Embryonic Wnt gene expression in the nitrofen-induced hypoplastic lung using 3-dimensional imaging

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Abstract
Purpose: Wnts have been reported to play a key role in the lung morphogenesis. We have previously reported that pulmonary gene expression of Wnt2 and Wnt7b is downregulated on day 15 of gestation in the nitrofen-induced congenital diaphragmatic hernia (CDH) model. However, the distribution pattern of gene expression of Wnts in the very early lung development remains unclear. Optical projection tomography (OPT) is a new technique for 3-dimensional imaging of small developing organs and gene distribution combined with whole-mount in situ hybridization. We designed this study to investigate the distribution pattern of Wnts gene expression in lung buds of nitrofen-induced CDH model using OPT.

Methods: Embryos from normal and nitrofen-treated dams were harvested on embryonic day 10 (E10), and divided into controls and nitrofen group, respectively. Whole-mount in situ hybridization to detect transcripts of Wnt2 and Wnt7b was performed, analyzed, and reconstructed using OPT.

Results: The expression of Wnt2 transcripts was detected in the lung bud mesenchyme and markedly diminished in nitrofen group compared to controls, whereas Wnt7b transcripts were expressed in the mesoderm of bronchi and the lung bud with no detectable difference between 2 groups.

Conclusion: We provide evidence for the first time that Wnt2 expression is downregulated at lung bud stage in the nitrofen model. Optical projection tomography is potentially a useful approach to visualize both gene expression and morphology during very early stages of lung development.

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Despite improved understanding of the pathophysiology of congenital diaphragmatic hernia (CDH) and advances in perinatal care, the prognosis of infants with severe CDH remains poor [1,2]. Hypoplastic lung and persistent pulmonary hypertension are the principle causes of the high morbidity and mortality in infants with CDH [1-3]. The affected infants show developmental retardation of lung, including fewer bronchial branches and alveoli, and retardation of alveolar development [4-6]. The pathogenesis of pulmonary hypoplasia associated with CDH is not fully understood. In the nitrofen-induced CDH animal model, some investigators reported that nonmechanical factors directory mediated by nitrofen play a significant role in the
pathogenesis of lung hypoplasia, causing abnormal regulation of early lung development [7,8]. Keijzer et al [8] proposed the dual-hit hypothesis to explain the observations on pulmonary hypoplasia in this model. This hypothesis proposes that the early retardation in lung development that occurs before the development of the diaphragmatic defect is caused by nitrofen, whereas the late-gestational increase in lung hypoplasia is caused by mechanical compression from herniated viscera. Recently, a role for Wnt signaling in lung development was suggested by the observation that several Wnt genes are expressed in the developing lung mesenchyme and/or epithelium [9-11]. Studies in several model systems have shown that Wnt signaling regulates multiple steps in organogenesis, including cell proliferation, differentiation, and lineage specification [12,13]. In particular, Wnt gene products have been shown to act as mediators of epithelial-mesenchymal interactions in the developing lung [13] where Wnt7b is required for normal lung mesenchymal proliferation in a narrow window of development before E15.5 in mice [14,15]. Wntb inactivation decreased airway branching, caused pulmonary hypoplasia, and decreased lung smooth muscle [14,15]. Wnt 7b null mutant mice died of respiratory failure at birth [14]. Other Wnt genes, such as Wnt2 and Wnt5a are expressed in the mesenchyme of the developing lung [16,17]. Wnt2 is expressed in mesoderm during branching morphogenesis and approximately 50% of Wnt2 knockout mice die soon after birth of respiratory failure [18].

We previously reported that the expression level of Wnt2 and Wnt7b genes was altered in nitrofen-treated hypoplastic lungs compared to normal lungs at early-gestational stages (E15), providing evidence that the Wnt signaling pathway is downregulated in the early stages of lung development [19]. However, the level of expression is only one aspect of the potential patterning activity of signaling molecules, with the precise spatiotemporal distribution of transcripts within the emerging tissue playing an important role in morphogenetic outcomes. Therefore, we hypothesized that not only the amount of expression but also the distribution of expression of Wnt7b and Wnt2 may be affected in the hypoplastic lungs of nitrofen-treated embryos. To test this hypothesis, we evaluated the 3-dimensional (3D) distribution pattern of Wnt genes expression in normal and hypoplastic lungs in the very early stages of lung development using a recently developed technique, optical projection tomography (OPT).

Optical projection tomography is a new approach for 3D imaging of small biologic specimens [20]. The principals of OPT is similar to that of x-ray computed tomography. It fills an imaging gap between magnetic resonance imaging and confocal microscopy, being most suited to specimens that are from 1 to 10 mm across [20,21]. In addition, it is the only technique that can record both colorimetric and fluorescent staining simultaneously with tissue morphology and therefore allows imaging of the 3D distribution of gene expression patterns as revealed by in situ techniques [20-22] to detect either RNA (in situ hybridization) or protein (immunolabeling). Application of the technique to mutant or disrupted embryos has the added potential of revealing anatomical abnormalities in detail including subtle aspects of the phenotype not readily detected from sectioning specimens where sections may be lost or distorted or simply may not reveal 3D details [20]. Thus, we performed OPT to study the precise expression pattern of Wnt2 and 7b genes in the context of the normal and disrupted developing lung in the nitrofen-induced CDH animal model with a view to elucidating the molecular mechanisms involved in lung hypoplasia.

1. Materials and methods

1.1. Animal model

Fetal pulmonary hypoplasia with coexistent diaphragmatic hernia was induced by gavaging time-mated pregnant CD-1 mice [23]. Females caged with males were checked each morning for the presence of a vaginal plug as an indication of mating and the time designated as 0.5 days postcoitum (dpc). At 8.5 dpc, 25-mg nitrofen (WAKO Chemical, Osaka, Japan) dissolved in olive oil was given as a single dose via a stomach tube under anesthesia. In control animals, the same dose of olive oil was given without nitrofen. Cesarean delivery was performed on 10.5dpc of gestation under general anesthesia. Embryos were harvested by laparotomy. Twenty nitrofen-treated embryos and 20 control embryos were subjected to OPT study (10 for Wnt2, 10 for Wnt7b for each group).

Embryos were collected in ice-cold phosphate buffered saline (PBS), separated from surrounding tissue and fixed overnight in 4% paraformaldehyde in PBS at 4°C before dehydration through a series of methanol washes (25%, 50%, 75% in PBS and 100%) and storage at −20°C. The Department of Health and Children approved all the animal experiments (reference B100/3697) under the Cruelty to Animal Act, 1876, as amended by European Communities Regulations 2002.

1.2. In situ hybridization

Antisense RNA probes to detect gene expression and control sense probes were generated by in vitro transcription from linearized plasmid DNA containing complementary DNA clones corresponding to Wnt7b and Wnt2 genes. The gene sequences used as probes correspond to nucleotide 93 to 1581 on genbank sequence NM_009528 (Wnt7b) and nucleotide 19 to 1493 on genbank sequence BC026373 (Wnt2), both kind gifts of A. McMahon, Harvard. RNA probes labeled with digoxigenin (DIG)-uridine triphosphate (UTP) were synthesized using the DIG RNA labeling mix and appropriate RNA polymerases (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s recommendations. Sense control probes were routinely used in
each experiment. In situ hybridization was carried out largely according to Wilkinson [24] with minor adjustments. Hybridization times were extended to 2 to 3 days at 65°C. Final posthybridization washes were in 0.2% saline-sodium citrate (SSC)/0.1% cholamidopropyl dimethlammonio porpnesulfonate (CHAPS), 3 times for 20 minutes at 65°C. The digoxigenin-labeled RNA was localized within the embryonic tissue using an alkaline phosphatase conjugated antidigoxigenin antibody (Roche Molecular Biochemicals, Mannheim, Germany). Colorimetric detection of alkaline phosphatase activity was performed with substrate solution containing 225 μg/mL of nitroblue tetrazolium and 87.5 μg/mL of 5-bromo-4-chloro-3-indolyl phosphate. The level of stain was monitored carefully, and the reaction was stopped by placement in PBS and fixation in 4% paraformaldehyde in PBS when the color had developed to a clearly visible level and background staining was still low, usually less than 2 hours. Note that very darkly stained specimens are not suitable for OPT scanning as light will not be transmitted through the tissue. Specimens were photographed before OPT scanning.

1.3. Optical projection tomography

Hybridized and photographed specimens were rinsed briefly in distilled water and embedded in an appropriate orientation in molten (below 37°C) low melting point agarose (1% in water). Once the agarose had solidified at room temperature, blocks were cut, glued to a metal mount, and dehydrated in methanol at room temperature over night. Specimens were then optically cleared in benzyl alcohol/benzyl benzoate (1:2) for a minimum of 5 hours and up to 2 days. The specimens were scanned as described by Sharpe et al [20] with a prototype OPT scanner built and provided by the MRC Human Genetics Unit, Edinburgh, installed in the Zoology Department, Trinity College Dublin. A Q imaging Retiga Exi camera was used to record images through a 360° rotation of the specimen viewed through a Leica MZ FLIII microscope with a plan 0.5× objective. Scans were performed under visible light provided by a 20 W halogen lamp (bright-field channel) using 700 or 750 nm filters and under UV light to capture autoflourescence from the specimen to represent embryo morphology. The 3D reconstructions were performed as described by Sharpe et al [20] using programs provided by the Edinburgh Mouse Atlas Project. The 3D reconstructions were viewed and analyzed using software (MA3DView and MAPaint) also provided by Edinburgh Mouse Atlas Project. Virtual sections shown here were captured directly from the 3D reconstructions using MAPaint.

2. Results

Superficial examination of whole mount in situ hybridized embryos showed the overall expression patterns of Wnt7b and Wnt2 in control (Fig. 1A and C) and nitrofen-treated embryos (Fig. 1B and D). Wnt7b expression in the developing lung buds is clear (Fig. 1A arrow). Wnt7b expression was also observed in the future brain, in the developing spinal cord, and the optic and otic vesicles. Superficially, Wnt2 expression was observed in a more widespread and diffuse domain in the region of the developing lungs (Fig. 1C, arrow) as well as in the heart, septum transversum, allantois, umbilicus, and in the lateral mesenchyme of the body wall between the fore and hind limb buds. Although expression of Wnt7b appeared to be similar in nitrofen-treated and control embryos, the expression of Wnt2 appeared to be less extensive in some nitrofen-treated embryos (Fig. 1C and D).

The OPT scanning of the specimens and 3D reconstruction of the expression patterns allowed a more thorough examination. Fig. 2 shows reconstructions that allow 3D representations of the lung-associated patterns of both Wnt7b and Wnt2 in normal embryos. These images are a combination of volume representations (left) highlighting the expression domains in white/light gray and surface representations at different threshold levels showing the expression domains (center) in isolation and the surface of the whole embryo.

Fig. 1 Whole mount in situ analysis of Wnt7b expression in control (A) and nitrofen-treated (B) E10.5 embryos. Arrows indicate the Wnt7b expression in the developing lungs. Wnt7b was expressed in an increasing gradient from the proximal-to-distal airway. (C) An E10.5 mouse embryo whole-mount stained for expression of the Wnt2 gene. An arrow indicates the Wnt2 expression in the lung. (D) The Wnt2 transcripts were localized to the outer layer. The Wnt2 expression was not detected in the lungs of E10.5 nitrofen-treated embryos (arrow).
Using this visualization, the shape and size of the expression domains of Wnt7b (Fig. 2A) and Wnt2 (Fig. 2B) are clear; the domains of Wnt7b in the endoderm of the lung buds and Wnt2 in the mesenchyme around the lung buds have been outlined. This extraction of the patterns allows complex 3D domains to be viewed more readily.

The 3D computer reconstructions of scanned embryos can also be rotated and virtually sectioned in any plane to view the internal distribution of expressing cells within the morphological context of the embryo. Figs. 3 and 4 shows sections of normal (A-C) and nitrofen-treated embryos (D-F) stained to reveal expression of Wnt7b (Fig. 3) and Wnt2 (Fig. 4). Coronal (B) and a series of transverse sections (C) show expression (white/light gray) domains in the lung buds for Wnt7b and mesenchyme surrounding the lungs for Wnt2 in normal embryos. This visualization of the patterns allows a more detailed comparison between treated and control embryos. This approach showed that there was no detectable difference in the distribution of Wnt7b expressing cells between control and treated embryos. On the other hand, nitrofen-treated embryos showed variability in the expression domains of Wnt2, specifically in the lung-associated mesenchyme.

In 4 of 10 nitrofen-treated embryos, Wnt2 expression was not detected in the lung mesenchyme (compare Fig. 4C and F) although expression in other sites such as the lateral body wall and the allantois are still clearly visible. In another 4 embryos, Wnt2 expression was very weak in the lung-associated mesenchyme, whereas in 2 of 10 nitrofen-treated embryos, the expression level of Wnt2 was similar to that in normal developing lungs.

3. Discussion

Despite intensive research, the molecular mechanisms involved in lung hypoplasia associated with CDH are still
unclear. According to the dual-hit hypothesis proposed by Keijzer et al [8] to explain the nitrofen-induced animal model of CDH, the early retardation in lung development that occurs before the development of the diaphragmatic defect is caused directly by nitrofen, whereas lung hypoplasia in late gestation is caused by mechanical compression from herniated viscera. To elucidate the mechanisms involved in hypoplastic lung in the early stages of gestation, we focused on Wnt7b and Wnt2.

Although the expression of Wnt7b and Wnt2 have been previously described in the developing lung [14,15,17], the detailed distribution of expressing cells in 3D had not previously been shown. The 3D patterns shown here emphasize the complementary expression of these 2 Wnt signaling molecule encoding genes in neighboring tissues of the endoderm and mesenchyme of the developing lungs. Gene inactivation studies have implicated both genes in the correct development of the lungs, although the link is clearer for Wnt7b where knockout mice die of respiratory failure at birth (16), whereas 50% of Wnt2 inactivated mice die soon after birth, probably because of respiratory failure (18). It is therefore likely that reciprocal Wnt signaling between these adjacent tissues is needed to guide correct development.

The use of OPT allowed a more detailed study of potential changes in the expression of these Wnt genes in response to nitrofen exposure; specifically, 3D reconstruction allowed the distribution of expressing cells to be analyzed. Because these genes encode signaling molecules that influence the development of surrounding cells, any changes in the localization of signal production could have profound effects on developmental processes.

The expression pattern of Wnt7b in all 10 nitrofen-treated embryos was similar to the expression pattern observed in the control group. This would suggest that nitrofen influences the amount of gene product but not the population of cells expressing Wnt7b gene; Wnt7b is still expressed at a level detectable by in situ hybridization in the lung bud epithelium. It is difficult to draw conclusions about the significance of this finding because we do not know the functional significance of different levels of Wnt7b transcripts. On the other hand, lack or reduced expression of Wnt2 was observed in the lung bud mesenchyme of nitrofen-treated embryos. Our results suggest that nitrofen altered the localization of Wnt2 production during very early stages of lung development. Although the precise role of Wnt2 expression in lung development is still unclear, these findings indicate that the impact of nitrofen on lung development may be at least in part mediated by alterations in the local production of Wnt2 signal.

This study illustrates the use of OPT to analyze and compare gene expression in an animal model of a congenital abnormality and shows the potential to simultaneously reveal morphology and gene expression changes. In this study, analysis was focused on early stages of
gestation, 1.5 days after nitrofen treatment, with the view to observing direct gene expression effects. It therefore observed changes before the closure of the diaphragm occurs. Because the anomalies that occur in CDH are complex (lung hypoplasia, defect of diaphragm, herniation of abdominal organ, and others), the technique of 3D visualization of the developing organs will provide additional information at later stages that may help to elucidate the mechanisms involved, for example, during formation of diaphragm. Although OPT holds great potential to look at morphological changes at later stages of development in the model, there are some challenges to observing gene expression in significantly larger embryos. This is related to the penetration of the molecular components required for in situ hybridization in more complex tissues. However, we have successfully visualized expression in visceral tissues up to E13.5 in embryos where the trunk has been dissected and imaged separately.

In conclusion, our findings demonstrate that OPT is a powerful approach to visualizing gene expression and morphology during very early stages of lung development. This technique may provide insights into molecular mechanisms involved not only in lung hypoplasia but also in other anomalies occurring in the nitrofen-induced CDH model. It has great potential for wider use in the analysis of development in animal models of congenital abnormalities.

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**References**

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