but ROCK inhibition experimentally prevented neonatal chronic lung disease (35). Nevertheless, the present findings could not answer the question whether prenatal increase in TGF-β and ROCK pathways after sustained tissue stretch by TO might favor postnatal lung damage due to mechanical ventilation and oxygen exposure.

Survival, neonatal outcomes and long-term morbidity following FETO for severe CDH are under investigation in a multicenter randomized control trial (www.totaltrial.eu), the primary completion date of which has not yet been reached. Despite the descriptive nature of the present study, it appears likely that experimental TO affects extracellular matrix remodeling in fetal rabbit lungs. Awaiting studies in human patients who are adequately matched for postnatal management, evaluation of fetal therapy should include close follow-up of postnatal respiratory function and routine autopsy where appropriate.
ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

A.V., S.H.-B., A.W.F., and J.C.J. designed the study and delineated the hypothesis. A.V., S.H.-B., A.W.F., I.M.R., and J.C.J. carried out animal experiments. A.V., C.V., V.S., A.B., J.M., D.N., M.D., and J.C.J. collected and processed human lung tissue and acquired clinical data. A.V. and I.M.R. performed laboratory assays. A.V., S.H.-B., A.W.F., and J.C.J. analyzed and interpreted the data, and drafted the manuscript. All authors edited the manuscript and approved the final version. A.W.F. and J.C.J. supervised the project.
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44. **Patel N, Moenkemeyer F, Germano S, Cheung MM.** Plasma vascular endothelial growth factor A and placental growth factor: novel biomarkers of pulmonary


50. **Ruano R, Yoshisaki CT, Da Silva MM, Cecon ME, Grasi MS, Tannuri U, Zugaib M.** A randomized controlled trial of fetal endoscopic tracheal occlusion versus postnatal


Figure 1. Expression of transforming growth factor (TGF)-β isoforms in human congenital diaphragmatic hernia (CDH) and control fetal lungs. A–C: Concentrations of total (latent plus mature) TGF-β peptides assessed by ELISA did not change between 22 and 39 weeks of gestation. Slopes between regression lines from control and CDH groups were not different. \( r \), Pearson’s correlation coefficient; NS, not significant. D&E: Immunohistochemical detection of TGF-β isoforms (brown stain) in non-parenchymal structures (D) and in the lung periphery (E). Because TGF-β1 and TGF-β3 co-localized, only representative lung sections immunostained for TGF-β1 were shown. Open and solid arrowheads respectively indicate medial and adventitial layers of pulmonary arteries. Open arrows show connective tissue septa or pleura. Insets are high-magnification views from the same field. Negative controls were obtained by primary antibody omission. Scale bars = 100 µm. a, parenchymal arteries; aw, conducting airways; bv, non-parenchymal blood vessels.

Figure 2. Lung abundance and activity of Smad2/3 in human congenital diaphragmatic hernia (CDH) and control fetuses from midterm onwards. A: Immunoblotting showed an apparent increase in phosphorylated Smad2/3 (p-Smad2/3) in both groups. Vertical white line indicates where the blot was cropped to exclude a band with protein degradation. B&C: Correspondent densitometric analysis using GAPDH as loading control confirmed these observations. D: The ratio of p-Smad2/3 over total Smad2/3 increased overtime, but failed to reach statistical significance in controls. There was, however, no difference between regression lines from CDH and control groups. A.U., arbitrary units; \( r \), Pearson’s correlation coefficient; NS, not significant. E: Similar immunolocalization of p-Smad2/3 in representative lung sections of control and CDH fetuses at saccular and alveolar stages. DAPI was used as nuclear stain (blue signal). p-Smad2/3 detection in conducting airway epithelium intensified with advanced gestation (upper row). The media of large and medium-sized arteries was labeled...
(intermediate row), whereas arterioles and pericytes encircling alveolar vessels were not
(lower row). In the lung periphery, p-Smad2/3 predominated in α-smooth muscle actin (α-
SMA)-negative interstitial cells (lower row). Arrowheads indicate unstained myofibroblasts
along primary septa. Arrows show a positive reaction for p-Smad2/3 in round-shaped
myofibroblasts at the tip of secondary crests. Negative controls were obtained by primary
antibody omission. Scale bars = 100 µm. a, parenchymal artery; aw, conducting airways; bv,
non-parenchymal blood vessel.

Figure 3. Increased lung growth and improved airway morphology after tracheal occlusion.
A: Hematoxylin and eosin staining of lung sections from the rabbit model of congenital
diaphragmatic hernia (CDH) and tracheal occlusion (TO), including SHAM (sham-operated
lungs), DH (hypoplastic lungs with diaphragmatic hernia), DH+TO (DH lungs treated with
tracheal occlusion), and TO (healthy lungs with intact diaphragm treated with tracheal
occlusion) groups. B–D: Lung-to-body weight ratios and airway morphometry in the rabbit
model. *P < 0.05 or ***P < 0.001 vs. DH; †P < 0.05, ††P < 0.01 or †††P < 0.001 vs. SHAM; ‡P
= 0.059 vs. SHAM (n = 8-10 per group). E: Lung-to-body weight ratio values for CDH
patients with expectant prenatal management or fetal endoscopic tracheal occlusion (FETO).
*P < 0.05 vs. expectant CDH. F: FETO lungs showed airway enlargement and remodeled
blood vessels with thick adventitia (blue stain), as indicated by Masson’s trichrome staining.
aw, conducting airways; bv, blood vessels. Scale bars = 100 µm in all pictures.

Figure 4. Postnatal lung expression of transforming growth factor (TGF)-β isoforms in the
rabbit model for congenital diaphragmatic hernia (CDH) and tracheal occlusion (TO) and in
CDH patients with expectant prenatal management or fetal endoscopic TO (FETO). A&B:
Real-time PCR quantification of mRNA levels of genes encoding TGF-β isoforms and
receptors (Tβ-RI, Tβ-RII) as well as mature TGF-β1 levels using ELISA in the rabbit model
(n = 6-10 and n = 3-5 per group, respectively). Bar graphs represent mean values expressed as
a percentage of sham-operated rabbits, which is set to 1. \( ^*P < 0.05 \) or \( ^{**}P < 0.01 \) vs. DH; \( ^*P < 0.05 \) or \( ^{††}P < 0.01 \) vs. SHAM. A.U., arbitrary unit; SHAM, sham-operated lungs; DH, hypoplastic lungs with diaphragmatic hernia; DH+TO, DH lungs treated with tracheal occlusion; TO, healthy lungs with intact diaphragm treated with tracheal occlusion. C&D: Correspondent gene and protein analyses in FETO (\( n = 2 \)) and untreated CDH lungs (\( n = 4 \)) with short-term mechanical ventilation (\( i.e. \) less than 72 hours). \( ^*P < 0.05 \) or \( ^{‡}P = 0.05 \) vs. expectant CDH. E: In untreated CDH and FETO lungs, TGF-β1 and TGF-β3 were immunodetected in the interstitium of terminal airways, and TGF-β2 in connective tissue septa (\textit{brown stain}). Immunostained macrophages were identified in FETO lungs (\textit{high-magnification insets}). Macrophage infiltration and TGF-β1 interstitial staining (\textit{arrows}) were obvious after sustained mechanical ventilation. F: Irrespective of fetal therapy, TGF-β1 and TGF-β3 co-localized in conducting airway epithelium and medial layers of pulmonary arteries (\textit{open arrows}). Only representative lung sections immunostained for TGF-β1 were therefore shown. TGF-β2 was visualized in conducting airway epithelium and adventitial layers of pulmonary arteries (\textit{solid arrows}). Scale bars = 100 µm. aw, conducting airways; bv, non-parenchymal blood vessel; MV, mechanical ventilation.

Figure 5. Postnatal lung activity of Smad2/3 in the rabbit model for congenital diaphragmatic hernia (CDH) and tracheal occlusion (TO) and in CDH patients with expectant prenatal management or fetal endoscopic TO (FETO). A–D: Immunoblotting for phosphorylated Smad2/3 (p-Smad2/3) and total Smad2/3 as well as correspondent densitometric analysis using GAPDH as loading control in rabbit (\( A&B \)) and human (\( C&D \)) lungs. Despite apparent lower levels for absolute and relative p-Smad2/3 in the TO group, differences between rabbit groups were not significant (\( n = 4 \) per group, distributed in two different gels processed in parallel). In case of short-term mechanical ventilation (\( i.e. \) less than 72 hours), FETO (\( n = 2 \)) reduced absolute and relative p-Smad2/3 levels as compared with untreated CDH (\( n = 4 \)).
Protein bands appeared less intense in FETO lungs with sustained mechanical ventilation, but statistical comparisons could not be performed owing to heterogeneity of patient’s characteristics. \(^*P < 0.05\) vs. expectant CDH. A.U., arbitrary unit; SHAM, sham-operated lungs; DH, hypoplastic lungs with diaphragmatic hernia; DH+TO, DH lungs treated with tracheal occlusion; TO, healthy lungs with intact diaphragm treated with tracheal occlusion.

\(E–H\): Immunolocalization of p-Smad2/3 using DAPI as nuclear stain (blue signal) in representative rabbit and human lung sections. In all rabbit groups (\(E\)), the labeling of alveolar walls was predominantly cytoplasmic with a few labeled nuclei (arrows). FETO apparently reduced p-Smad2/3 immunoreactivity of alveolar walls irrespective of the duration of mechanical ventilation (\(F\&G\)). In high-magnification views, which correspond to the magnified area demarcated by hatched lines in upper photomicrographs, accumulation of septal myofibroblasts (α-SMA-positive cells) after FETO was not associated with p-Smad2/3 expression. Finally, untreated CDH and FETO lungs displayed similar labeling of conducting airway epithelium and pulmonary arterial media, whereas small parenchymal arterioles were not immunoreactive (\(H\)). Negative controls were obtained by primary antibody omission.

Scale bars = 100 µm in all pictures. a, parenchymal artery; aw, conducting airways; bv, non-parenchymal blood vessel; MV, mechanical ventilation.

**Figure 6.** Postnatal expression of Rho-associated protein kinase (ROCK) 1 and 2 in the rabbit model of congenital diaphragmatic hernia (CDH) and tracheal occlusion (TO) and in human CDH lungs treated or not by fetal endoscopic TO (FETO). \(A\&C\): Whole lung immunoblotting for Rho-associated protein kinase (ROCK) 1 and 2 in rabbit (\(A\)) and human (\(C\)) fetuses. \(B\&D\): Correspondent densitometry using GAPDH as loading control. ROCK1 levels increased after experimental TO (\(n = 4\) per group, distributed in two different gels processed in parallel). Data are expressed as a percentage of sham-operated rabbits, which is set to 1. \(^*P < 0.05\) vs. DH; \(^{†}P < 0.05\) vs. SHAM (SHAM, sham-operated lungs; DH, hypoplastic lungs with
diaphragmatic hernia; DH+TO, DH lungs treated with tracheal occlusion; TO, healthy lungs with intact diaphragm treated with tracheal occlusion). In case of short-term mechanical ventilation (i.e. less than 72 hours), FETO ($n = 2$) increased ROCK1 and 2 levels as compared with untreated CDH ($n = 4$). Protein bands looked more intense in FETO lungs with sustained respiratory support, but comparisons could not be realized due to heterogeneity of patients’ characteristics. $^*P < 0.05$ or $^{**}P < 0.01$ vs. expectant CDH. $E$: Correlation between protein levels for p-Smad2/3 and ROCK1 in human lung samples. $r$, Pearson’s correlation coefficient; A.U., arbitrary units; MV, mechanical ventilation.

**Figure 7.** Expression of downstream targets of transforming growth factor (TGF)-β in the rabbit model of congenital diaphragmatic hernia (CDH) and tracheal occlusion (TO) and in human CDH lungs treated or not by fetal endoscopic TO (FETO). $A&B$: Relative mRNA levels of collagen I (COL1A1), collagen III (COL3A1), fibronectin (FN), caveolin-1 (CAV1), and surfactant proteins (SPA, SPB, and SPC) in rabbit groups. Data are expressed as a percentage of sham-operated rabbits, which is set to 1. $^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ vs. DH; $^†P < 0.05$, $^{††}P < 0.01$ or $^{†††}P < 0.001$ vs. SHAM ($n = 6-10$ per group; SHAM, sham-operated lungs; DH, hypoplastic lungs with diaphragmatic hernia; DH+TO, DH lungs treated with tracheal occlusion; TO, healthy lungs with intact diaphragm treated with tracheal occlusion). $C$: Alveolar immunolocalization (*brown stain*) of collagens I and III in the rabbit model. Insets are high-magnification views from the same field. $D$: Accumulation of interstitial collagen fibers (*arrows*; Masson’s trichrome staining) was obvious in FETO lungs subject to long-term mechanical ventilation. Slight changes were visualized even in the absence of sustained respiratory assistance. Insets are high-magnification views from the same field. $E–H$: Lung hysteresivity as the ratio of tissue damping and tissue elastance and significant correlations with genes encoding extracellular matrix components in the rabbit
Figure 8. Myofibroblast differentiation after tracheal occlusion (TO) in human and experimental congenital diaphragmatic hernia (CDH). A: α-SMA mRNA levels in rabbit lungs \((n = 6-10\) per group; SHAM, sham-operated lungs; DH, hypoplastic lungs with diaphragmatic hernia; DH+TO, DH lungs treated with tracheal occlusion; TO, healthy lungs with intact diaphragm treated with tracheal occlusion). B&C: Whole lung immunoblotting for α-SMA in the rabbit model and CDH neonates treated or not by fetoscopic endoluminal TO (FETO). D: Densitometry using GAPDH as loading control in rabbit groups \((n = 4\) per group, distributed in two different gels processed in parallel) and human lungs with short-term mechanical ventilation \((n = 2-4)\). ** \(P < 0.01\) vs. DH; † \(P < 0.05\), † † \(P < 0.05\) or † † † \(P < 0.001\) vs. SHAM. E–H: Correlations between mature TGF-β1 peptide and transcripts encoding α-SMA and matrix molecules produced by myofibroblasts in the rabbit model \((r, \text{Pearson’s correlation coefficient})\). I&J: Immunolocalization of α-SMA in the periphery of rabbit and human lungs subject to prenatal tissue stretch. Note the presence of elongated α-SMA deposits in FETO lungs with short-term mechanical ventilation, which strikingly increase after sustained respiratory assistance \((\text{arrows})\). A.U., arbitrary unit; a, small parenchymal artery; MV, mechanical ventilation. Scale bars = 100 µm in all pictures.
Table 1. Clinical characteristics of controls and fetuses with congenital diaphragmatic hernia (CDH)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (n = 16)</th>
<th>CDH (n = 12)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male/female</td>
<td>8/7*</td>
<td>7/5</td>
<td>0.795</td>
</tr>
<tr>
<td>Prenatal steroids</td>
<td>0/16</td>
<td>0/12</td>
<td>N.A.</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>31 ± 4</td>
<td>28 ± 5</td>
<td>0.113</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>1271 (1003−1934)</td>
<td>857 (734−1537)</td>
<td>0.316</td>
</tr>
<tr>
<td>Lung-to-body weight ratio, %</td>
<td>2.1 ± 0.4</td>
<td>0.7 ± 0.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Postmortem delay, h</td>
<td>9 (4−24)</td>
<td>24 (5−24)</td>
<td>0.281</td>
</tr>
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</table>

Data are presented as number, mean ± SD or median (P25−P75). *Sex unknown for one control fetus. N.A., not applicable.
**Table 2.** Clinical characteristics of newborns with isolated left-sided congenital diaphragmatic hernia (CDH) with or without temporary fetoscopic endoluminal tracheal occlusion (FETO).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Expectant management ($n = 6$)</th>
<th>FETO ($n = 5$)</th>
<th>$P$ value</th>
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<tr>
<td>Sex, male/female</td>
<td>4/2</td>
<td>3/2</td>
<td>0.82</td>
</tr>
<tr>
<td>Age at birth, weeks</td>
<td>37 ± 3</td>
<td>34 ± 3</td>
<td>0.15</td>
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<tr>
<td>Body weight, g</td>
<td>2956 (2520–3500)</td>
<td>2660 (1699–2961)</td>
<td>0.32</td>
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<tr>
<td>O/E LHR at diagnosis, %</td>
<td>21.8 ± 5.9</td>
<td>23.5 ± 6.4</td>
<td>0.66</td>
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**Prenatal treatments**

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<th>$P$ value</th>
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<tr>
<td>Steroids</td>
<td>2</td>
<td>3</td>
<td>0.82</td>
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<tr>
<td>FETO, days</td>
<td>N.A.</td>
<td>33.8 ± 14.5</td>
<td>N.A.</td>
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<tr>
<td>O/E LHR after FETO, %</td>
<td>N.A.</td>
<td>35.9 ± 10.8</td>
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**Postnatal treatments**

<table>
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<th>Treatments</th>
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<th>FETO</th>
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<tr>
<td>Ventilation, days</td>
<td>1.5 (1–3)</td>
<td>6 (0–27.5)</td>
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<td>Rescue HFOV</td>
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<tr>
<td>Inhaled nitric oxide</td>
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<tr>
<td>Extracorporeal support</td>
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**Death**

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<td>Age at death, days</td>
<td>1.5 (1–3)</td>
<td>6 (0–30)</td>
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<td>Failure of FETO removal</td>
<td>N.A.</td>
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</tr>
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<td>Air leak</td>
<td>2</td>
<td>0</td>
<td>0.26</td>
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<tr>
<td>Hypoxic respiratory failure</td>
<td>6</td>
<td>2</td>
<td>0.13</td>
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<tr>
<td>Compartment syndrome</td>
<td>0</td>
<td>1</td>
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<table>
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<th>Hypoplastic left heart</th>
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<td>23 (21–24)</td>
<td>24 (10–24)</td>
<td>0.92</td>
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Data are presented as number, mean ± SD or median (P25–P75). The two FETO patients with delay of emergency retrieval of balloon were manually ventilated and died in the delivery room. CMV, conventional mechanical ventilation; HFOV, high-frequency oscillatory ventilation; N.A., not applicable; O/E LHR, observed to expected lung area to head circumference ratio.
Table 3. Newly designed primer sequences used in human and rabbit lungs

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<thead>
<tr>
<th>Gene symbol</th>
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<th>E</th>
<th>R²</th>
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<tr>
<td>COL1A1</td>
<td>Rabbit</td>
<td>F: CTGAGCCAGCAGATTGAGAA   59.27</td>
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<td></td>
<td></td>
<td>R: ACTCTCCGCTCTTCCAGTCA    60.14</td>
<td></td>
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<td>Amplicon size: 109 bp</td>
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<td>COL3A1</td>
<td>Rabbit</td>
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<td>Amplicon size: 57 bp</td>
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<tr>
<td>FN</td>
<td>Rabbit</td>
<td>F: CTGGGGAATGGAAAGGAG      59.46</td>
<td>2.005</td>
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<td>R: AGCAGATGGCACCAGATAC     60.25</td>
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<td>Tm</td>
<td>E</td>
<td>R²</td>
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**F**, forward primer; **R**, reverse primer; **Tm**, melting temperature; **bp**, number of base pairs; **E**, real-time PCR efficiency; **R²**, coefficient of determination; **COL1A1**, collagen I α1; **COL3A1**, collagen III α1; **FN**, fibronectin; **ACTA2**, α-smooth muscle actin; **CAV1**, caveolin-1; **TGFB1**, transforming growth factor-β1; **TGFB2**, transforming growth factor-β2; **TGFB3**, transforming growth factor-β3; **TGFB1**, transforming growth factor-β receptor type I; **TGFB2**, transforming growth factor-β receptor type II.
Table 4. Literature overview about the consequences of prenatal tracheal occlusion on TGF-β signaling, extracellular matrix, and epithelial homeostasis

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<thead>
<tr>
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<th>Effect of experimental TO</th>
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<tr>
<td><strong>TGF-β pathway</strong></td>
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<td>TGF-β isoforms</td>
<td>Increase in CDH and healthy sheep (47, 64)</td>
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<td>Thrombospondin-1</td>
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<td>pSmad2/3</td>
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<td>microRNA-200b</td>
<td>No evidence so far</td>
<td>Increase in lung fluid (45)</td>
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<td>ROCK2</td>
<td>Increase in mouse with oligohydramnios (13)</td>
<td>No evidence so far</td>
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<td><strong>Alveolar epithelium</strong></td>
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<td>Surfactant proteins</td>
<td>Decrease in CDH mouse and sheep (9, 17)</td>
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<td>Decrease in healthy rat (39)</td>
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<td>Type II cells</td>
<td>Decrease in healthy and CDH rabbit (18, 66)</td>
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<td>TTF-1</td>
<td>Decrease in CDH rat (9)</td>
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<td><strong>Extracellular matrix</strong></td>
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<td>α-SMA</td>
<td>Increase in healthy mouse (51)</td>
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<td>Collagens</td>
<td>Increase in healthy sheep and mouse (40, 53)</td>
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<td>Tropoelastin</td>
<td>Increase in CDH sheep and rabbit (6, 62)</td>
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<td>Increase in healthy sheep and mouse (30, 53)</td>
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<td>Fibronectin</td>
<td>Increase in healthy rat and sheep (39, 53)</td>
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<td>TIMPs</td>
<td>Increase in TIMP-1 in CDH rabbit (62)</td>
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<td>CTGF</td>
<td>Increase in healthy rat and sheep (39, 53)</td>
<td>No evidence so far</td>
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</table>

α-SMA, smooth muscle actin; CDH, congenital diaphragmatic hernia; CTGF, connective tissue growth factor; FETO, temporary fetoscopic endoluminal tracheal occlusion; MMP,
matrix metalloproteinase; ROCK, Rho-associated kinase; TGF-β, transforming growth factor-
β; TIMP, tissue inhibitor of matrix metalloproteinase; TO, tracheal occlusion; TTF-1, transcription thyroid factor-1.