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Expression of Prx1 and Tcf4 is decreased in the diaphragmatic muscle connective tissue of nitrofen-induced congenital diaphragmatic hernia

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ABSTRACT

Background/purpose: Pleuroperitoneal folds (PPFs) are the source of the primordial diaphragm's muscle connective tissue (MCT), and developmental mutations have been shown to result in congenital diaphragmatic hernia (CDH). The protein paired-related homeobox 1 (Prx1) labels migrating PPF cells and stimulates expression of transcription factor 4 (Tcf4), a novel MCT marker that controls morphogenesis of the fetal diaphragm. We hypothesized that diaphragmatic Prx1 and Tcf4 expression is decreased in the nitrofen-induced CDH model.
Methods: Time-mated rats were exposed to either nitrofen or vehicle on gestational day 9 (D9). Fetal diaphragms were microdissected on D13, D15, and D18, and divided into control and nitrofen-exposed specimens. Gene expression levels of *Prx1* and *Tcf4* were analyzed by qRT-PCR. Immunofluorescence double staining for Prx1 and Tcf4 was performed to evaluate protein expression and localization.
Results: Relative mRNA expression of *Prx1* and *Tcf4* was significantly downregulated in PPFs (D13), developing diaphragms (D15) and fully muscularized diaphragms (D18) of nitrofen-exposed fetuses compared to controls. Confocal laser scanning microscopy revealed markedly diminished Prx1 and Tcf4 expression in diaphragmatic MCT of nitrofen-exposed fetuses on D13, D15, and D18 compared to controls.
Conclusions: Decreased expression of Prx1 and Tcf4 in the fetal diaphragm may cause defects in the PPF-derived MCT, leading to development of CDH in the nitrofen model.
Level of evidence: Level 2c (Centre for Evidence-Based Medicine, Oxford).

Congenital diaphragmatic hernia (CDH) is a relatively common malformation with international incidence rates currently ranging between 1.93 and 2.3 cases per 10,000 births [1,2]. Severe pulmonary hypoplasia and persistent pulmonary hypertension are considered to be the main reasons for the life-threatening respiratory distress in newborn infants with diaphragmatic defects [3,4]. Despite substantial advances in postnatal resuscitation and modern lung-protective strategies, CDH remains one of the major therapeutic challenges in modern neonatal intensive care, causing high mortality and long-term morbidity for survivors [5–7].

The origin of diaphragmatic defects is considered to lie in the non-muscular parts of the fetal diaphragm. In fact, a proliferative abnormality of the mesenchymal-derived pleuroperitoneal folds (PPFs) has recently been reported in rats with CDH [8,9]. Mesenchymal elements in the developing diaphragm have been shown to mainly comprise of muscle connective tissue (MCT) [9]. Furthermore, there is strong evidence that developmental mutations that inhibit the formation of normal diaphragmatic MCT can result in CDH [10,11]. It has also been suggested that decreased expression of important regulatory proteins for mesenchymal cell proliferation during diaphragmatic morphogenesis leads to defective PPFs and eventually diaphragmatic defects [12]. Although the pathogenesis of CDH has been extensively studied, the exact molecular basis of abnormal MCT formation is not clearly understood.

The protein paired-related homeobox 1 (Prx1), which labels migrating PPF cells and stimulates the expression of transcription factor 4 (Tcf4), a novel MCT marker that controls growth of the fetal diaphragm, has recently been identified to play a key role during diaphragmatic development [13]. It has further been demonstrated that PPFs are the only source of the primordial diaphragm's MCT and therefore significantly contribute to its myogenesis [13–15]. Mutations in PPF-derived cells have been shown to cause CDH [16,17]. The aim of this study was to investigate the hypothesis that the expression of Prx1 and Tcf4 is decreased in the developing diaphragm in the nitrofen-induced CDH.

1. Material and methods

1.1. Animals, drugs and experimental design

Pathogen-free adult Sprague–Dawley rats® (Harlan Laboratories, Shardlow, UK) were mated overnight and the presence of spermatozoids in the vaginal smear of females was considered as embryonic day 0.5 (D0.5). Pregnant animals were then randomly divided into two experimental groups: “nitrofen” and “control”. On D9, dams were

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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 with a gastric tube. Fetuses were delivered via caesarean section under anesthesia on selected time-points D13 (PPFs), D15 (developing diaphragms) and D18 (fully muscularized diaphragms), and sacrificed by decapitation. After laparotomy, D18 fetuses were inspected under a Leica S8AP0 stereomicroscope (Leica Microsystems AG, Heerbrugg, Switzerland) for diaphragmatic defects. Fetal diaphragms from nitrofen-exposed animals with CDH and controls were dissected under sterile conditions via thoracotomy and stored in a TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) at −20 °C. Additionally, whole D13 and D15 fetuses as well as D18 trunks were fixed in 10% paraformaldehyde (PFA) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) overnight. In total, 72 fetal diaphragms were used for this study ($n = 12$ per time-point and experimental group, respectively).

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. Total RNA isolation

Following fixation in 10% PFA, paraffin-embedded D13 and D15 fetuses were transversely sectioned at a thickness of 10 μm and mounted on PEN membrane glass slides® (MDS Analytical Technologies, Sunnyvale, CA, USA) in order to obtain total RNA from PPFs and developing diaphragms. All appropriate sections were deparaffinized with xylene, rehydrated through ethanol and distilled water, stained with hematoxylin and dehydrated. D13 PPFs and developing D15 diaphragms were dissected from 9 consecutive sections per fetus by laser capture microdissection (Arcturus XT® Instrument, MDS Analytical Technologies, Sunnyvale, CA, USA) and total RNA was extracted 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 D18 diaphragms, total RNA was extracted from the TRIzol® suspension using the acid guanidinium thiocyanate-phenol-chloroform extraction method. Spectrophotometrical quantification of total RNA was performed with a NanoDrop ND-1000 UV–Vis® Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, NC, USA).

1.3. Complementary DNA synthesis and quantitative real-time polymerase chain reaction

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 protocol. The resulting cDNA 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 pairs are listed in Table 1. Following 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 *Prx1* and *Tcf4* 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
Gene-specific primer sequences for quantitative real-time polymerase chain reaction.

Gene	Sequence (5′-3′)	Product size (bp)
Prx1		
Forward	CCA CAT GTG CCA ACA ATA GC	106
Reverse	GGC ACC TGG TTC CTC TGT AA	
Tcf4		
Forward	CGA ATC ACA TGG GTC AGA TG	124
Reverse	AAA CGG GGT TAA GGA GCA GT	
β-actin		
Forward	TTG CTG ACA GGA TGC AGA AG	108
Reverse	TAG AGC CAC CAA TCC ACA CA	

1.4. Immunofluorescence double staining and confocal laser scanning microscopy

After 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, MO, 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 to improve cell permeabilization. Sections were then washed in PBS + 0.05% Tween (Sigma-Aldrich, St. Louis, MO, USA) and subsequently blocked with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, 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 Prx1 (rabbit polyclonal, sc-21,948-R; 1:100), Tcf4 (goat polyclonal, sc-8631, 1:100) and GATA4 (mouse polyclonal, sc-25,310, 1:100) (Santa Cruz Biotechnology Inc., Heidelberg, Germany) overnight at 4 °C. On the next day, sections 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. Following another washing step in PBS + 0.05% Tween, sections were counterstained with a DAPI antibody (10,236,276,001, 1:1000) (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.

1.5. 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 distribution or a Mann–Whitney *U* test when the data deviated from normal distribution. A P value <0.05 was considered as statistically significant.

2. Results

2.1. Relative mRNA expression of Prx1 and Tcf4 in rat PPFs and fetal diaphragms

The relative mRNA expression levels of *Prx1* and *Tcf4* were significantly downregulated in PPFs of nitrofen-exposed fetuses on D13, developing diaphragms of nitrofen-exposed fetuses on D15 and fully