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ORIGINAL ARTICLE

Expression of dispatched RND transporter family member 1 is decreased in the diaphragmatic and pulmonary mesenchyme of nitrofen-induced congenital diaphragmatic hernia

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Abstract

Purpose Congenital diaphragmatic hernia (CDH) and associated pulmonary hypoplasia (PH) are thought to be caused by a malformation of the diaphragmatic and pulmonary mesenchyme. Dispatched RND transporter family member 1 (Disp-1) encodes a transmembrane protein that regulates the release of cholesterol and palmitoyl, which is critical for normal diaphragmatic and airway development. Disp-1 is strongly expressed in mesenchymal compartments of fetal diaphragms and lungs. Recently, *Disp-1* mutations have been identified in patients with CDH. We hypothesized that diaphragmatic and pulmonary Disp-1 expression is decreased in the nitrofen-induced CDH model.

Methods Time-mated rats received nitrofen or vehicle on gestational day 9 (D9). Fetal diaphragms and lungs were microdissected on selected endpoints D13, D15 and D18; and divided into control and nitrofen-exposed specimens ($n=12$ per sample, time-point and experimental group). Diaphragmatic and pulmonary *Disp-1* expression was evaluated by qRT-PCR. Immunofluorescence double staining for Disp-1 was combined with diaphragmatic and pulmonary mesenchymal markers Wt-1 and Sox-9 to localize protein expression in fetal diaphragms and lungs.

Results Relative mRNA levels of *Disp-1* were significantly decreased in pleuroperitoneal folds/primordial lungs on D13 (0.18 ± 0.08 vs. 0.46 ± 0.41; $p<0.05$/1.06 ± 0.27 vs. 1.34 ± 0.79; $p<0.05$), developing diaphragms/lungs on D15 (0.18 ± 0.06 vs. 0.44 ± 0.23; $p<0.05$/0.73 ± 0.36 vs. 1.16 ± 0.27; $p<0.05$) and fully muscularized diaphragms/differentiated lungs on D18 (0.22 ± 0.18 vs. 0.32 ± 0.23; $p<0.05$/0.56 ± 0.16 vs. 0.77 ± 0.14; $p<0.05$) of nitrofen-exposed fetuses compared to controls. Confocal laser scanning microscopy demonstrated markedly diminished Disp-1 immunofluorescence predominately in the diaphragmatic and pulmonary mesenchyme of nitrofen-exposed fetuses on D13, D15 and D18, associated with a clear reduction of proliferating mesenchymal cells.

Conclusions Decreased Disp-1 expression during diaphragmatic development and lung branching morphogenesis may interrupt mesenchymal cell proliferation, thus leading to diaphragmatic defects and PH in the nitrofen-induced CDH model.

Keywords Disp-1 · Diaphragm · Lung · Congenital diaphragmatic hernia · Pulmonary hypoplasia · Nitrofen

Introduction

Congenital diaphragmatic hernia (CDH) is a relatively common and life-threatening birth defect, with incidence rates ranging between 1.9 and 2.3 per 10,000 newborns in the United States and Europe [1, 2]. Despite significant advance in postnatal resuscitation and lung-protective treatment strategies [3], neonatal mortality and long-term morbidity from CDH continue to be high with an overall survival rate of approximately 70% [4].

Recent studies on the embryological origin of CDH suggested that the defect may arise from a malformation of the so-called pleuroperitoneal folds (PPFs) [5, 6], allowing

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intrathoracic herniation of abdominal organs and thereby disturbing normal lung and diaphragm development. There is also strong evidence that diaphragmatic and pulmonary morphogenesis requires the structural integrity of its underlying mesenchymal tissue, and developmental mutations that inhibit normal mesenchymal formation have been shown to result in CDH and pulmonary hypoplasia (PH) [7, 8]. Using an experimental CDH model, Clugston et al. [9] have demonstrated that the primary mechanism of malformed PPFs and associated PH is caused by the decreased proliferation of mesenchymal cells. However, the exact molecular mechanisms that regulate mesenchymal cell proliferation in fetal diaphragms and lungs remain unknown.

The toxicologically induced nitrofen model of CDH is a frequently used animal model and has provided new insights into the complex pathogenesis of this birth defect [10]. Administration of the herbicide nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl ether) to pregnant rats during midgestation has been shown to result in a high rate of CDH and bilateral PH in their offspring, both remarkably similar to the actual human situation [11, 12].

Dispatched RND transporter family member 1 (Disp-1) encodes a transmembrane protein that regulates release of cholesterol and palmitoyl, which is known to be critical for normal diaphragmatic and respiratory development [13, 14]. Disp-1 was recently found to be strongly expressed in the mesenchymal compartments of fetal diaphragms and lungs [13]. Additionally, Kentarci et al. [15] have identified *Disp-1* mutations in CDH patients, highlighting its important role for diaphragmatic and associated airway formation.

We, therefore, designed this study to investigate the hypothesis that diaphragmatic and pulmonary Disp-1 expression is decreased in the nitrofen-induced CDH model.

Materials and methods

Animals, drugs and experimental design

Following acclimatization, pathogen-free Sprague–Dawley rats® (Harlan Laboratories, Shardlow, UK) were naturally mated overnight, separated and checked for plugging. The presence of spermatozoids in the vaginal smear was considered as proof of pregnancy and termed as embryonic day 0.5 (D0.5) of gestation. Pregnant animals were then randomly divided into two experimental groups ("Nitrofen" and "Control"). On D9, dams were 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 intragastrically. All fetuses were delivered via caesarean section under anesthesia on selected endpoints D13, D15 and D18, and euthanatized by decapitation. Whole fetuses from D13 and D15 animals were fixed in 10% paraformaldehyde (PFA) (Santa Cruz Biotechnology Inc, Heidelberg, Germany) overnight, whereas D18 fetuses underwent laparotomy and inspection for CDH using a Leica S8AP0 stereomicroscope (Leica Microsystems AG, Heerbrugg, Switzerland) under sterile conditions. Microdissected diaphragms and lungs from nitrofen-exposed animals with a diaphragmatic defect and controls were either stored in TRIzol® reagent (Invitrogen, Carlsbad, USA) at − 20 °C or also fixed in 10% PFA overnight. In total, 72 fetal diaphragms and 72 fetal lungs were used for this study ($n = 12$ per time-point and experimental group, respectively).

Total RNA isolation

After fixation in 10% PFA, D13 and D15 fetuses were paraffin-embedded, transversely sectioned at a thickness of 10 µm and mounted on PEN membrane glass slides® (MDS Analytical Technologies, Sunnyvale, USA) to obtain total RNA from PPFs, primordial lungs and developing diaphragms. All appropriate sections were deparaffinized with xylene, rehydrated through ethanol and distilled water, stained with hematoxylin and dehydrated. D13 PPFs and primordial lungs, as well as developing D15 diaphragms were dissected from nine consecutive sections by laser capture microdissection (Arcturus XT® Instrument, MDS Analytical Technologies, Sunnyvale, USA). Following dissection, total RNA was isolated using a High Pure FFPE RNA Micro Kit® (Roche Diagnostics, West Sussex, UK) according to the manufacturer's protocol. After thawing and homogenization of the fully muscularized diaphragms (D18) and fetal lungs (D15 and D18), total RNA was extracted from the TRIzol® suspension using the acid guanidinium thiocyanate–phenol–chloroform extraction method. RNA quantification was performed with a NanoDrop ND-1000 UV–Vis® Spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, USA).

Complementary DNA synthesis and quantitative real-time polymerase chain reaction

Reverse transcription of 1 µg 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 protocol. The resulting single-stranded cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) using a LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Gene-specific primer pairs are listed in Table 1. After an initialization phase at 95 °C for 5 min, 55 amplification cycles were carried out under the following conditions: Each cycle included

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

Gene	Sequence (5′–3′)	Product size (base pairs)
Disp-1		183
Forward	AGA CTG AGG GCC TGT AGA GA	
Reverse	TAG GGT TGG TAG TGC TCG TG	
β-actin		108
Forward	TTG CTG ACA GGA TGC AGA AG	
Reverse	TAG AGC CAC CAA TCC ACA CA	

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 *Disp-1* were measured with a Light Cycler® 480 instrument (Roche Diagnostics, West Sussex, UK) and normalized against the housekeeping gene *β-actin*. All experiments were run in duplicate for each sample and primer pair.

Histological examination, immunofluorescence double staining and confocal laser scanning microscopy

Following fixation in 10% PFA, whole fetuses from D13 and D15 animals as well as microdissected diaphragms and lungs from D18 fetuses were paraffin-embedded, transversely sectioned at a thickness of 5 µm and mounted on polylysine-coated slides (VWR International, Leuven, Belgium). Adequate tissue sections were deparaffinized with xylene and rehydrated through ethanol and distilled water. Conventional hematoxylin and eosin staining (Sigma Aldrich, Saint Louis, USA) was used to investigate the diaphragmatic and pulmonary histology. All remaining sections were incubated with phosphate-buffered saline (PBS) containing 1.0% Triton X-100 (Sigma Aldrich Ltd, Arklow, Ireland) at room temperature for 20 min to improve cell permeabilization. Sections were then washed in PBS + 0.05% Tween (Sigma Aldrich, Saint Louis, USA) and subsequently 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 affinity-purified primary antibodies against Disp-1 (rabbit polyclonal, ab124192, 1:100) (Abcam plc, Cambridge, UK) and Wt-1 (mouse polyclonal, sc-7385; 1:100) or Sox-9 (goat polyclonal, sc-17341, 1:100) (Santa Cruz Biotechnology Inc, Heidelberg, Germany) at 4 °C overnight. On the next day, sections were washed in PBS + 0.05% Tween and incubated with corresponding secondary antibodies (donkey anti-rabbit Alexa 647-A150067, 1:250; donkey anti-mouse Alexa 488-A150109, 1:250 and donkey anti-goat Alexa 555-A21432, 1:250) (Abcam plc, Cambridge, UK) at room temperature for 1 h. After another washing step in PBS + 0.05% Tween, sections were counterstained with a DAPI antibody (10236276001, 1:1000) (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min, washed again, mounted and coverslipped using Sigma Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA). For analysis, all sections were scanned with a ZEISS LSM 700 confocal microscope (Carl Zeiss Micro-Imaging GmbH, Jena, Germany) and evaluated by two independent investigators.

Table 2 Relative mRNA expression levels of *Disp1* in PPFs and primordial lungs (D13), developing diaphragms and lungs (D15) and fully muscularized diaphragms and differentiated lungs (D18)

Endpoints	*Disp1*	
	Control	Nitrofen
Diaphragms		
D13	0.46 ± 0.41	0.18 ± 0.08*
D15	0.44 ± 0.23	0.18 ± 0.06*
D18	0.32 ± 0.23	0.22 ± 0.18*
Lungs		
D13	1.34 ± 0.79	1.06 ± 0.27*
D15	1.16 ± 0.27	0.73 ± 0.36*
D18	0.77 ± 0.14	0.56 ± 0.16*

*$p < 0.05$ vs. control

Statistical analysis

All numerical data are 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 distribution or a Mann–Whitney *U* test when the data deviated from normal distribution. Statistical significance was accepted at *p* values of less than 0.05.

Results

Relative mRNA expression of Disp-1 in fetal diaphragms and lungs

Following qRT-PCR, *Disp-1* gene expression was significantly decreased in PPFs and primordial lungs on D13, in developing diaphragms and lungs on D15, as well as in fully muscularized diaphragms and differentiated lungs on D18 of nitrofen-exposed fetuses compared to controls (Table 2).

Histological analysis and evaluation of mesenchymal Disp-1 immunofluorescence in fetal diaphragms and lungs

On D13, PPFs in the control group appeared as triangular-shaped structures protruding out from the lateral body wall, whereas nitrofen-exposed fetuses had severely abnormal PPFs that were characterized by an absence of the dorsally projecting point of the triangular PPF. In addition, hematoxylin and eosin staining demonstrated that developing diaphragms on D15 and also fully muscularized diaphragms on D18 of nitrofen-exposed fetuses were markedly hypogenic compared to controls.

Immunofluorescence staining for Disp-1 was combined with the mesenchymal markers Wt-1 and Sox-9, respectively; to localize Disp-1 protein expression in fetal diaphragms and lungs. Confocal laser scanning microscopy revealed a clear co-expression of Disp-1 with Wt-1 and Sox-9 in PPFs and primordial lungs on D13, in developing diaphragms and lungs on D15 as well as in fully muscularized diaphragms and differentiated lungs on D18. Furthermore, this study confirmed the qRT-PCR results by showing a strikingly diminished Disp-1 immunofluorescence mainly in the diaphragmatic mesenchyme of nitrofen-exposed PPFs and primordial lungs on D13 as well as the diaphragmatic and pulmonary mesenchyme of nitrofen-exposed fetuses on D15 and D18 compared to controls (Fig. 1). This finding was

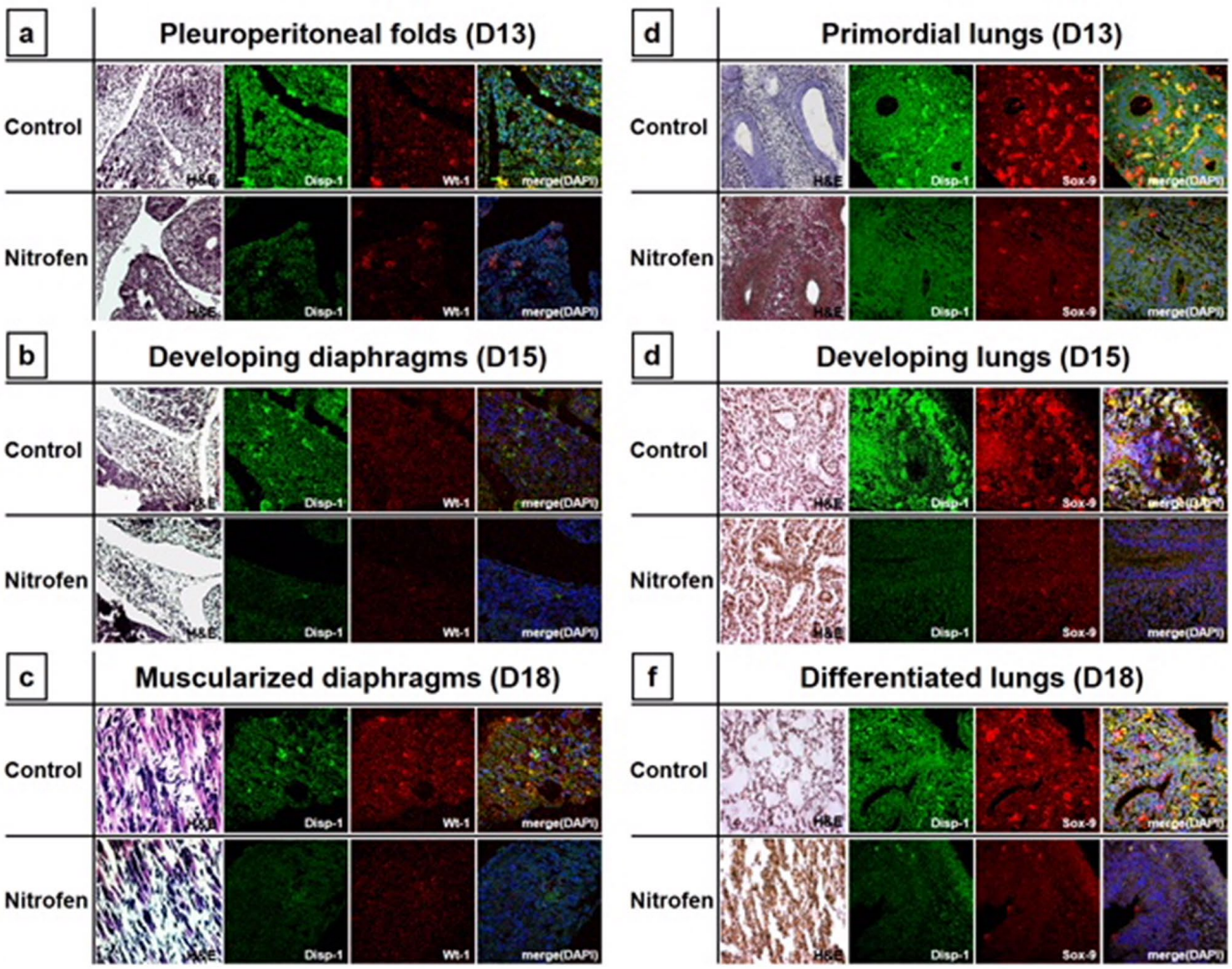

Fig. 1 Hematoxylin and eosin (H&E) staining (left image of each time-point and experimental group, respectively) and immunofluorescence evaluation of Disp-1 (green staining) and Wt-1 or Sox-9 (red staining) combined with DAPI (blue staining). Confocal laser scanning microscopy showed co-expression of Disp-1 and Wt-1 mainly in diaphragmatic mesenchymal cells and revealed strikingly diminished Disp-1 immunofluorescence in **a** PPFs (D13), **b** developing diaphragms (D15) and **c** fully muscularized diaphragms (D18) of nitrofen-exposed CDH fetuses compared to controls. Disp-1 expression in **d** primordial lungs (D13), **e** developing lungs (D15) and **f** differentiated lungs (D18) was co-located with Sox-9 primarily in pulmonary mesenchymal cells and revealed markedly diminished Disp-1 immunofluorescence in nitrofen-exposed CDH fetuses compared to controls

also associated with a reduced proliferation of mesenchymal cells in nitrofen-exposed PPFs and fetal CDH diaphragms and lungs on D13, D15 and D18 compared to controls.

Discussion

The development of fetal diaphragms and lungs is a complex process, which is temporally and spatially orchestrated by a plethora of intricate gene and tissue interactions. Fetal lungs require specific cross-talk interactions between epithelium and mesenchyme, especially during airway branching morphogenesis [16, 17]. It has recently been shown that disrupted epithelial–mesenchymal interactions during lung branching morphogenesis contribute to the development of PH in rodents with nitrofen-induced CDH [18]. Although the embryological origins of the diaphragmatic defect and associated PH have widely been studied, the exact processes involved in the regulation of diaphragmatic mesenchymal formation and abnormal airway branching in CDH remains not fully understood.

CDH is assumed to originate from embryological defects in the mesenchymal compartments of PPFs, which mainly comprise muscle connective tissue (MCT) [19, 20]. The fetal diaphragm, therefore, depends on the correct formation of its MCT and underlying extracellular matrix [5, 6]. There is also strong evidence that developmental mutations that inhibit normal development of the diaphragmatic mesenchyme can lead to CDH [7, 8]. Previous studies from our laboratory have demonstrated that the expression of various regulatory factors for the normal development of diaphragmatic mesenchyme are decreased in nitrofen-induced CDH [21, 22].

The sonic hedgehog (Shh) pathway is known to play an important role during fetal lung and diaphragm morphogenesis [23]. Mutation of the transcription factor Gli, which is a downstream target of Shh signaling, were found to contribute to diaphragmatic defects in CDH and associated PH [24, 25]. Kinesin family member 7 (Kif7), a further essential component of the Shh cascade, has recently been identified to play a crucial role in diaphragmatic and pulmonary development by controlling the proliferation of mesenchymal cells [26, 27]. In addition, it has been suggested that decreased mesenchymal Kif7 expression in PPFs may lead to defective mesenchymal proliferation, ultimately resulting in diaphragmatic defects and hypoplastic lungs [21]. Disp-1, which is strongly expressed in mesenchymal compartments of fetal diaphragms and lungs [13], actually represents another vital component of the Shh signaling pathway. It encodes a transmembrane protein that controls release of cholesterol and palmitoyl, which are required for normal diaphragmatic and airway formation [13, 14], and recently, *Disp-1* mutations have been identified in patients with CDH [15].

In the present study, we demonstrated for the first time that diaphragmatic and pulmonary *Disp-1* gene expression is significantly decreased in PPFs and primordial lungs on D13, in developing diaphragms and lungs on D15 as well as in fully muscularized diaphragms and differentiated lungs on D18 of nitrofen-exposed rat fetuses compared to their control littermates. Additionally, immunofluorescence staining for Disp-1 showed a co-localization with Wt-1 and Sox-9, which are crucial transcription factors during diaphragmatic and lung development and both strongly expressed by mesenchymal [28–31]. Confocal laser scanning microscopy revealed a markedly diminished Disp-1 expression in the diaphragmatic mesenchyme of nitrofen-exposed PPFs and primordial lungs as well as diaphragmatic and pulmonary mesenchyme of CDH fetuses on D15 and D18. These results confirmed that the quantitative decrease in diaphragmatic and lung *Disp-1* mRNA transcripts was also translated to the respective protein level. Furthermore, we identified a clear reduction of proliferating mesenchymal cells in nitrofen-exposed PPFs and fetal CDH diaphragms and lungs on D13, D15 and D18, which again may indicate a disrupted development of its underlying extracellular matrix. These findings provide new insights into the pathomechanisms underlying CDH and associated PH. However, it remains unclear to what extent Disp-1 actually contributes to the observed mesenchymal malformations in diaphragms and lungs of nitrofen-exposed fetuses. Further investigations of the Shh signaling pathway may be helpful to elucidate the pathogenesis of abnormal diaphragm formation and impaired airway branching in CDH.

In conclusion, our results suggest that decreased Disp-1 expression during diaphragmatic development and lung branching morphogenesis may interrupt mesenchymal cell proliferation, thus leading to diaphragmatic defects and PH in the nitrofen-induced CDH model.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal procedures in this study were carried out according to the current guidelines for management and welfare of laboratory animals and the experimental protocol was fully 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).

Informed consent For this type of study informed consent was not required.

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