

Investigation of molecular mechanisms of diaphragmatic defects in the nitrofen-induced rat model of congenital diaphragmatic hernia

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**Investigation of molecular mechanisms of
diaphragmatic defects in the nitrofen-induced rat
model of congenital diaphragmatic hernia**

A thesis submitted by

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for the degree of
Master of Science by research
October 2015

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Professor Ray Stallings

Candidate thesis declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Master of Science by research, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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A list of abbreviations

CDH	Congenital diaphragmatic hernia
CTR1	Cu-uptake transporter 1
Cu	Copper
DAPI	4',6-diamidino-2-phenylindole
ECM	Extracellular matrix
FREM1	FRAS1-related extracellular matrix 1
FREM2	FRAS1-related extracellular matrix 2
Kif7	Kinesin family member 7
Lox	Lysyl oxidase
MPC	Muscle precursor cell
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PPF	Pleura-peritoneal fold
qRT-PCR	Quantitative real-time polymerase chain reaction
Shh	<i>Sonic hedgehog</i>

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Summary

Developmental mutations that inhibit normal formation of extracellular matrix (ECM) in fetal diaphragms have been identified in congenital diaphragmatic hernia (CDH). *FRAS1*-related extracellular matrix 1 (*FREM1*) plays a critical role in the development of the fetal diaphragm. It has been demonstrated that a deficiency of *FREM1* can lead to CDH both in humans and mice. Furthermore, *FREM1*-deficient fetuses exhibit a decreased level of mesenchymal cell proliferation in their developing diaphragms. *FRAS1* and *FRAS1*-related extracellular matrix 2 (*FREM2*), which encode important ECM proteins, are secreted by mesenchymal cells during diaphragmatic development. The *FRAS1/FREM2* gene unit has been shown to form a ternary complex with *FREM1*, which plays a crucial role during formation of human and rodent diaphragms.

The first objective of this work was to investigate the morphological changes in the normal and abnormal diaphragm in the nitrofen rat model. The pleura-peritoneal folds (PPFs) in the control group were triangular-shaped structures protruding out from the lateral body wall, whereas nitrofen-exposed fetuses had an abnormal PPF structure, characterized by the absence of the dorsally projecting point of the triangular PPF.

The second objectives was to investigate the expression levels and distribution of *FREM1*, *FRAS1* and *FREM2* genes and their proteins in the normal and abnormal diaphragm. In nitrofen-exposed fetuses, relative mRNA expression of *FREM1*, *FRAS1* and *FREM2* were significantly reduced in developing diaphragms compared to controls. Confocal laser scanning microscopy revealed markedly diminished *FREM1*, *FRAS1* and *FREM2*

immunofluorescence in diaphragmatic mesenchyme, which was associated with reduced proliferation of mesenchymal cells in nitrofen-exposed fetuses compared to controls.

Our results suggest that decreased mesenchymal expression of *FREM1*, *FRAS1* and *FREM2* in the nitrofen-induced CDH model may cause failure of the *FREM1/FRAS1/FREM2* gene complex, disturbing the formation of diaphragmatic ECM and thus contributing to the development of diaphragmatic defects in CDH.

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Chapter 1

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Introduction

1.1 Congenital diaphragmatic hernia

1.1.1 Definition

Congenital diaphragmatic hernia (CDH) is a congenital malformation characterized by the absence of the diaphragm. The most common type of CDH is Bochdalek hernia also known as a postero-lateral diaphragmatic hernia and accounts for over 95% cases. CDH causes intrathoracic herniation of abdominal organs and thus disturbing normal lung development. The size of the defect varies from small to very large, involving most of the hemidiaphragm. The most common abnormalities associated with CDH are cardiovascular anomalies, followed by skeletal, central nervous system, genitourinary, gastrointestinal, craniofacial, abdominal wall defects, and chromosomal and syndromic defects. Despite significant advances in neonatal resuscitation and intensive care, newborn infants with CDH continue to have high mortality. Infants with associated anomalies have much lower survival rates than those with isolated CDH. The high mortality and morbidity in CDH is mainly attributed to pulmonary hypoplasia and persistent pulmonary hypertension.

1.1.2 Historical background

Diaphragmatic hernia was first described in 1575 by the French surgeon Ambroise Pare, who reported two autopsy cases of traumatic diaphragmatic hernia [1]. The first description of congenital diaphragmatic hernia was reported by the French physician Lazarus Riverius as an incidental finding at post-mortem examination in a 24-year-old man [2]. Sir Charles Holt in 1701 first reported a case of CDH in a child [3]. In his 1769 monograph, the Italian anatomist Giovanni Battista Morgagni differentiated various types of CDHs, including the anterior defect that bears his name [4]. Sir Astley Paston Cooper published in 1827 the first comprehensive report on

classification, symptoms and pathology of CDH [5]. In 1834, the French physician Rene Laennec demonstrated that the diagnosis of CDH could easily be made by chest auscultation and also suggested that laparotomy might be correct approach for hernia repair [6]. Henry Bowditch collected the first cohort series of patients with CDH in 1847 at the Massachusetts General Hospital in Boston, emphasizing the clinical criteria for making the diagnosis [7].

In 1848, the Czech anatomist Vincent Alexander Bochdalek accurately described a posterolateral defect in the diaphragm. This hernia carries his name today [8]. He speculated that hernia resulted from an intrauterine rupture of the membrane in the lumbocostal triangle, but his understanding of the embryology of CDH was not supported by modern embryological studies.

In 1888, the Swedish surgeon Naumann made the first recorded attempt at laparotomy for reduction of CDH, but was unsuccessful [9]. The American surgeon O'Dwyer in 1890 performed the first, but unsuccessful repair in a 3-year-old infant with CDH via an abdominal approach [10]. The German surgeon Aue reported the first successful repair in an adult in 1901 [11] and Heidenhain successfully repaired a CDH in a 9-year-old boy in 1905 [12].

In 1925 the American surgeon Hedblom reviewed 44 CDH cases and showed that 75% of untreated cases with CDH died in the newborn period, suggesting that an earlier intervention might improve survival [13]. In 1929 Bettman and Hess presented the youngest patient with CDH, who had successfully been operated on aged 3.5 months [14]. In the same year, Greenwald and Steiner reviewed symptoms of infants and children with CDH, concluding that this condition might not be as infrequent as it was generally believed [15]. Successful surgical repair of CDH were rare until 1940 when Ladd and Gross reported 9 of 16 patients surviving surgery, including the

youngest being 40 hours old [16]. In 1946 Robert Gross performed the first successful repair in a neonate with CDH less than 24 hours after birth [17]. In 1950, Koop and Johnson proposed a transthoracic approach as a means of closing the CDH under more direct vision [18]. As the surgical expertise improved further, several innovative techniques were introduced to address large diaphragmatic defects, including the use of muscle flaps and prosthetic patches [19,20].

The first important paper describing pulmonary hypoplasia (PH) as the underlying pathophysiological abnormality in CDH was by Campanale and Rowland [21] in 1953. Areechon and Reid [22] in 1963 first recognized the relationship between PH and the high mortality in infants with CDH. In 1971, Murdock et al. [23] and Rowe and Uribe [24] reported that the major pathophysiological abnormality in CDH is persistent pulmonary hypertension (PPH). Barlett et al. [25] in 1976 reported the first survivors of PPH treated with extracorporeal membrane oxygenation (ECMO). Since then there have been a number of reports of use of ECMO in patients with CDH complicated by PPH [26]. There are many other innovations which have been employed in the treatment of CDH patients who develop respiratory distress soon after birth and these include high frequency ventilation [27], delay of surgery [28] and foregoing chest tubes [29].

Historically, PH in CDH was believed to be the result of compression of the lungs by the herniating intrathoracic abdominal organs. However, our understanding of abnormal pulmonary development in relation to CDH has significantly improved because of data obtained in the nitrofen model of CDH. Using this model, it was demonstrated that pulmonary development is already affected prior to development of the diaphragmatic hernia, implicating that the lungs are primarily disturbed in their development, before mechanical compression can happen [30,31]. The dual-hit hypothesis, which explains PH in CDH as the result of 2 developmental insults, was led

to be postulated by this [32]. This hypothesis proposes that early retardation of lung development affecting both lungs occurs before closure of the diaphragm, probably attributed to nitrofen. The second insult affects only the ipsilateral lung and is the result of interference of fetal breathing movements of this lung caused by the herniation of abdominal organs into the thorax [31].

Although none of the surgical, transgenic, and toxicologic models for CDH and its associated PH and PPH have succeeded in completely explaining the developmental and biological basis of this congenital anomaly, many new insights into the pathogenesis have been deduced from the results of studies using these models so far.

While the historical perspective on CDH stretching over 400 years has seen many innovative and elegant procedures for the management of this condition, the mortality in CDH is still exceptionally high. The challenge for the future is to develop novel therapeutic approaches to improve the survival in neonates with the anomaly.

1.1.3 Classification

CDHs can be classified to several subtypes depending on the location of the defect. The most common type of CDH is a posterolateral Bochdalek-type (~90-95%) with the majority occurring on the left-sided (80%) (**Figure 1**): less frequently on the right-sided (19%) (**Figure 2**) or bilateral (1%) [33]. The other types are an anterior Morgagni-type (~2%) and a central, septum transversum-type (very rare). Approximately 80% of the hernias are left-sided, 19% right-sided and 1% bilateral [34]. However, close scrutiny of various diaphragmatic defects has demonstrated wide phenotypic variations in shape, size and location, suggesting that a clear distinction among the different types can be problematic [35].

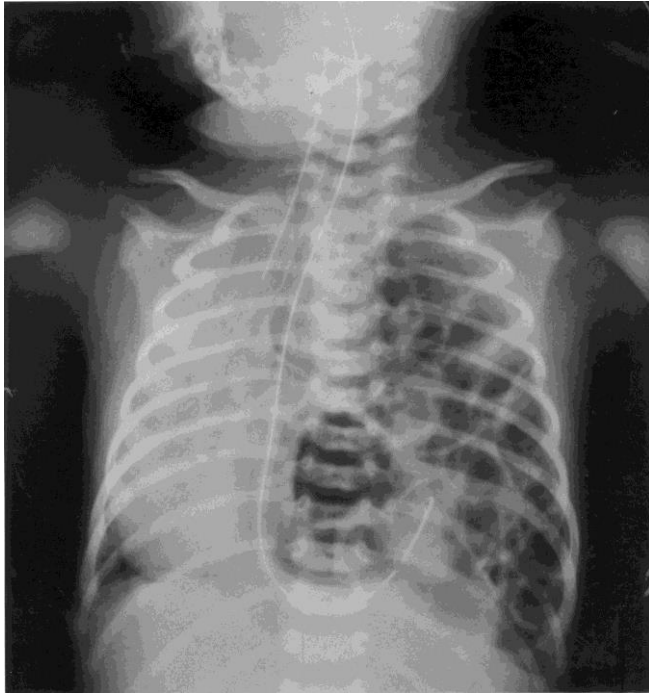


Figure 1 X-ray of the chest in a newborn showing large left-sided Congenital Diaphragmatic Hernia with Mediastinal shift to the right

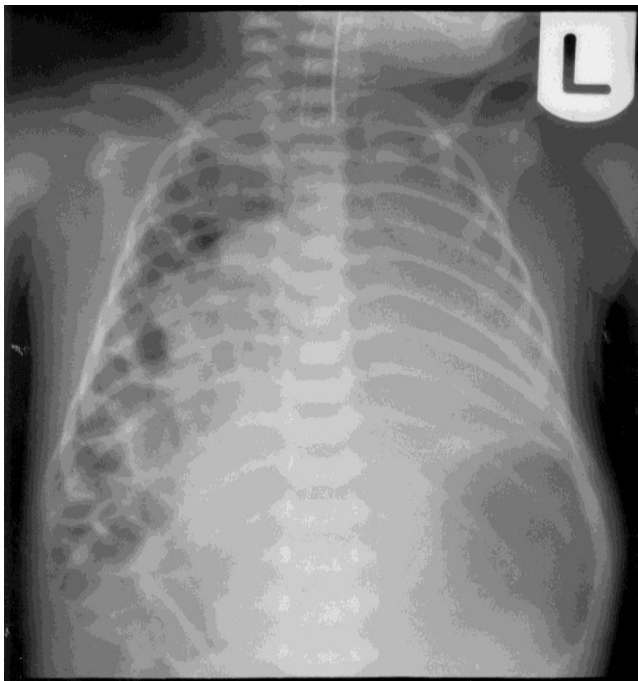


Figure 2 X-ray of the chest and upper abdomen showing large right-sided diaphragmatic hernia

1.1.4 Epidemiology

CDH is a relatively common birth defect currently affecting 1 in 2000 to 4000 newborns, which accounts for approximately 8% of all major congenital malformation, an incidence similar to cystic fibrosis [36,37]. Prevalence rates ranging between 2.4 and 3.8 cases per 10,000 total births have recently been found by population-based studies from the USA and Western Australia [38-40]. European registry-based studies reported similar prevalence rates, 2.3 per 10,000 live births [41]. However, the true incidence of CDH is considerably higher than seen in the neonatal surgical practice. The reason is because the incidence of CDH in stillborns and abortions seem to be less well documented [42]. Approximately one third of CDH infants with the associated fetal congenital anomalies can result in stillborn [43,44]. Therefore, hidden mortality (stillbirths, abortions) continues to underscore true outcomes with recent population based surveys reporting a persistently high mortality when all antenatal and perinatal cases of CDH are included [45].

1.2 Normal embryological development of the diaphragm

Formation of the primordial diaphragm is essential for normal diaphragmatic development. The diaphragm develops from multiple embryonic sources [46]. The muscle and its associated connective tissue and central tendon develop from three sources: the pleura-peritoneal folds (PPFs), septum transversum and the somites [47]. The PPFs is a wedge-shaped structure that tapers medially from the lateral cervical wall to the esophageal mesentery and fuses ventrally with the septum transversum [48]. The PPF is of particular

importance in diaphragm embryogenesis, because it is the target for migrating diaphragmatic muscle precursor cells (MPCs) [49]. MPCs migrate to the PPF to form the muscular components of the primordial diaphragm, and then, they expand to form the fetal diaphragm [50].

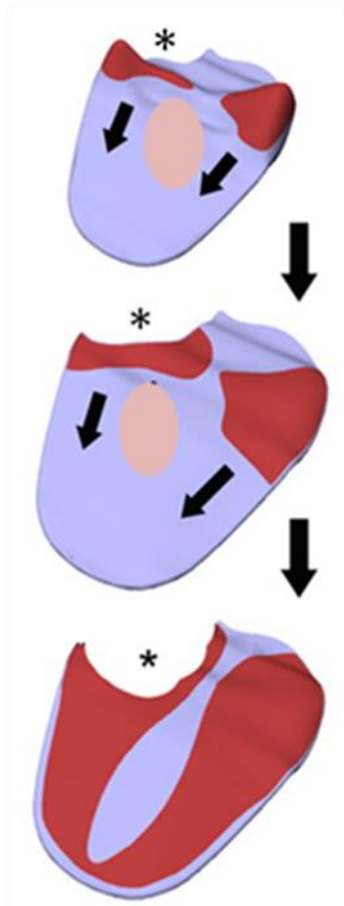
1.3 Pathogenetical and –physiological aspects of congenital diaphragmatic hernia

The embryogenesis of the PPF has become a focus for elucidating the pathogenesis of CDH [48]. The PPF has been shown to be abnormal in the nitrofen-induced CDH rodent model, eventually leading to the formation of diaphragmatic defects (**Figure 3**) [50,51].

Furthermore, recent evidence shows that diaphragmatic anomalies arise from a defect in the amuscular mesenchymal component, which mainly comprises of fibrous connective tissue [47,48]. It has been demonstrated that diaphragmatic morphogenesis requires the structural integrity of connective tissue, and developmental mutations that inhibit the formation of extracellular matrix (ECM) result in CDH [52,53].



● MPCs



■ MPCs

Figure 3 Muscle precursor cells (MPCs) migrate to the PPF to form the muscular components of the primordial diaphragm, and then, they expand to form the fetal diaphragm [50].

1.4 Animal models of congenital diaphragmatic

Hernia

Much of the current understanding of the pathophysiology of CDH originates from experimental animal studies. Three types of CDH animal models have been developed over the years: surgically created model, genetic model and nitrofen model [54,55].

1.4.1 Surgical models

Surgical models are based on a surgical intervention making a diaphragmatic defect in fetal rabbits and sheep. This CDH models are mainly suitable to investigate interventional strategies in CDH. Examples of investigated interventions are administration of corticosteroids, in utero repair of the diaphragmatic defect and fetal tracheal occlusion or a combination of the two [56,57]. However, since this animal CDH model is surgically created during fetal life, it is unable to yield any insights into the embryogenesis of CDH.

1.4.2 Genetic models

Several mutant phenotypes (knock out model for *Wt1* [46], *Shh* [58], *Gli2/Gli3* [59], *Slit3* [60], *Fog2* [61], *Gata4/Gata6* [62,63], *COUP-TFII* [64], *Pdgfra* [65] and *RARs* [66]) have been described in which diaphragmatic hernia may feature but they have a range of often far more frequent and significant malformations that make them phenotypically different from human CDH. Only a mutation in *FOG2* has so far been demonstrated in a single patient with nonsyndromic CDH [65].

1.4.3 Nitrofen model

Toxicological studies revealed that while the herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) was relatively nontoxic to adult rats, it produced a number of developmental abnormalities of lung, heart, skeletal, and diaphragmatic tissues in fetuses exposed prenatally [67,68]. Subsequent studies demonstrated that the maldevelopment of the diaphragm was prominent if nitrofen was administered as a single dose to pregnant rats or mice between days 8 and 11 after conception. The range and location of diaphragmatic defects produced by nitrofen in the perinatal rat are remarkably similar to those observed in the human infant with CDH. When nitrofen is administered to pregnant rats on gestational day 9, approximately 70% of the offspring show CDH and 100% of the offspring have pulmonary hypoplasia (PH) [69,70]. In addition, previous work by Migliazza et al. [71-73] has demonstrated that there is striking similarity in the incidence and nature of associated cardiovascular and skeletal defects in human CDH patients and nitrofen-exposed rats. Thus in view of the similarities between the pathologies observed in the nitrofen induced CDH rat model and infants with CDH, nitrofen model has been used widely as an experimental model to investigate the pathogenesis of CDH [70]. Furthermore, while there is no clear evidence suggesting that nitrofen-like compounds or any other environmental factors are involved in the etiology of CDH in humans, an understanding of the mechanisms by which nitrofen is exerting its actions (e.g., affecting some aspects of endogenous hormone or transcription factor function) may lead to insights into the etiology of the disorder. During the past several years our research group has been investigating structural and molecular basis of CDH using this model [74].

1.5 Malformation of the diaphragmatic mesenchyme

The development of fetal diaphragms is a complex process, temporally and spatially orchestrated by multiple gene and tissue interactions. Normal diaphragmatic morphogenesis requires muscle progenitors to migrate from the somites to the developing diaphragm and that the muscle connective tissue forms with proper structural integrity [47,75]. Developmental mutations that inhibit the formation of ECM have been shown to result in CDH [47].

Copper (Cu) is an important element during diaphragm morphogenesis by participating in cross-linking of collagen and elastin fibers [52,53]. It has been reported that Cu is required for the activity of many enzymes that play an important role during development of the fetal diaphragm [76]. Cu transport is strictly regulated by two membrane proteins: Cu-uptake transporter 1 (CTR1) and the Cu-efflux pump ATP7A [77]. It has been demonstrated that elevated CTR1 and ATP7A levels result in an increased intracellular Cu uptake, which upregulates the activity of Cu-dependent enzymes and thus contributes to crosslink of ECM [78]. Animals lacking Cu-dependent enzymes exhibit abnormal connective tissue with diaphragmatic defects [52,53].

Lysyl oxidase (Lox) is an extracellular Cu -dependent enzyme that catalyzes the cross-linking of ECM proteins [52,79]. These cross-links are essential for the tensile strength of collagens and the rubber-like properties of elastin, both abundant ECM proteins that are necessary for the structural integrity and function of connective tissue in developing diaphragms [53,80]. The expression of Lox has been demonstrated to be markedly increased in fibrotic tissues, including fetal diaphragms [53,81]. Thus, Lox appears to be critical for the integrity of newly developing collagen and elastic fibers by contributing to the structural stability of connective tissue formation during diaphragmatic development [47,52,53]. It has been reported

that *lox* knockout mice exhibit abnormal connective tissue with diaphragmatic defects [52,53]. Inactivation of the mouse *lox* gene has recently been shown to lead to perinatal death caused by diaphragmatic rupture [82]. This diaphragmatic rupture has been identified in D18.5 *lox* knockout mice at the site of the collagen-rich diaphragmatic central tendon, which allows abdominal contents to enter the thoracic cavity and thus disturbing formation of the respiratory system [52]. These findings in the *lox* knockout mice strongly suggest that a reduction in Lox activity may have a critical effect on the pathogenesis of CDH [52,53].

We recently demonstrated that diaphragmatic expression of Lox is decreased in the nitrofen-induced CDH model [83]. In addition, we also recently reported that the Lox deficiency and our findings of decreased expression CTR1 and ATP7A in the developing diaphragm indicate that the loss of connective tissue integrity may be a cause of CDH [84]. Disruption of the Cu-deficient signalling pathway may impair cross-linking of elastin and collagen which is essential for the proper structural integrity of connective tissue and its tensile strength.

The origin of CDH is assumed to lie in a malformation of the amuscular primordial diaphragm [75]. It is known that fetal diaphragmatic development requires the structural integrity of its underlying mesenchymal tissue [47]. Developmental mutations that inhibit the formation of normal diaphragmatic mesenchyme have been shown to cause CDH [47,75]. Kinesin family member 7 (*Kif7*), an essential component of the *Sonic hedgehog* (*Shh*) signaling cascade, has recently been identified to play a crucial role in diaphragmatic development by controlling the proliferation of mesenchymal cells [85-87]. In addition, the loss of *Kif7* has been reported to result in diaphragmatic defects [85]. Furthermore, it has been demonstrated that the diaphragmatic expression of *Kif7* was

decreased in the nitrofen-induced CDH model, thus suggesting that decreased *Kif7* expression during diaphragmatic development may interfere with mesenchymal cell proliferation, leading to defective PPFs, and resulting in diaphragmatic defects in this model [88].

FRAS1-related extracellular matrix 1 (FREM1) encodes an extracellular matrix protein. Recently a case of a female child with an isolated left-sided posterolateral CDH was reported that carried a *FREM1* deletion [89,90]. In addition, it has been reported that *FREM1* is expressed in the anterior portion of the developing diaphragm and that *FREM1* deficiency causes anterior CDH in mice [89,91]. Moreover, *FREM1*-deficient fetuses exhibit a decreased level of mesenchymal cell proliferation in their developing diaphragms [89]. These results confirm that FREM1 plays a critical role in the development of the fetal diaphragm and that FREM1 deficiency can cause CDH in both humans and mice. Moreover, FREM1 is known to form a ternary complex in the basement membrane with FRAS1 and FRAS1-related extracellular matrix 2 (FREM2) (**Figure 4**) [92]. Recently, FRAS1 and FREM2 gene have been identified as the causative genes in human Fraser syndrome which is infrequently associated with CDH [93,94]. *FRAS1* and *FREM2* also encode essential ECM proteins, which are both expressed in the fetal diaphragm [89]. Although CDH has not yet been reported in *FRAS1*- or *FREM2*-deficient mice, failure to form a FREM1/FRAS1/FREM2 complex may predispose to the development of diaphragmatic defects.

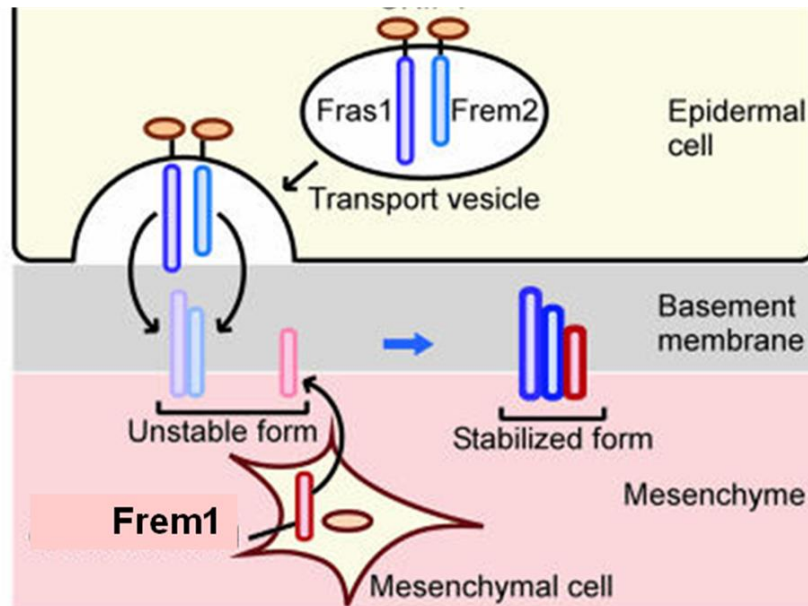


Figure 4 A model for the reciprocal stabilization of the Fras1, Frem2, and Frem1 through complex formation at the epidermal basement membrane [92].

1.6 Aims and objectives

In this project we aimed to investigate the molecular mechanism underlying the development of CDH by examining the expression of key candidate genes at a critical time in the development of diaphragm.

The first objective of this work was to investigate the morphological changes in the normal and abnormal diaphragm in the nitrofen rat model.

The second objectives was to investigate the expression levels and distribution of candidate genes and proteins associated with CDH (i.e. FREM1, FRAS1 and FREM2) in the normal and abnormal diaphragm.

Chapter 2

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Material and Methods

2.1 Animal model and experimental design

2.1.1 Animal protocol

After obtaining ethical approval (Ref. REC668b) from the local research ethics committee, pathogen-free adult Sprague-Dawley® rats (Harlan Laboratories, Sharnlow, UK) were kept in a well-controlled environment (50-55% humidity, 19-21°C, 12-h light period, food and water *ad libitum*), and males and females were mated overnight. Day 0 of Pregnancy will be taken as the day on which a vaginal plug was found and confirmed by the presence of spermatozoa in a vaginal swab. On day 9 of gestation, the rat was carefully restrained with its neck extended, and a stainless steel straight cannulae (75mm x 16G) was passed gently down the esophagus to the stomach and 100mg of Nitrofen (2,4-dichlorophenyl-p-nitrophenylether, WAKO Chemicals GmbH, Neuss, Germany) dissolved in 1 ml of olive oil was administered. This dose of Nitrofen has been established to give the highest proportion of embryos in a litter with CDH. After this procedure, although no adverse effects had been described on the dam rat after gastric administration of Nitrofen, the rat was closely monitored twice daily for signs of distress. Each rat in the control group received equivalent volume of olive oil without nitrofen via the same route and technique.

Nitrofen is the common name of the compound 2,4-dichlorophenyl-p-nitrophenyl ether (International Chemical safety Cards #0929) that was previously used as a contact herbicide agent. Field handlers of the herbicide were subject to inhalation and dermal contact exposure during application procedures, causing irritation to the respiratory tract and dermatitis. For these reasons Nitrofen is no longer used as an herbicide. No short-term adverse effects have been described if intragastrically given to a rat; however, it is highly teratogenic if given to a pregnant rat and this is the effect we are

expecting to achieve in this research. Nitrofen was stored well closed, separated from food and properly labelled. Protective clothes, gloves and face mask was used when handling Nitrofen avoiding all contact or spilling.

Harvesting of Study fetuses was carried out under terminal anaesthesia. On gestational day 13, 15 and 18 each rat from the control and experimental group was carefully restrained and anaesthesia was induced with Isoflurane 2% in order to sedate it. After that, an intracardiac injection of Pentobarbitol Sodium 100mg was given in order to humanely kill the dam and its foetuses. Following, under aseptic conditions, the embryos were recovered by caesarean section with microsurgical technique and fixed according to each limb of the study.

The Department of Health and Children approved the protocol of these animal experiments (ref. B100/4378) under the Cruelty to Animals Act, 1876; as amended by European Communities Regulations 2002 and 2005.

2.1.2 Preparation for diaphragm samples

Fetuses were harvested by cesarean section on selected time-points D13, D15 and D18 and were inspected for diaphragmatic defects (**Figure 5**). All diaphragmatic samples ($n=72$) were dissected under a stereomicroscope (Leica Microsystems AG, Heerbrugg, Switzerland) and divided in control and nitrofen-exposed specimens ($n=12$ per time-point and experimental group). Samples were either stored in TRIzol[®] reagent (Invitrogen, Carlsbad, USA) for subsequent RNA isolation, or fixed in 10% paraformaldehyde (PFA) (Santa Cruz Biotechnology Inc, Heidelberg, Germany) for histologic processing.

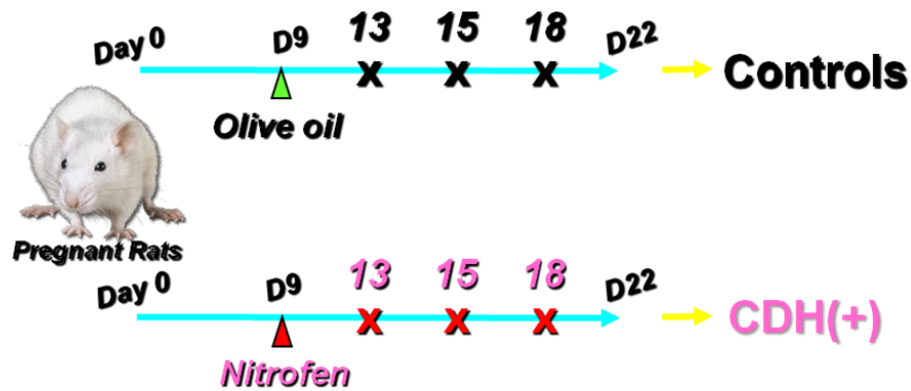


Figure 5 Experimental design

2.2 Total RNA isolation and complementary DNA synthesis

In order to obtain total RNA from PPFs from D13 fetuses and developing diaphragms from D15 fetuses, paraffin-embedded whole animals were transversely sectioned at a thickness of 10 μm and mounted on PEN membrane glass slides[®] (MDS Analytical Technologies, Sunnyvale, USA). After deparaffinization, rehydration, hematoxylin staining, and dehydration, primordial diaphragms were dissected by laser capture microdissection (Arcturus XT[®] Instrument, MDS Analytical Technologies, Sunnyvale, USA). Total RNA was extracted using a High Pure FFPE RNA Micro Kit[®] (Roche Diagnostics, West Sussex, UK) according to the manufacturer's protocol. Total RNA from fetal diaphragm (D18) samples were extracted with the acid guanidinium thiocyanate-phenol-chloroform extraction method using a TRIzol[®] reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Total RNA quantification was performed spectrophotometrically (NanoDrop ND-1000 UV-Vis[®] Spectrophotometer, Wilmington, USA), and RNA

solution was stored at -20°C. Synthesis of cDNA was performed using a Transcript High Fidelity cDNA Synthesis Kit[®] (Roche Diagnostics, Grenzach-Whylen, Germany) according to the manufacturer's protocol. All cDNA samples were stored at 4°C until further use.

2.3 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction was performed using a LightCycler[®] 480 SYBR Green I Master Mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Gene-specific primer pairs used in this study 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 sec, an annealing step at 60°C for 15 sec and an elongation step at 72°C for 10 sec. The final elongate temperature was 65°C for 1 min. Relative mRNA expression levels of *FREM1*, *FRAS1* and *FREM2* 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 duplicated for each sample and primer pair.

Table 1 Primer sequences for quantitative real-time polymerase chain reaction

Gene	Sequence (5'-3')	Product size (bp)
<i>FREM1</i>		
Forward	CAC AGC AGC CAT CAC AAG TT	125
Reverse	AGC ATG GAC CCT TGG ATC AA	
<i>FRAS1</i>		
Forward	GCT TCA GAA ACC TCC ACA GC	179
Reverse	TCA GGC CAT CTG TGA CTG AG	
<i>FREM2</i>		
Forward	ACC CAG GAT GAA GTG GAC AG	180
Reverse	GGA CAC GCC CTT ACT TAC CA	
<i>β-actin</i>		
Forward	TTG CTG ACA GGA TGC AGA AG	108
Reverse	TAG AGC CAC CAA TCC ACA CA	

2.4 Histological examination, immunofluorescence staining and confocal laser scanning microscopy

Following fixation in 10% PFA, whole D13 and D15 fetuses as well as D18 trunks were paraffin-embedded, transversely sectioned at a thickness of 5 µm, and mounted on polylysine-coated slides (VWR International, Leuven, Belgium). Resulting 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 histology.

All sections for immunofluorescence staining were incubated with phosphate-buffered saline (PBS) containing 1.0% Triton X-100 (Sigma Aldrich Ltd, Arklow, Ireland) for 20 min at room temperature 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 either against FREM1 (rabbit polyclonal, sc-98447; 1:100), FRAS1 (goat polyclonal, sc-79244, 1:100), FREM2 (mouse polyclonal, sc-376555, 1:100) and GATA4 (rabbit polyclonal, sc-9053; 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 antibodies (donkey anti-rabbit Alexa 647-A150067, 1:250, donkey anti-goat Alexa 555-A21432, 1:250 and donkey anti-mouse Alexa 488-A150109, 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 antibody (10236276001, 1:1,000) (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min, washed again, and mounted with glass coverslips using Sigma Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA).

All sections were scanned with a ZEISS LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) and independently evaluated by two investigators.

2.5 Statistical analysis

All numerical data are presented as means \pm standard error of the mean. Differences between two 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.

Chapter 3

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Results

3.1 The morphological changes in the normal and abnormal PPF in the nitrofen rat model

The PPF in controls was triangular-shaped structures protruding out from the lateral body wall (**Figure 6**). Consistent with previous reports, nitrofen-exposed fetuses had an abnormal PPF structure, characterized by the absence of the dorsally projecting point of the triangular PPF (**Figure 7**).

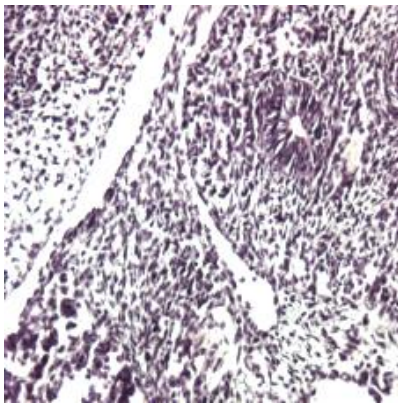


Figure 6 Hematoxylin- and eosin-staining in developing fetal diaphragms on D13. PPFs in the control group were triangular-shaped structures protruding out from the lateral body wall.

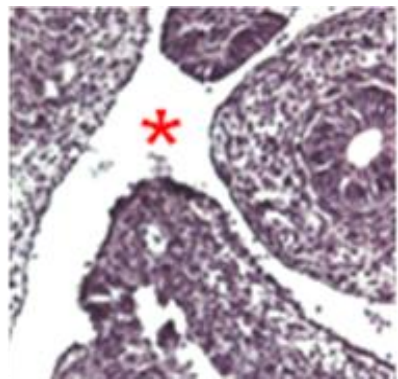


Figure 7 Hematoxylin- and eosin-staining in developing fetal diaphragms on D13. Nitrofen-exposed fetuses had an abnormal PPF structure, characterized by the absence of the dorsally projecting point (asterisk).

3.2 Relative mRNA expression of *FREM1*, *FRAS1* and *FREM2* in rat PPFs and fetal diaphragms

Relative mRNA expression of *FREM1* was significantly reduced in PPFs of nitrofen-exposed fetuses on D13 (0.30 ± 0.23 vs. 0.83 ± 0.19 ; $p<0.05$), developing diaphragms of nitrofen-exposed fetuses on D15 (0.54 ± 0.22 vs. 1.19 ± 0.28 ; $p<0.05$) and fully muscularized diaphragms of nitrofen-exposed fetuses on D18 (0.49 ± 0.37 vs. 0.97 ± 0.53 ; $p<0.05$) in comparison with controls (Figure 8).

In addition, relative mRNA expression of *FRAS1* (Figure 9) and *FREM2* (Figure 10) were significantly reduced in PPFs of nitrofen-exposed fetuses on D13 (1.76 ± 0.86 vs. 3.09 ± 1.15 ; $p<0.05$ and 0.47 ± 0.26 vs. 0.82 ± 0.36 ; $p<0.05$), developing diaphragms of nitrofen-exposed fetuses on D15 (1.45 ± 0.80 vs. 2.63 ± 0.84 ; $p<0.05$ and 0.41 ± 0.16 vs. 1.02 ± 0.49 ; $p<0.05$) and fully muscularized diaphragms of CDH fetuses on D18 (1.35 ± 0.75 vs. 2.32 ± 0.92 ; $p<0.05$ and 0.37 ± 0.24 vs. 0.70 ± 0.32 ; $p<0.05$) compared to controls.

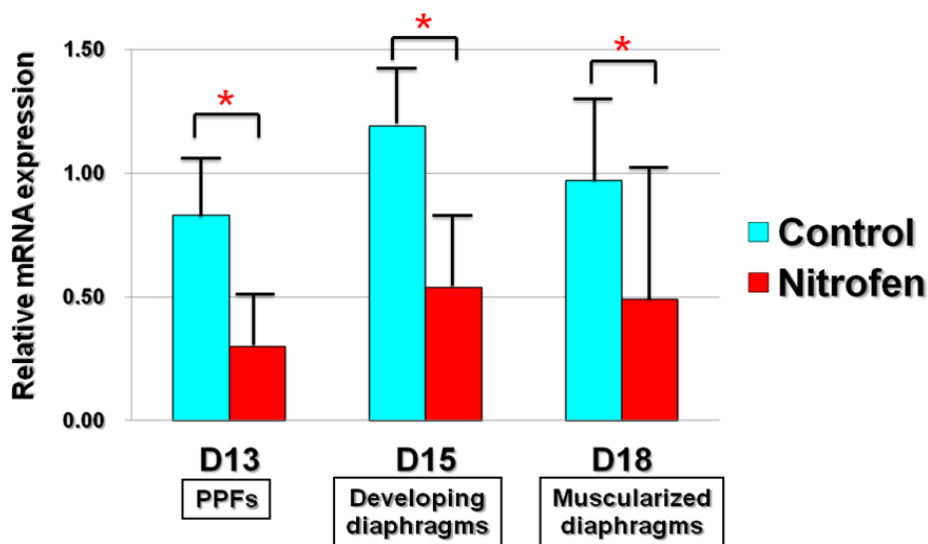


Figure 8 *FREM1* expression in pleuroperitoneal folds (PPFs), developing diaphragms and fully muscularized diaphragms. Gene levels were normalized to the housekeeping gene β -actin. (12 samples per time point and per treatment)

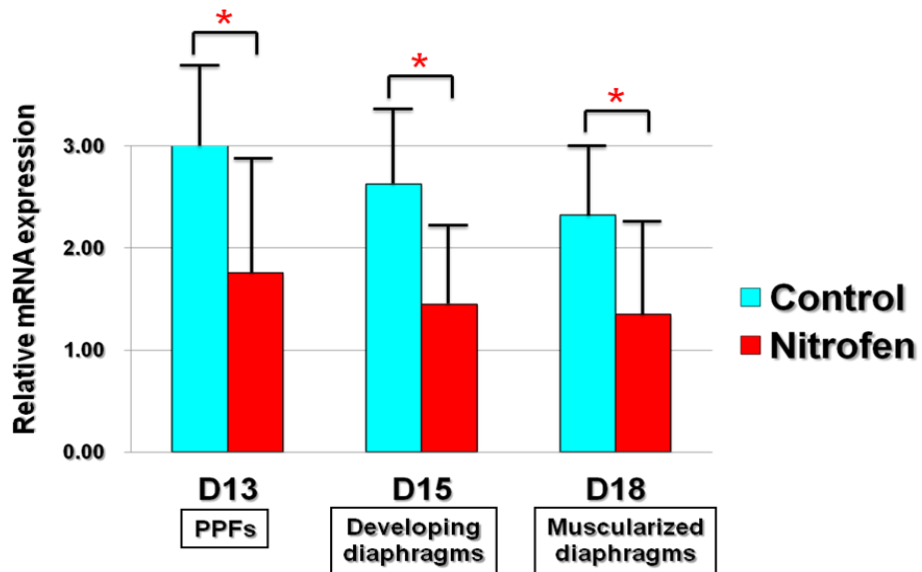


Figure 9 *FRAS1* expression in pleuroperitoneal folds (PPFs), developing diaphragms and fully muscularized diaphragms. Gene levels were normalized to the housekeeping gene β -actin. (12 samples per time point and per treatment)

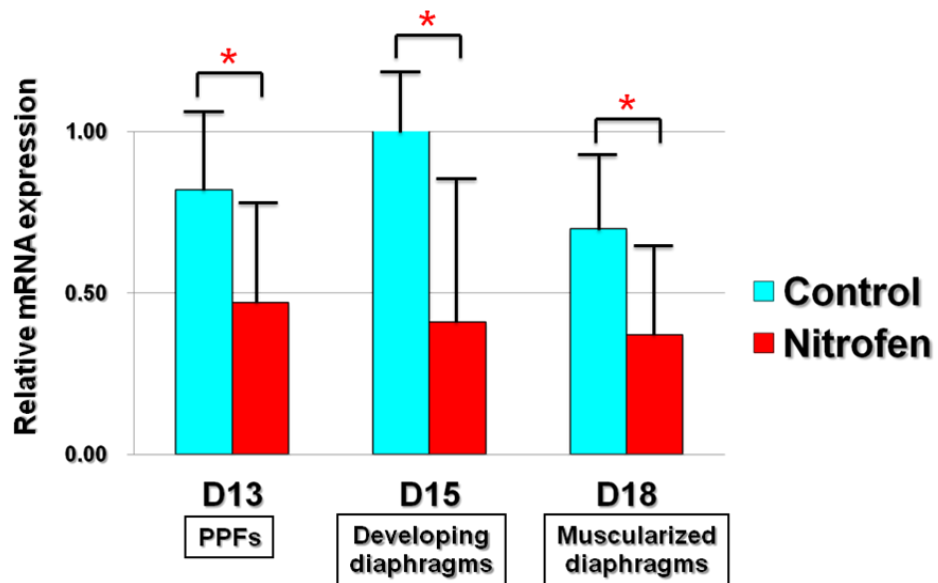


Figure 10 *FREM2* expression in pleuroperitoneal folds (PPFs), developing diaphragms and fully muscularized diaphragms. Gene levels were normalized to the housekeeping gene β -actin. (12 samples per time point and per treatment)

3.2 Immunofluorescence of FREM1 and GATA4 in fetal rat diaphragms

Immunofluorescence staining for FREM1 was performed to evaluate whether the decreased amount of *FREM1* transcripts were also reflected in a decreased amount of FREM1 proteins. Immunofluorescence staining for FREM1 was further combined with the mesenchymal marker GATA4 in order to localize FREM1 protein expression and tissue distribution in developing fetal diaphragms. Confocal laser scanning microscopy revealed a strong diaphragmatic FREM1 immunofluorescence in control fetuses on D13, D15 and D18, which was co-localized with GATA4 immunofluorescence. However, the diaphragmatic FREM1 immunofluorescence was markedly diminished in nitrofen-exposed fetuses on D13, D15 and D18 compared to controls, which was associated with a reduced proliferation of diaphragmatic mesenchymal cells (**Figure 11**).

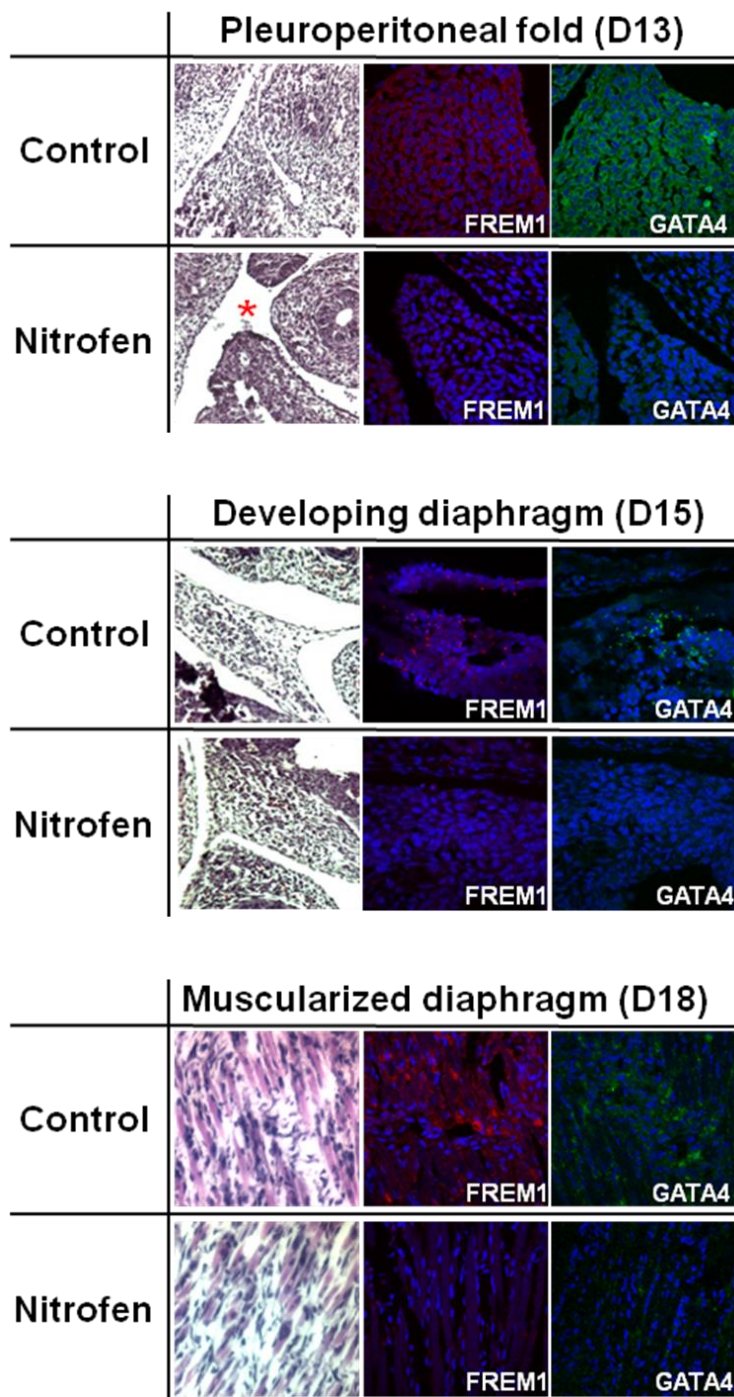


Figure 11 Hematoxylin- and eosin-staining in developing fetal diaphragms on D13, D15 and D18 (left column of each time-point and experimental group). FREM1 (red staining) and GATA4 immunofluorescence (green staining) with DAPI (blue staining) in developing fetal diaphragms on D13, D15 and D18: Control fetuses showed a strong FREM1, which was co-localized with the mesenchymal marker GATA4. Nitrofen-exposed fetuses exhibited a markedly diminished FREM1 immunofluorescence, which was associated with a reduced proliferation of diaphragmatic mesenchymal cells. (12 samples per time point and per treatment)

3.3 Immunofluorescence evaluation of FRAS1, FREM2 and GATA4 in rat PPFs and fetal diaphragms

Immunofluorescence staining for FRAS1 and FREM2 was combined with the mesenchymal marker GATA4 in order to evaluate FRAS1 and FREM2 protein expression and localization in PPFs and fetal diaphragmatic tissue on D13, D15 and D18. Confocal laser scanning microscopy revealed a co-expression of FRAS1 and FREM2 with GATA4, and confirmed the qRT-PCR results by showing a markedly diminished FRAS1 (**Fig. 12**) and FREM2 (**Fig. 13**) immunofluorescence in the diaphragmatic mesenchyme of nitrofen-exposed PPFs and CDH fetuses on D13, D15 and D18 compared to controls. This finding was associated with a reduced proliferation of mesenchymal cells in nitrofen-exposed PPFs and fetal CDH diaphragms on D13, D15 and D18 compared to controls.

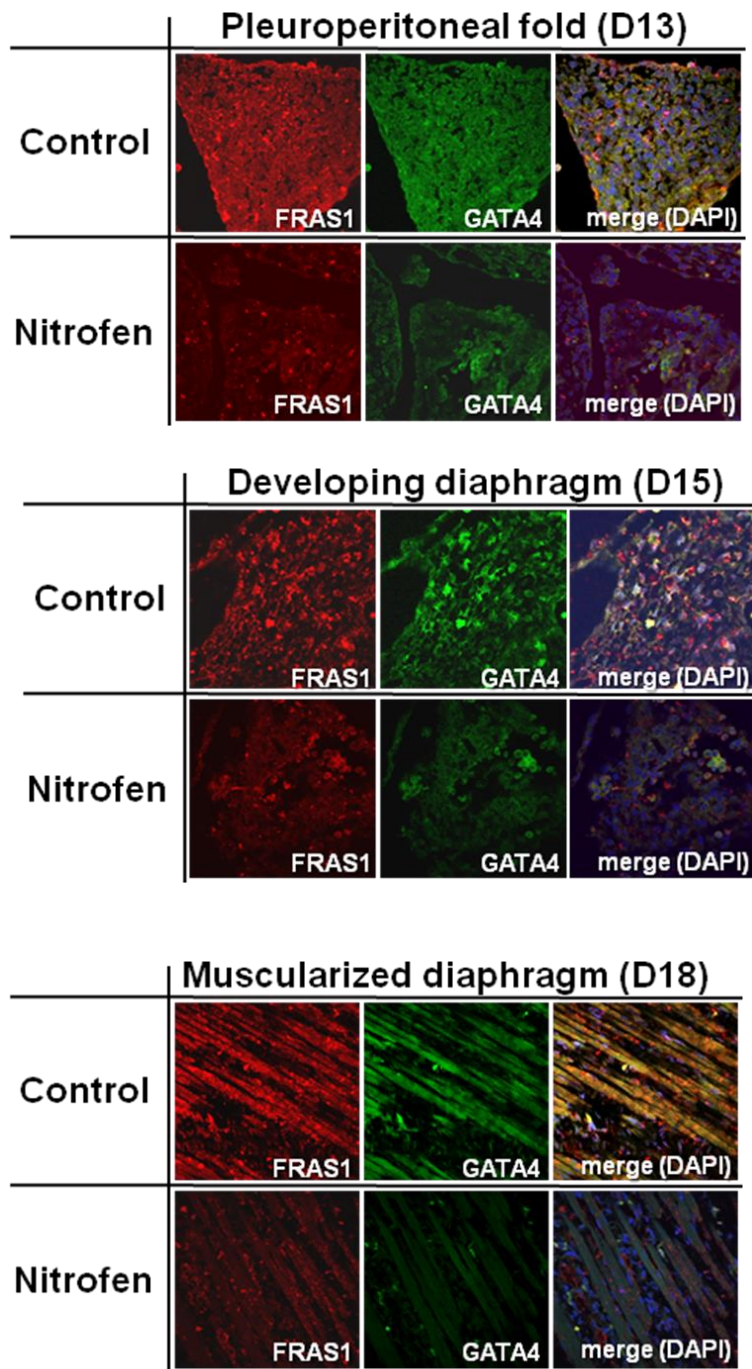


Figure 12 Immunofluorescence evaluation of PPFs and fetal diaphragms for FRAS1 (*red staining*) and GATA4 (*green staining*) with DAPI (*blue staining*). Confocal laser scanning microscopy showed co-expression of FRAS1 and GATA4 primarily in diaphragmatic mesenchymal cells and revealed strikingly diminished FRAS1 immunofluorescence in PPFs (D13), developing diaphragms (D15) and fully muscularized diaphragms (D18) of nitrofen-exposed CDH fetuses compared to controls. (12 samples per time point and per treatment)

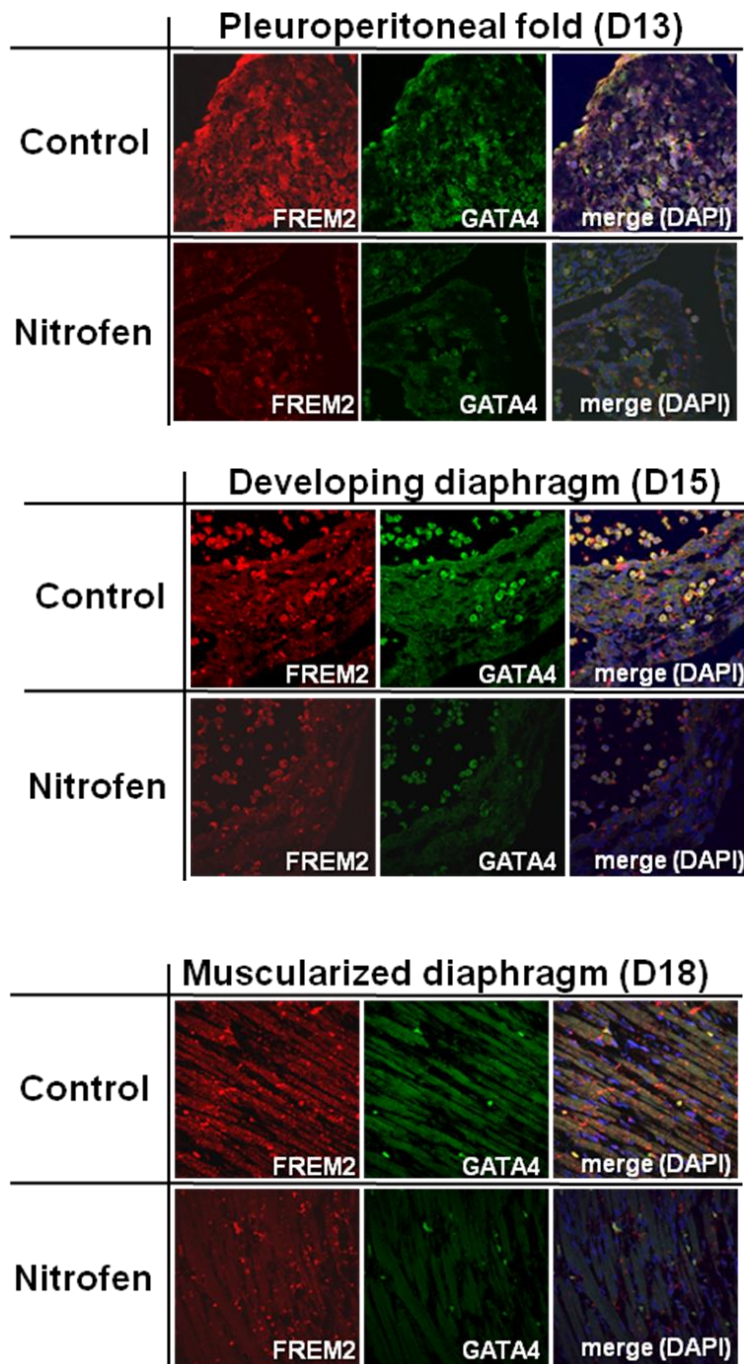


Figure 13 Immunofluorescence evaluation of PPFs and fetal diaphragms for FREM2 (*red staining*) and GATA4 (*green staining*) with DAPI (*blue staining*). Confocal laser scanning microscopy demonstrated co-localization of FREM2 and GATA4 mainly in diaphragmatic mesenchymal cells and further showed a markedly diminished FREM2 immunofluorescence in PPFs (D13), developing diaphragms (D15) and fully muscularized diaphragms (D18) of nitrofen-exposed CDH fetuses compared to controls. (12 samples per time point and per treatment)

Chapter 4

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Discussion

4.1 Discussion

Given the frequency with which CDH occurs, an understanding of the genetic, cellular and morphogenetic mechanisms regulating diaphragm development, both normally and during herniation, is critical. Many fundamental questions about diaphragm development remain unanswered. The morphogenesis of the diaphragm's muscle connective tissue and their relationship to the transverse septum and PPF remain poorly understood.

Amuscular mesenchymal component of the PPF is defective and does not provide a complete foundation for the formation of diaphragmatic musculature [47,75]. Although the pathogenesis of diaphragmatic defects has been extensively studied, the molecular basis of the abnormal ECM formation in CDH remains unclear. Investigating the expression levels and distribution of candidate genes and proteins involved in ECM formation in CDH in the normal and abnormal diaphragm in the early gestation should provide new insights into the pathogenesis of CDH.

We recently reported that the diaphragmatic expression of Copper (Cu)-dependent enzymes lysyl oxidase, Cu-uptake transporter 1 and Cu-efflux pump ATP7A were decreased in the nitrofen-induced CDH model [83,84]. These results indicated that disruption of the Cu-deficient signalling pathway may impair cross-linking of elastin and collagen, which is essential for the proper structural integrity of the diaphragmatic mesenchymal tissue. Furthermore, it has been demonstrated that the diaphragmatic expression of an essential component of the *Sonic hedgehog* signalling cascade, kinesin family member 7 (Kif7) was decreased in the nitrofen-induced CDH model, thus suggesting that decreased Kif7 expression during diaphragmatic development may interfere with mesenchymal cell proliferation, leading to defective PPFs, and resulting in diaphragmatic defects in this model [88].

FREM1 encodes an extracellular matrix protein. Recently a case of a female child with an isolated left-sided posterolateral CDH was reported that carried a *FREM1* deletion [89,90]. In addition, it has been reported that *FREM1* is expressed in the anterior portion of the developing diaphragm and that *FREM1* deficiency causes anterior CDH in mice [89,91]. Moreover, *FREM1*-deficient fetuses exhibit a decreased level of mesenchymal cell proliferation in their developing diaphragms [89]. These results confirm that FREM1 plays a critical role in the development of the fetal diaphragm and that FREM1 deficiency can cause CDH in both humans and mice. Moreover, FREM1 is known to form a ternary complex in the basement membrane with FRAS1 and FREM2 [92]. Recently, FRAS1 and FREM2 gene have been identified as the causative genes in human Fraser syndrome which is infrequently associated with CDH [93,94]. *FRAS1* and *FREM2* also encode essential ECM proteins, which are both expressed in the fetal diaphragm [89]. Although CDH has not yet been reported in *FRAS1*- or *FREM2*-deficient mice, failure to form a FREM1/FRAS1/FREM2 complex may predispose to the development of diaphragmatic defects.

In the present study, we demonstrated for the first time that the diaphragmatic *FREM1*, *FRAS1* and *FREM2* gene expression is significantly reduced in PPFs on D13, developing diaphragms on D15 and fully muscularized diaphragms on D18 in the nitrofen-induced CDH model compared to control littermates. Additionally, immunofluorescence staining for FREM1, FRAS1 and FREM2 showed a co-localization with GATA4, which is a crucial transcription factor during diaphragmatic development and strongly expressed by mesenchymal cells in developing fetal diaphragms [62,95]. Confocal laser scanning microscopy revealed a markedly diminished FREM1, FRAS1 and FREM2 expression in the diaphragmatic mesenchyme of nitrofen-exposed PPFs and CDH fetuses on D13, D15 and D18

compared to controls. Hence, these results confirmed that the quantitative decrease in diaphragmatic *FREM1*, *FRAS1* and *FREM2* mRNA transcripts were also translated to the protein level. A previous study from our laboratory has provided strong evidence that the diaphragmatic expression of GATA4 is downregulated in the nitrofen model, suggesting that a decreased GATA4 expression may impair the diaphragmatic development in nitrofen-induced CDH [96]. In addition, it has recently been demonstrated that *FREM1* and GATA4 interact genetically in the development of lung lobulation defects [97]. Besides the markedly diminished *FREM1*, *FRAS1* and *FREM2* expression in nitrofen-exposed PPFs and fetal diaphragms with CDH, we also found a reduced proliferation of mesenchymal cells in nitrofen-exposed PPFs and fetal CDH diaphragms on D13, D15 and D18, which indicates a disrupted formation of its underlying ECM.

4.2 Future directions

Newborns born with CDH hernia are at high risk of mortality and significant long-term morbidity. The nitrofen rat model has proved to be a good model for studying this malformation and its pathogenesis. This research will help determining the potential mechanism that leads to the development of diaphragmatic defect in CDH.

Further studies on connective tissue formation and the structural integrity of the developing diaphragm are required and should provide new insights into the pathogenesis underlying diaphragmatic defects in CDH.

As with other congenital anomalies, an improved understanding of the pathogenesis of CDH may help to design new treatment modalities targeted at specific developmental insults.

Ultimately, the goal is to positively modulate the natural course of the disease and maybe even help to prevent it from happening at all.

4.3 Conclusions

Our results suggest that decreased mesenchymal expression of *FREM1*, *FRAS1* and *FREM2* in the nitrofen-induced CDH model may cause failure of the *FREM1/FRAS1/FREM2* gene complex unit, disturbing the formation of diaphragmatic ECM and thus contributing to the development of diaphragmatic defects in CDH. These findings may therefore provide new insights into the pathomechanisms underlying CDH.

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