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ORIGINAL ARTICLE

Lower $NPAS_3$ expression during the later stages of abnormal lung development in rat congenital diaphragmatic hernia

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Abstract
Purpose Congenital diaphragmatic hernia (CDH) is characterized by a developmental defect in the diaphragm, pulmonary hypoplasia and pulmonary hypertension. $NPAS_3$ is a PAS domain transcription factor regulating *Drosophila* tracheogenesis. $NPAS_3$ null mice develop pulmonary hypoplasia in utero and die after birth due to respiratory failure. We aimed to evaluate $NPAS_3$ expression during normal and abnormal lung development due to CDH.
Methods CDH was induced by administering 100 mg/ml nitrofen to time-pregnant dams on embryonic day (E) 9 of gestation. Lungs were isolated on E15, E18 and E21 and $NPAS_3$ localization was determined by immunohistochemistry and quantified using Western blotting.
Results We found that only E21 hypoplastic CDH lungs have reduced expression of $NPAS_3$ in the terminal saccules. Western blotting confirmed the down-regulation of $NPAS_3$ protein in the nitrofen-induced hypoplastic lungs.
Conclusions We demonstrate for the first time that nitrofen-induced hypoplastic CDH lungs have reduced $NPAS_3$ expression in the terminal saccules during the later stages of abnormal lung development. Our findings suggest that $NPAS_3$ is associated with pulmonary hypoplasia in CDH.

Keywords Congenital diaphragmatic hernia · Pulmonary hypoplasia · Lung development · Nitrofen · $NPAS_3$

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Introduction

A developmental diaphragmatic defect characterizes congenital diaphragmatic hernia (CDH) and CDH is associated with pulmonary hypoplasia and pulmonary hypertension. Although it is relatively easy to repair the diaphragmatic defect in newborns, the morbidity and mortality of CDH patients remains high [1]. A better understanding of the molecular mechanisms regulating lung development will help to better understand abnormal lung development in CDH and improve the outcome of babies with this devastating disease [2–4].

$NPAS_3$ is a neuronal PAS domain protein 3 gene that encodes a basic helix–loop–helix (bHLH) transcription factor that is expressed broadly in the developing neuroepithelium [5, 6]. Members of this protein family contain a bHLH DNA binding domain located on the amino-terminal side of a PAS domain. The PAS domain is approximately 260 amino acids in length, contains two direct repeats of about 60 amino acids [7] and functions in diverse physiological contexts including environmental adaptation to hypoxia and circadian regulation [8]. $NPAS_3$ regulates *Drosophila* tracheogenesis and is associated with lung development in vertebrates [9]. The human $NPAS_3$ protein is evolutionarily conserved among mammals, including the mouse, rat and chimpanzee, by having more than 90 %

conserved sequence identity in the peptide sequence [10]. There is only one study reporting the importance of $NPAS_3$ for lung development: $NPAS_3$ null mice develop pulmonary hypoplasia in utero and die after birth from respiratory failure. Moreover, $NPAS_3$ is essential for normal lung development, playing a crucial role in maintaining lung homeostasis and is also expressed and important for postnatal lung pathologies [9]. Little is known about the embryonic expression of the human $NPAS_3$ gene [11]. $NPAS_3$ has also been reported by others to be associated with several disorders including schizophrenia, but most work has focussed on the brain [12–17].

We hypothesized that abnormal $NPAS_3$ expression underlies abnormal lung development in CDH and, therefore, we aimed to evaluate the expression of $NPAS_3$ in normal and abnormal CDH lungs in the nitrofen rat model of CDH.

Materials and methods

Animal experiments were performed and approved by the Bannatyne Animal Care Committee of the University of Manitoba. Animals were housed according to the recommendations of NIH guidelines and the Guide for the Care and Use of Laboratory Animals, published by the National Academy Press [18, 19].

Animal and experimental design

Adult Sprague–Dawley female rats were mated overnight and checked the following morning to confirm a vaginal plug. Plug confirmation was considered embryonic day zero (E0). On E9, randomly selected pregnant rats, received either 100 mg of nitrofen (2,4-dichlorophenyl-*p*-nitrophenylether) dissolved in 1 ml of olive oil, or olive oil alone by orally gavaging them under a short anesthesia. On E15, E18 and E21 dams were euthanized, and fetuses were harvested by Cesarean section and killed by decapitation. Lungs were dissected and divided into two groups (control and hernia) from three different mothers for each gestational time point ($n = 5$ for each group). Lungs were then either fixed in 4 % paraformaldehyde in PBS at 4 °C overnight for immunohistochemistry or snap frozen in liquid nitrogen for Western blot analysis.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded lung tissues from different stages of lung development as a qualitative method. Immunohistochemistry was carried out on 5-μm sections. Tissue sections were deparaffinized in xylene and rehydrated through a graded alcohol series. Sections were incubated with the antibody for $NPAS_3$ (ProSci incorporated, 4109) overnight at 4 °C (1:1000 dilution) as previously described [20]. Negative control reactions were performed by omission of the primary antibody. The secondary antibody was a horseradish peroxidase-labeled anti-rabbit antibody (Jackson Labs, 111-175-144) and this was applied for 1 h in a 1:400 dilution at room temperature. Sections were then developed with a diaminobenzidine (DAB) H_2O_2 substrate complex and counterstained with hematoxylin. Subsequently, the sections were dehydrated, and the slides mounted with glass coverslips. Results are representative of embryos from three different mothers. Results were quantified in a blinded fashion and confirmed by Western blot.

Western blot

Nitrofen and control lungs were washed and protein was extracted using lysis buffer: 10 mM Tris–HCl (pH 6.8), 5 μM β-glycerophosphate, 20 μM EDTA, 5 % SDS, a protease inhibitor cocktail tablet and phosphatase inhibitors (1 mM sodium orthovanadate, 2 mM EGTA, 10 mM sodium pyrophosphate, 30 mM sodium chloride). Protein concentration was determined using RC DC™ Protein Assay (Bio-Rad). Fifteen μg of total proteins were reduced with β-mercaptoethanol, size fractionated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). $NPAS_3$ was detected with an $NPAS_3$ antibody (ProSci incorporated) using a 1:1000 dilution and a horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody (Bio-Rad) in a 1:5000 dilution. For loading control, blots were probed with anti-GAPDH (Abcam, MA, USA) in a 1:10000 dilution, using a horseradish peroxidase-conjugated goat-anti-mouse secondary antibody in a 1:6000 dilution (Bio-Rad). Exposed films were scanned and densitometry was performed using ImageJ software (Wayne Rasband, NIH, USA).

Statistical analysis

All quantitative data are presented as mean ± standard error. Differences between control lungs and hypoplastic CDH lungs at each gestational time point were evaluated using one-way-ANOVA Bonferroni test. Statistical significance was set at $p < 0.05$.

Immunohistochemistry studies

$NPAS_3$ protein was present at all gestational time points (E15, E18 and E21) in control and nitrofen-induced hypoplastic lungs. At E15 and E18, $NPAS_3$ expression was similar between control and hypoplastic CDH lungs (Fig. 1a, b). $NPAS_3$ protein was expressed in the airway epithelium and mesothelium as well as in the mesenchymal

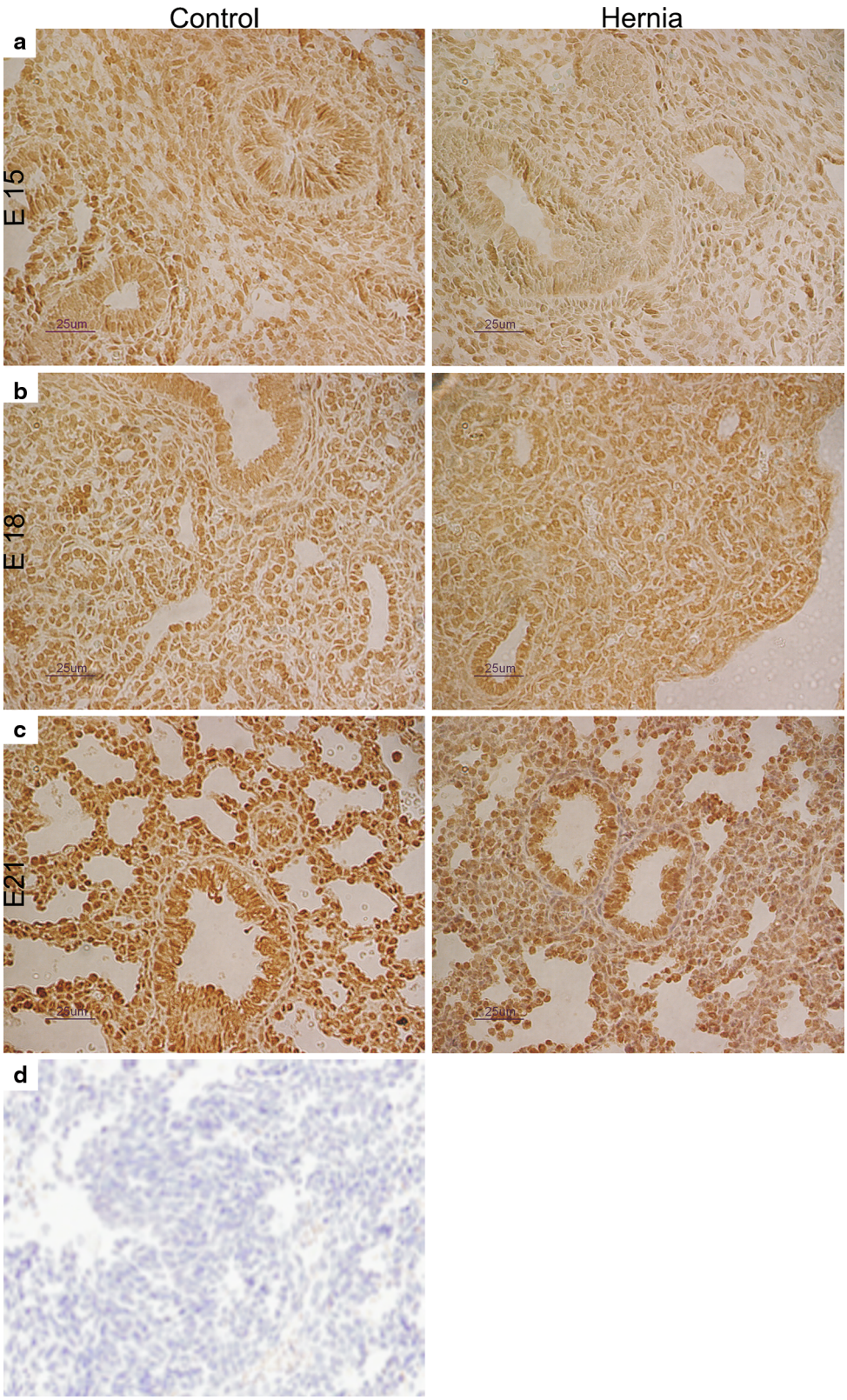

Fig. 1 $NPAS_3$ expression in normal and nitrofen-induced hypoplastic lungs during fetal rat lung development. $NPAS_3$ is present throughout all stages of lung development from early E15 until late E21. **a** E15. **b** E18. **c** E21. **d** Immunohistochemistry negative control—omission of the primary antibody

cells. $NPAS_3$ is mainly nuclear. In E21 CDH lungs, $NPAS_3$ was lower in the peribronchial mesenchymal cells and in the mesenchyme (Fig. 1c).

Western blot studies

We quantified $NPAS_3$ expression levels using Western blot. Western blot analysis was in agreement with IHC observations and revealed that $NPAS_3$ was expressed throughout all studied gestational ages in normal and CDH fetal lungs. $NPAS_3$ expression was similar between normal and CDH lungs on E15 and E18 (early and late pseudoglandular stage, respectively, Fig. 2a, b) and lower on E21 (saccular stage) in CDH lungs when compared to controls (Fig. 2c).

Discussion

As $NPAS_3$ is important for lung development and growth, we aimed to evaluate if $NPAS_3$ expression is different in nitrofen-induced hypoplastic CDH lungs, to determine whether this protein is associated with abnormal lung development and CDH. If that is the case it will help to better understand the disease and address the mechanisms involved in hypoplastic lung development. We observed that $NPAS_3$ expression is similar during the earlier stages of lung development in normal and nitrofen-induced CDH lungs. In contrast, $NPAS_3$ expression was decreased in CDH lungs prior to birth suggesting that $NPAS_3$ plays a role during the later stages of lung development. These findings are supported by published data from others demonstrating that $NPAS_3$ is not only a regulator of proximal lung development, but also appears to play a role during postnatal lung pathology. Moreover, in normal lung development, FGF-10 induces the dynamic expression of its inhibitor Spry2 and FGF-10 expression decreases [21]. Furthermore, exogenous administration of FGF10 in $NPAS_3$ knockout mice, rescues abnormal lung branching [9]. The mechanism by which NPAS3 controls lung development is complex, although FGF10 seems to be associated with the regulation of NPAS3 expression.

$NPAS_3$ knockout mice have reduced branching morphogenesis early in gestation with diminished alveolarization, decreased Shh, FGF9, FGF10 and Bmp4 and increased Spry2 consistent with reduced FGF signaling, resulting in death due to respiratory distress after birth. Hypoplastic CDH lungs also display abnormal branching morphogenesis. However, in our current study we observed normal $NPAS_3$ expression during the early stages of lung development when normal and nitrofen-induced hypoplastic CDH lungs were compared (Fig. 1a, b) [19]. These data suggest that $NPAS_3$ does not play a key role during the abnormal branching morphogenesis of nitrofen-induced hypoplastic CDH lungs. This might be due to CDH being a multifactorial disease or that the mechanism by which $NPAS_3$ controls lung development is more complex [9]. Moreover, $NPAS_3$ heterozygous knockout mice do not display reduced branching morphogenesis [9]. We are the first to report a reduction of $NPAS_3$ expression during the saccular stages of nitrofen-induced abnormal lung development. This suggests that in the nitrofen model, $NPAS_3$ can be a regulator of late abnormal lung development interfering with different signaling pathways.

It would be interesting in future studies using $NPAS_3$ knockout mice, to investigate if retinoic acid, Bmp, and or Shh exogenous administration will rescue abnormal branching and lung growth. This experiment will demonstrate if $NPAS_3$ expression is mediated through any of these signaling pathways that are known to be involved in lung branching [19, 21–24].

$NPAS_3$ expression is reduced in the mesenchymal cells of the nitrofen-induced hypoplastic CDH lung and we did not observe any expression in the peribronchial mesenchyme (Fig. 1c). Previous studies observed that $NPAS_3$ heterozygous mice at the saccular stage of lung

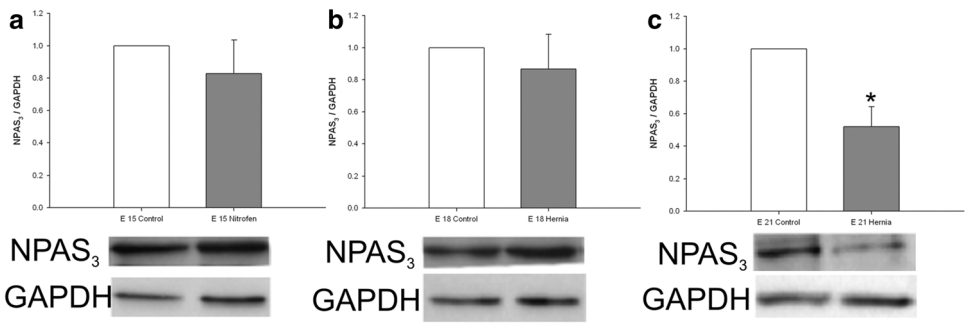

Fig. 2 $NPAS_3$ is lower in nitrofen-induced hypoplastic CDH lungs during the later stages of fetal rat lung development. Western blot analysis of $NPAS_3$ performed in several gestational ages from E15 to E21. Representative blot examples are shown. All protein levels were normalized to GAPDH. **a** E15. **b** E18. **c** E21. Values represent mean ± SE. $p < 0.05$

development have widespread defects in alveolar septation. $NPAS_3$ knockout mice are more severely affected with only patches of future alveolar tissue interspersed with dilated airways resulting in bronchiectasis [9].

In summary, we found that $NPAS_3$ expression is lower during the saccular stages of nitrofen-induced abnormal lung development. Our findings suggest that abnormal $NPAS_3$ expression is associated with pulmonary hypoplasia in CDH.

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Conflict of interest The manuscript does not contain clinical studies or patient data. The authors declare that they have no conflict of interest.

Ethical standard All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

1. Gosche JR, Islam S, Boulanger SC (2005) Congenital diaphragmatic hernia: searching for answers. Am J Surg 190(2):324–332
2. Leeuwen L, Fitzgerald DA (2014) Congenital diaphragmatic hernia. J Paediatr Child Health 50(9):667–673
3. Pereira-Terra P, Deprest JA, Kholdebarin R, Khoshgoo N, DeKoninck P, Boerema-De Munck AA, Wang J, Zhu F, Rottier RJ, Iwasiow BM, Correia-Pinto J, Tibboel D, Post M, Keijzer R (2015) Unique tracheal fluid microRNA signature predicts response to FETO in patients with congenital diaphragmatic hernia. Ann Surg [Epub ahead of print]
4. Peixoto FO, Pereira-Terra P, Moura RS, Carvalho-Dias E, Correia-Pinto J, Nogueira-Silva C (2015) Role of ephrins-B1 and -B2 during fetal rat lung development. Cell Physiol Biochem 35(1):104–115
5. Montedonico S, Nakazawa N, Puri P (2008) Congenital diaphragmatic hernia and retinoids: searching for an etiology. Pediatr Surg Int 24(7):755–761
6. Brunskill EW, Witte DP, Shreiner AB, Potter SS (1999) Characterization of $NPAS_3$, a novel basic helix–loop–helix PAS gene expressed in the developing mouse nervous system. Mech Dev 88(2):237–241
7. Zhou Y, Barnard M, Tian H, Li X, Ring HZ, Francke U, Shelton J, Richardson J, Russell DW, McKnight L (1997) Molecular characterization of two mammalian bHLH-PAS domain proteins selectively expressed in the central nervous system. Proc Natl Acad Sci USA 94(2):713–718
8. McIntosh AR, Kovacevic N, Lippe S, Garrett D, Grady C, Jirsa V (2010) The development of a noisy brain. Arch Ital Biol 148(3):323–337
9. Zhoua S, Degana S, Pottsb EN, Fosterb WM, Sunday ME (2009) $NPAS_3$ is a trachealess homolog critical for lung development and homeostasis. Proc Natl Acad Sci USA 106(28):11691–11696
10. Gould P, Kamnasaran D (2011) Immunohistochemical analyses of $NPAS_3$ expression in the developing human fetal brain. J Vet Med 40:196–203
11. Kamm GB, López-Leal R, Lorenzo JR, Franchini LF (2013) A fast-evolving human $NPAS_3$ enhancer gained reporter expression in the developing forebrains of transgenic mice. Philos Trans R Soc Lond B Biol Sci 368(1632):20130019
12. Kamnasaran D, Muir WJ, Ferguson-Smith MA, Cox DW (2003) Disruption of the neuronal PAS_3 gene in a family affected with schizophrenia. J Med Genet 40(5):325–332
13. Huang J, Perlis RH, Lee PH, Rush AJ, Fava M, Sachs GS, Lieberman J, Hamilton SP, Sullivan P, Sklar P, Purcell S, Smoller JW (2010) Cross-disorder genomewide analysis of schizophrenia, bipolar disorder, and depression. Am J Psychiatry 167(10):1254–1263
14. Kempermann G, Krebs J, Fabel K (2008) The contribution of failing adult hippocampal neurogenesis to psychiatric disorders. Curr Opin Psychiatry 21(3):290–295
15. Pickard BS, Pieper AA, Porteous DJ, Blackwood DH, Muir WJ (2006) The $NPAS_3$ gene emerging evidence for a role in psychiatric illness. Ann Med 38(6):439–448 (Review)
16. Brunskill EW, Ehrman LA, Williams MT, Klanke J, Hammer D, Schaefer TL, Sah R, Dorn GW, Potter SS, Vorhees CV (2005) Abnormal neurodevelopment, neurosignaling and behavior in $NPAS_3$-deficient mice. Eur J Neurosci 22(6):1265–1276
17. Erbel-Sieler C, Dudley C, Zhou Y, Wu X, Estill SJ, Han T, Diaz-Arrastia R, Brunskill EW, Potter SS, McKnight SL (2004) Behavioral and regulatory abnormalities in mice deficient in the $NPAS_1$ and $NPAS_3$ transcription factors. Proc Natl Acad Sci USA 101:13648–13653
18. Doi T, Sugimoto K, Ruttenstock E, Dingemann J, Puri P (2010) Prenatal retinoic acid upregulates pulmonary gene expression of PI3K and AKT in nitrofen-induced pulmonary hypoplasia. Pediatr Surg Int 26:1011–1015
19. Baptista MJ, Melo-Rocha G, Pedrosa C, Gonzaga S, Teles A, Estevão-Costa J, Areias JC, Flake AW, Leite-Moreira AF, Correia-Pinto J (2005) Antenatal vitamin A administration attenuates lung hypoplasia by interfering with early instead of late determinants of lung underdevelopment in congenital diaphragmatic hernia. J Pediatr Surg 40(4):658–665
20. Chi V, Chandy KG (2007) Immunohistochemistry: paraffin sections using the Vectastain ABC kit from vector labs. J Vis Exp 8:308
21. Morrisey EE, Hogan BL (2010) Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell 18:8–23
22. Moura RS, Coutinho-Borges JP, Pacheco AP, Damota PO, Correia-Pinto J (2011) FGF signalling pathway in the developing chick lung: expression and inhibition studies. PLoS One 6:e17660
23. Metzger RJ, Klein OD, Martin GR, Krasnow MA (2008) The branching programme of mouse lung development. Nature 453:745–751
24. Correia-Pinto J, Gonzaga S, Huang Y, Rottier R (2010) Congenital lung lesions-underlying molecular mechanisms. Semin Pediatr Surg 19:171–179