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# Follistatin-like 1 expression is decreased in the alveolar epithelium of hypoplastic rat lungs with nitrofen-induced congenital diaphragmatic hernia

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ABSTRACT

*Background/purpose:* Pulmonary hypoplasia (PH), characterized by incomplete alveolar development, remains a major therapeutic challenge associated with congenital diaphragmatic hernia (CDH). Follistatin-like 1 (Fstl1) is a crucial regulator of alveolar formation and maturation, which is strongly expressed in distal airway epithelium. *Fstl1*-deficient mice exhibit reduced airspaces, impaired alveolar epithelial cell differentiation, and insufficient production of surfactant proteins similar to PH in human CDH. We hypothesized that pulmonary Fstl1 expression is decreased during alveolarization in the nitrofen-induced CDH model.

*Methods:* Timed-pregnant rats received nitrofen or vehicle on gestational day 9 (D9). Fetal lungs were harvested on D18 and D21 and divided into control −/nitrofen-exposed specimens. Alveolarization was assessed using morphometric analysis techniques. Pulmonary gene expression of *Fstl1* was determined by qRT-PCR. Immunofluorescence-double-staining for Fstl1 and alveolar epithelial marker surfactant protein C (SP-C) was performed to evaluate protein expression/localization.

*Results:* Radial alveolar count was significantly reduced in hypoplastic lungs of nitrofen-exposed fetuses with significant down regulation of *Fstl1* mRNA expression on D18 and D21 compared to controls. Confocal-laser-scanning-microscopy revealed strikingly diminished Fstl1 immunofluorescence and SP-C expression in distal alveolar epithelium of nitrofen-exposed fetuses with CDH-associated PH on D18 and D21 compared to controls.

*Conclusions:* Decreased expression of Fstl1 in alveolar epithelium may disrupt alveolarization and pulmonary surfactant production, thus contributing to the development of PH in the nitrofen-induced CDH model.

*Level of evidence:* 2b (Centre for Evidence-Based Medicine, Oxford).



Congenital diaphragmatic hernia (CDH) is a relatively common condition, with a reported incidence of 2.3 cases for 10,000 births [1]. Despite significant advances in postnatal resuscitation and modern lung-protective strategies, CDH remains one of the major therapeutic challenges in modern neonatal intensive care [2,3]. The high mortality and long-term morbidity rates in patients born with CDH are mainly attributable to the severity of pulmonary hypoplasia (PH) and persistent pulmonary hypertension [4,5]. Hypoplastic lungs are characterized by immaturity and smaller size with a decreased number of terminal airways, thickened alveolar walls, increased interstitial tissue, diminished alveolar airspaces and reduced gas-exchange surface area [6].

Experimental animal models of CDH have a long history and are frequently used to investigate the pathogenesis of CDH-associated anomalies in fetal lungs [7]. The teratogenic nitrofen CDH model is widely used as timing of the diaphragmatic insult and PH are similar to the human situation [8]. When nitrofen is administered to pregnant rats on gestational day 9, approximately 70% of the offspring develop CDH and 100% show PH [9].

Formation of primordial alveolar air sacs and maturation of distal airspaces are fundamental developmental processes during fetal lung development that require the expression of multiple regulatory factors, which in turn stimulate alveolar growth [10]. Decreased alveolarization and reduced surfactant phospholipids synthesis have been observed in the nitrofen model, suggesting that these factors may contribute to the development of PH in CDH [5,11,12]. Although the pathophysiological mechanisms of PH in CDH have been extensively studied, the exact molecular basis of the underlying structural abnormalities in nitrofen-induced PH remains unclear.

Follistatin-like 1 (Fstl1) is a crucial regulator of alveolar formation and maturation, which is strongly expressed in distal airway epithelium [13–15]. *Fstl1*-deficient mice exhibit reduced airspaces, impaired alveolar epithelial cell differentiation and insufficient production of

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surfactant proteins similar to PH in human CDH [16]. The aim of this study was to investigate the hypothesis that pulmonary Fstl1 expression is decreased during alveolarization in the nitrofen-induced CDH model.

## 1. Material and methods

### 1.1. Animals, drugs and experimental design

Pathogen-free Sprague–Dawley rats® (Harlan Laboratories, Shardlow, UK) were mated overnight and females were checked daily for spermatozoids in their vaginal smear. The day of plugging was considered as proof of pregnancy and defined as embryonic day 0.5 (D0.5). Timed-pregnant animals were randomly divided into two experimental groups: "Nitrofen" and "Control". On D9, dams were briefly anesthetized with 2% volatile isoflurane (Piramal Healthcare Ltd., Morpeth, UK) and either 100 mg of nitrofen (WAKO Chemicals GmbH, Neuss, Germany), dissolved in 1 ml of olive oil, or vehicle alone was administered *via* oral-gastric lavage. On the selected time-points D18 and D21, animals were anesthetized and their fetuses were delivered *via* caesarean section. After laparotomy, fetal diaphragms were inspected under a Leica S8AP0 stereomicroscope (Leica Microsystems AG, Heerbrugg, Switzerland) for CDH. Whole lungs of nitrofen-exposed fetuses with a diaphragmatic defect ($n = 12$ per time-point) and controls ($n = 12$ per time-point) were dissected under sterile conditions *via* thoracotomy and stored either in a TRIzol® reagent (Invitrogen, Carlsbad, USA) for total RNA isolation or fixed in 10% paraformaldehyde (PFA) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) for morphological analysis or immunofluorescence double staining. In total, 48 fetal animals from 8 different dams were used in this study.

All animal procedures were carried out according to the current guidelines for management and welfare of laboratory animals. The experimental protocol was approved by the local research ethics committee (REC668b) and the Department of Health and Children (Ref. B100/4378) under the Cruelty to Animals Act, 1876 (as amended by European Communities Regulations 2002 and 2005).

### 1.2. Assessment of fetal lung morphometry and alveolarization

Two independent investigators unaware of the experimental group performed fetal lung morphometry, which was objectively assessed by determining the radial alveolar count on hematoxylin- and eosin-stained (Sigma Aldrich, Saint Louis, USA) sections. Fifty randomly selected, non-overlapping fields from serial sections were investigated under a Leica DC300F digital camera (Leica Microsystems AG, Heerbrugg, Switzerland). For each field, the number of alveoli was counted visually and radial alveolar count was performed by identifying respiratory bronchioles, as previously described [17]. Briefly, the number of distal air sacs that were transacted by a line drawn from a terminal respiratory bronchiole to the nearest pleural surface was counted. No counts were made if the respiratory bronchiole was nearer to the edge of the slide than to the nearest connective tissue septum. All images were analyzed with ImageJ 1.47a (National Institute of Health, Bethesda, USA), a public domain, Java™-based image processing and analysis software program.

### 1.3. Total RNA isolation and synthesis of complementary DNA

Total RNA was isolated from fetal lungs with the acid guanidinium thiocyanate-phenol-chloroform extraction method using a TRIzol® reagent according to the manufacturer's protocol. Concentration and purity of total RNA was determined with a NanoDrop ND-1000 UV–Vis® Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA).

Reverse transcription of total RNA was carried out at 85 °C for 3 min (denaturation), at 44 °C for 60 min (annealing) and at 92 °C for 10 min (reverse transcriptase inactivation) using a Transcript High Fidelity cDNA Synthesis Kit® (Roche Diagnostics, Grenzach-Whylen, Germany) according to the manufacturer's instruction. The resulting complementary DNA was stored at 4 °C until further use.

### 1.4. Quantitative real-time polymerase chain reaction

The synthesized complementary DNA was used for quantitative real-time polymerase chain reaction using a LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Gene-specific primer sequences are listed in Table 1. After an initialization phase at 95 °C for 5 min, 55 amplification cycles were carried out. Each cycle included an initial denaturation step at 95 °C for 10 s, an annealing step at 60 °C for 15 s and an elongation step at 72 °C for 10 s. The final elongate temperature was 65 °C for 1 min. Relative mRNA expression levels of *Fstl1* were measured with a Light Cycler® 480 instrument (Roche Diagnostics, West Sussex, UK) and gene levels were normalized to the housekeeping gene *β-actin*. All experiments were run in duplicate for each sample and primer pair.

### 1.5. Immunofluorescence double staining and confocal laser scanning microscopy

Following overnight fixation in 10% PFA, fetal lungs were paraffin-embedded, transversely sectioned at a thickness of 5 μm and mounted on polylysine-coated slides (VWR International, Leuven, Belgium). Tissue sections were deparaffinized with xylene and rehydrated through ethanol and distilled water. To improve cell permeabilization, sections were incubated with phosphate-buffered saline (PBS) containing 1.0% Triton X-100 (Sigma Aldrich Ltd., Arklow, Ireland) for 20 min at room temperature. Sections were then washed in PBS + 0.05% Tween (Sigma Aldrich, Saint Louis, USA) and blocked with 3% bovine serum albumin (Sigma Aldrich, Saint Louis, USA) for 30 min to avoid non-specific absorption of immunoglobulin. The blocking solution was rinsed off and sections were incubated with polyclonal goat and rabbit antibodies against Fstl1 (sc-22650, 1:100) and SP-C (sc-13979, 1:100) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) overnight at 4 °C. On the next day, sections were washed in PBS + 0.05% Tween and incubated with corresponding secondary donkey anti-goat and donkey anti-rabbit antibodies: Alexa Fluor® 555 (A21432, 1:250) and Alexa Fluor® 647 (A150067, 1:250) (Abcam plc, Cambridge, UK) for 1 h at room temperature. After another washing step in PBS + 0.05% Tween, sections were counterstained with a DAPI (10,236,276,001, 1:1000) (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min to visualize double-stranded DNA, washed again and mounted with glass coverslips using Sigma Mounting Medium (Sigma-Aldrich, St. Louis, USA). All sections were scanned with a ZEISS LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) and independently evaluated by two investigators.

### 1.6. Statistical analysis

All numerical data is presented as means ± standard error of the mean. Differences between the two experimental groups were tested using an unpaired Student's *t* test when the data had normal

**Table 1**
Gene-specific primer sequences for quantitative real-time polymerase chain reaction.

| Gene | Sequence (5'-3') | Product size (bp) |
|---|---|---|
| ***Fstl1*** | | 110 |
| Forward | GAG GAA ATA GGG GGA GCT TG | |
| Reverse | | |
| ***β-actin*** | CTG TGG GGA TCA AAA GGA GA | 124 |
| Forward | TTG CTG ACA GGA TGC AGA AG | |
| Reverse | TAG AGC CAC CAA TCC ACA *CA* | |