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Fetal lung transcriptome patterns in an *ex vivo* compression model of diaphragmatic hernia

Zachary D. Fox, BA,[a,1] *Guihua Jiang, MS,*[a,1] *Kenneth K.Y. Ho, BE,*[b] *Kendal A. Walker, BS,*[a] *Allen P. Liu, PhD,*[b] *and Shaun M. Kunisaki, MD, MSc*[a,*]

[a] *Department of Surgery, Michigan Medicine, University of Michigan, Ann Arbor, Michigan*
[b] *Mechanical Engineering, Michigan Medicine, University of Michigan, Ann Arbor, Michigan*

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ABSTRACT

Background: The purpose of this study was to employ a novel *ex vivo* lung model of congenital diaphragmatic hernia (CDH) to determine how a mechanical compression affects early pulmonary development.

Methods: Day-15 whole fetal rat lungs (n = 6-12/group) from nitrofen-exposed and normal (vehicle only) dams were explanted and cultured *ex vivo* in compression microdevices (0.2 or 0.4 kPa) for 16 h to mimic physiologic compression forces that occur in CDH *in vivo*. Lungs were evaluated with significance set at $P < 0.05$.

Results: Nitrofen-exposed lungs were hypoplastic and expressed lower levels of surfactant protein C at baseline. Although compression alone did not alter the α-smooth muscle actin (*ACTA2*) expression in normal lungs, nitrofen-exposed lungs had significantly increased *ACTA2* transcripts (0.2 kPa: 2.04 ± 0.15; 0.4 kPa: 2.22 ± 0.11; both $P < 0.001$). Nitrofen-exposed lungs also showed further reductions in surfactant protein C expression at 0.2 and 0.4 kPa (0.53 ± 0.04, $P < 0.01$; 0.69 ± 0.23, $P < 0.001$; respectively). Whereas normal lungs exposed to 0.2 and 0.4 kPa showed significant increases in periostin (*POSTN*), a mechanical stress–response molecule (1.79 ± 0.10 and 2.12 ± 0.39, respectively; both $P < 0.001$), nitrofen-exposed lungs had a significant decrease in *POSTN* expression (0.4 kPa: 0.67 ± 0.15, $P < 0.001$), which was confirmed by immunohistochemistry.

Conclusions: Collectively, these pilot data in a model of CDH lung hypoplasia suggest a primary aberration in response to mechanical stress within the nitrofen lung, characterized by an upregulation of *ACTA2* and a downregulation in *SPFTC* and *POSTN*. This *ex vivo* compression system may serve as a novel research platform to better understand the mechanobiology and complex regulation of matricellular dynamics during CDH fetal lung development.

* *Corresponding author*. Section of Pediatric Surgery, C.S. Mott Children's Hospital, Michigan Medicine, University of Michigan, 1540 E. Hospital Drive, SPC 4211, Ann Arbor, MI 48109. Tel.: +1 734 936 8464; fax: +1 734 936 9784.
E-mail address: shaunkun@umich.edu (S.M. Kunisaki).
[1] These authors contributed equally to this work.
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Introduction

Pulmonary hypoplasia and pulmonary hypertension continue to be major problems in neonates born with congenital diaphragmatic hernia (CDH). Despite ongoing advances in state-of-the-art care, including fetal tracheal occlusion, extracorporeal membrane oxygenation, and permissive hypercapnia, mortality rates for infants with CDH remain in excess of 30%.[1-4] Among survivors, the pulmonary morbidity associated with this disease can be quite significant, as validated by the spawning of multidisciplinary clinics involving surgeons, pulmonologists, and other specialists at major children's hospitals nationwide.[5] A better understanding of the CDH disease pathogenesis is needed if further inroads are to be made in the management of this debilitating condition.

Until recently, CDH lung hypoplasia has been thought to be primarily caused by mechanical trauma after impaired closure of the pleuroperitoneal canal at 8-wk gestation.[6] The herniated abdominal contents subsequently lead to extrinsic mechanical compression of the lung during the pseudoglandular and canalicular stages of its development. Evidence for this is supported by surgical creation of a diaphragmatic defect in fetal lambs, which has been shown to induce lung hypoplasia.[7] Nevertheless, this classical paradigm has been challenged as being too simplistic based on several important observations.[8] First, despite the fact that the diaphragmatic defect almost always occurs in only one hemidiaphragm, the observed pulmonary hypoplasia and pulmonary hypertension at birth also affect the contralateral lung, albeit to a lesser degree.[9] Second, other space-occupying lesions because of which there is extrinsic mass effect on the early fetal lung (e.g., congenital lung malformations) do not typically result in significant lung hypoplasia and pulmonary hypertension at birth.[10] Finally, teratogenic rodent models of CDH point toward primary aberrations of retinoic acid signaling within the mesenchyme itself as a causative factor in CDH.[11,12]

Given these observations, we sought to better understand the relative contribution of mechanical forces in the CDH lung by exploring lung development in the nitrofen rat model using a novel *ex vivo* mechanical compression microdevice. Our hypothesis was that an *ex vivo* mechanical compression would cause more significant impairment of pulmonary epithelial and mesenchymal development in nitrofen lungs than control lungs.

Methods

Nitrofen CDH rat model

This study was approved by the University of Michigan Unit for Laboratory Animal Medicine under the protocol PRO00007385 in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. To induce fetal lung hypoplasia, timed pregnant Sprague-Dawley rats (Charles River, Wilmington, MA) were gavage fed 100 mg of nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether; Sigma-Aldrich, Cat# 33,374, St. Louis, MO), a banned herbicide and known retinoic acid synthesis inhibitor, dissolved in 1 mL of olive oil on gestational day 9 (E9) as previously described in our laboratory.[13] Additional dams were gavage fed 1 mL of olive oil (control vehicle) to provide fetal lung controls (also referred to as "normal lungs"). On day 15 (E15) or day 21 (E21), dams were deeply anesthetized, and all fetuses without regard to sex were harvested by cesarean section. Lungs were dissected out intact in Hank's buffered saline under a dissecting microscope (Fig. 1A).

Rat lung compression device

A microdevice capable of applying compressive stress to fetal rat lung explants was specially designed based on a previously described apparatus (Fig. 1B).[14] Briefly, whole fetal rat lungs were encapsulated in Matrigel (BD, Franklin Lakes, NJ) and placed in a Transwell six-well plate on top of a 0.4-μm pore size PET Transwell filter (07-200-170; Fisher Scientific, Hampton, NH) to allow for nutrient and oxygen diffusion. A sterilized PDMS mold with 24-mm holes was used to make a 1% agarose (UltraPure Agarose, #16500-100; Invitrogen, Carlsbad, CA) cushion overlay. To apply a constant stress (in kPa), a fixed weight was applied over the cushion (Fig. 1C). Although the agarose gel cushion gradually compacts over a 16-h period before stabilizing, this system allows for the total stress of the agarose-explant interface to remain constant throughout the experiments. The explants were randomly divided into six groups (n = 6-12 per group): normal 0 kPa, normal 0.2 kPa, normal 0.4 kPa, nitrofen 0 kPa, nitrofen 0.2 kPa, and nitrofen 0.4 kPa. The pressure settings were selected based on estimates of *in vivo* mechanical forces induced by abdominal herniation into the thorax[15,16] as well as preliminary data demonstrating nonviable lung tissue at 0.6-1.0 kPa.

Lung morphometric analyses

Explants were photographed under an inverted phase-contrast microscope (×4 magnification). Digital images at day 0 (E15), 24 h (E15 + 1), and 48 h (E15 + 2) were blindly analyzed for total lung surface area and terminal lung budding using ImageJ software as previously described.[17] For lung surface area calculations, the outline of the lung explant was manually traced and integrated using the imaging software. For a branching analysis, a terminal bud was defined as a single acinus separated by distinct septa at the periphery of the explant.

Histology

To confirm the CDH phenotype, euthanized fetuses were chosen at random and fixed in 4% paraformaldehyde for 24 h. Coronal sections were performed for hematoxylin and eosin staining. For immunofluorescence analyses, tissue fixation was performed in the same manner for 30 min before paraffin embedding. Five-micron-thick sections underwent antigen retrieval, were incubated with conjugated primary antibodies, and counterstained with 4',6-diamidino-2-phenylindole. Primary antibodies were against E-cadherin (ECAD, 1:500; BD), α-smooth muscle actin (α-SMA/ACTA2, 1:1000; Sigma), Ki67 (1:200; Millipore, Burlington, MA), cleaved caspase-3 (Cas3, 1:200; Cell Signaling Technology, Danvers, MA), and periostin (POSTN, 1:2000; Abcam, Cambridge, UK). Fluorescence