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# Expression of *Iroquois* genes is up-regulated during early lung development in the nitrofen-induced pulmonary hypoplasia

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**Abstract**

**Background/Purpose:** *Iroquois* homeobox (*Irx*) genes have been implicated in the early lung morphogenesis of vertebrates. *Irx1-3* and *Irx5* gene expression is seen in fetal lung in rodents up to day (D) 18.5 of gestation. Fetal lung in *Irx* knockdown mice shows loss of mesenchyme and dilated airspaces, whereas nitrofen-induced hypoplastic lung displays thickened mesenchyme and diminished airspaces. We hypothesized that the *Irx* genes are up-regulated during early lung morphogenesis in the nitrofen-induced hypoplastic lung.

**Methods:** Pregnant rats were exposed either to olive oil or nitrofen on D9. Fetal lungs harvested on D15 were divided into control and nitrofen groups; and the lungs harvested on D18 were divided into control, nitrofen without congenital diaphragmatic hernia (CDH[−]), and nitrofen with CDH (CDH[+]). *Irx* gene expression levels were analyzed by reverse transcriptase polymerase chain reaction. Immunohistochemistry was performed to evaluate protein expression of Irx family.

**Results:** Pulmonary *Irx1-3* and *Irx5* messenger RNA expression levels were significantly up-regulated in nitrofen group compared with controls at D15. On D15, Irx immunoreactivity was increased in nitrofen-induced hypoplastic lung compared with controls.

**Conclusion:** Overexpression of *Irx* genes in the early lung development may cause pulmonary hypoplasia in the nitrofen CDH model by inducing lung dysmorphogenesis with thickened mesenchyme and diminished airspaces.



Congenital diaphragmatic hernia (CDH) is life-threatening congenital anomaly, occurring in approximately 1 in 2500 births [1]. Despite recent advances in antenatal diagnosis and postnatal management, the mortality rate in patients with CDH remains high [2]. Hypoplastic lung and persistent pulmonary hypertension are the principle causes of the high morbidity

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and mortality in infants with CDH [1,3]. Much of the current understanding of pathogenesis of pulmonary hypoplasia in CDH originates from experimental studies. The administration of nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl ether) in rodents during specific time in gestation results in a high rate of CDH and associated pulmonary hypoplasia to their fetuses, which is strikingly similar to the condition seen in the human [4,5]. Several studies from our laboratory using nitrofen-induced CDH rodent model have provided important insights into the molecular mechanisms of pulmonary hypoplasia associated with CDH [6,7]. However, the exact molecular mechanism by which nitrofen induces hypoplastic lung in this model still remains unclear.

Lung development is a highly regulated process directed by mesenchymal-epithelial interactions, which coordinate the temporal and spatial expression of multiple regulatory factors required for proper lung formation [8]. The *Iroquois* homeobox (*Irx*) genes have been implicated in the patterning and specification of several *Drosophila* and vertebrate organs, including lung [9,10]. In fetal rat, *Irx1-3* and *Irx5* messenger RNA (mRNA) is confined to the branching lung epithelium, whereas *Irx4* is not expressed in the developing lung. It has been recently reported that *Irx* genes are involved in the regulation of proximodistal morphogenesis of the developing lung [9]. The antisense knockdown of all pulmonary *Irx* genes together results in loss of lung mesenchyme and dilated airspaces [9]. Contrary to the morphological phenotype seen in the *Irx* knockdown lung, the nitrofen-induced hypoplastic lung has been known to display thickened mesenchyme and diminished airspaces [11]. We designed this study to investigate the hypothesis that the pulmonary *Irx1-3* and *Irx5* are up-regulated during branching lung morphogenesis in early gestation in the nitrofen-induced hypoplastic lung.

## 1. Materials and methods

### 1.1. Animals and drugs

Adult Sprague-Dawley rats were mated, and the presence of spermatozoids in the vaginal smear was verified and was considered as gestational day (D) 0. Pregnant female rats were then randomly divided into 2 groups. Animals in the experimental group received intragastrically 100 mg of nitrofen (Wako Chemicals, Osaka, Japan) dissolved in 1 mL of olive oil under short anesthesia on D9 of gestation (term, 22 days), whereas those in the control group received only vehicle. Fetuses were then recovered by cesarean delivery on gestational D15 and D18. Fetuses exposed to nitrofen were divided into 2 groups: nitrofen without CDH group (CDH[−]) and nitrofen with CDH group (CDH[+]) (n = 8 at each time point, respectively). The control group (n = 8 at each time point) consisted of animals that received only vehicle. The Department of Health and Children approved the protocol of these animal experiments (ref B100/4022) under the Cruelty to Animals Act of 1876 as amended by European Communities Regulations 2002 and 2005, and all animals were treated according to the current guidelines of animal care.

### 1.2. RNA isolation and real-time reverse transcription polymerase chain reaction

The total RNA of each lung derived from fetuses was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to recommended protocol. Total RNA quantification was performed spectrophotometrically (NanoDrop ND-1000 UV-Vis Spectrophotometer). Total RNA (1 $\mu$g) was reverse-transcribed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, United Kingdom) according to manufacturer's instruction. Following reverse transcription at 44°C for 60 minutes, polymerase chain reaction was performed using a LightCycler 480 SYBR Green I Master (Roche Diagnostics) according to the manufacturer's protocol. Gene-specific primers are listed in Table 1. The specific primer pairs used in this study are same sequences as described in our previous article. After initial denaturation step of 5 minutes at 95°C, 45 cycles of amplification for each primer pair were carried out. Each cycle included a denaturation step, 10 seconds at 95°C; an annealing step, 15 seconds at 60°C; and an elongation step, 10 seconds at 72 °C. Final elongation temperature was 65°C for 1 minute. Relative levels of gene expression were measured using a LightCycler 480 (Roche Diagnostics) according to the manufacturer's instructions. The relative changes in the expression levels of *Irx* genes were normalized against the level of $\beta$-actin gene expression in each sample. Experiments were carried out at least in duplicate for each data point.

### 1.3. Immunohistochemistry

The paraffin-embedded fetal rat lungs were sectioned at a thickness of 5 $\mu$m, and the sections were deparaffinized with xylene and then rehydrated through ethanol and distilled water. Tissue sections were immersed in target retrieval solution (DAKO Ltd, Cambridgeshire, United Kingdom) heated for 20 minutes at 97°C followed by incubation in 0.3% $H_2O_2$ for 30 minutes to block endogenous peroxidase activity. Sections were incubated overnight at 4°C with each of the primary antibodies including a 1:100 dilution of a goat polyclonal antibody against IRX1 (Lot: sc-22578; Santa Cruz Biotechnology, Santa Cruz, CA), IRX2 (Lot: sc-22586; Santa Cruz Biotechnology), IRX3 (Lot: sc-22581; Santa Cruz Biotechnology), and a rabbit polyclonal antibody against IRX5 (Lot: sc-98397; Santa Cruz Biotechnology). Sections were then treated with horseradish peroxidase–labeled donkey anti-goat secondary antibody (Lot: sc-2033; Santa Cruz Biotechnology) for IRX1, IRX2, and IRX3 and with horseradish peroxidase–labeled goat anti-rabbit