

# Hypoxia inducible factor 2 $\alpha$ (HIF2 $\alpha$ /EPAS1) is associated with development of pulmonary hypertension in severe congenital diaphragmatic hernia patients

Yadi Huang<sup>1</sup>, Anne Boerema-de Munck<sup>1,2</sup>, Marjon Buscop-van Kempen<sup>1,2</sup>, Ilona Sluiter<sup>1</sup>, Ronald de Krijger<sup>3</sup>, Dick Tibboel<sup>1</sup> and Robbert J. Rottier<sup>1,2</sup>

<sup>1</sup>Department of Pediatric Surgery, Erasmus MC-Sophia Children's Hospital, Rotterdam, The Netherlands; <sup>2</sup>Department of Cell Biology, Erasmus MC, Rotterdam, The Netherlands; <sup>3</sup>Department of Pathology, Reinier de Graaf Hospital, Delft, and Princess Maxima Center for Pediatric Oncology, Utrecht, the Netherlands

## Abstract

We show that hypoxia inducible factor 2 $\alpha$  (HIF2 $\alpha$ ) is highly expressed in patients with pulmonary hypertension (PH). HIF2 $\alpha$  is expressed in every patient with congenital diaphragmatic hernia, while only half of the controls express HIF2 $\alpha$ . Our data suggest that HIF2 $\alpha$  is a link between hypoxia and the development of PH.

## Keywords

hypoxia inducible factor, pulmonary hypertension, pulmonary vascular development and epithelium

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## Introduction

Idiopathic pulmonary hypertension in newborns (PHN) is a life-threatening condition and requires intensive clinical support.<sup>1,2</sup> PHN is frequently associated with congenital disorders, such as congenital heart diseases and congenital diaphragmatic hernia (CDH).<sup>2</sup> The underlying causes of PHN are still largely unknown, although several potential genetic and epigenetic mechanisms have been described.<sup>2,3</sup> Prolonged exposure to hypoxia causes pulmonary arterial hypertension (PAH), and hypoxia inducible factors (HIF) are the key component of the cellular response to hypoxia.<sup>4</sup> HIFs are heterodimeric transcription factors composed of two subunits, a stable HIF1 $\beta$  and one of three oxygen-sensitive subunits HIF1 $\alpha$ , HIF2 $\alpha$ , or HIF3 $\alpha$ .<sup>4</sup>

HIFs have been associated with lung development and PH.<sup>5</sup> In humans, genome-wide studies showed a positive correlation of HIF2 $\alpha$  with adaptation to hypobaric hypoxia in Tibetan highlanders.<sup>6,7</sup> In rodents, both Hif1 $\alpha$  and Hif2 $\alpha$  appeared to modulate hypoxia-induced PH in gene ablation studies.<sup>5–12</sup> Two reports described that endothelial-specific ablation of prolyl-4-hydroxylase 2 (PHD2), which targets

HIFs for degradation by hydroxylating proline residues in HIF2 $\alpha$  under normoxic conditions, results in a HIF2 $\alpha$ -dependent adult PAH.<sup>10,11</sup> Using an endothelial-specific inactivation of HIF2 $\alpha$ , Cowburn et al. showed that HIF2 $\alpha$  is involved in hypoxia-induced PAH.<sup>12</sup> Since PAH is different from PHN, we examined HIF2 $\alpha$  protein expression in a developmental series of normal lung tissue, as well as in the lungs of a cohort of CDH patients and patients with idiopathic PH.

## Materials and methods

### Human lung tissue collection

With the approval of the Erasmus MC Medical Ethical Committee and the informed consent of parents, lung

Corresponding author:

Robbert J. Rottier, Erasmus MC-Sophia Children's Hospital, Department of Pediatric Surgery / Cell Biology, PO Box 2040, 3000 CA Rotterdam, The Netherlands.

Email: r.rottier@erasmusmc.nl

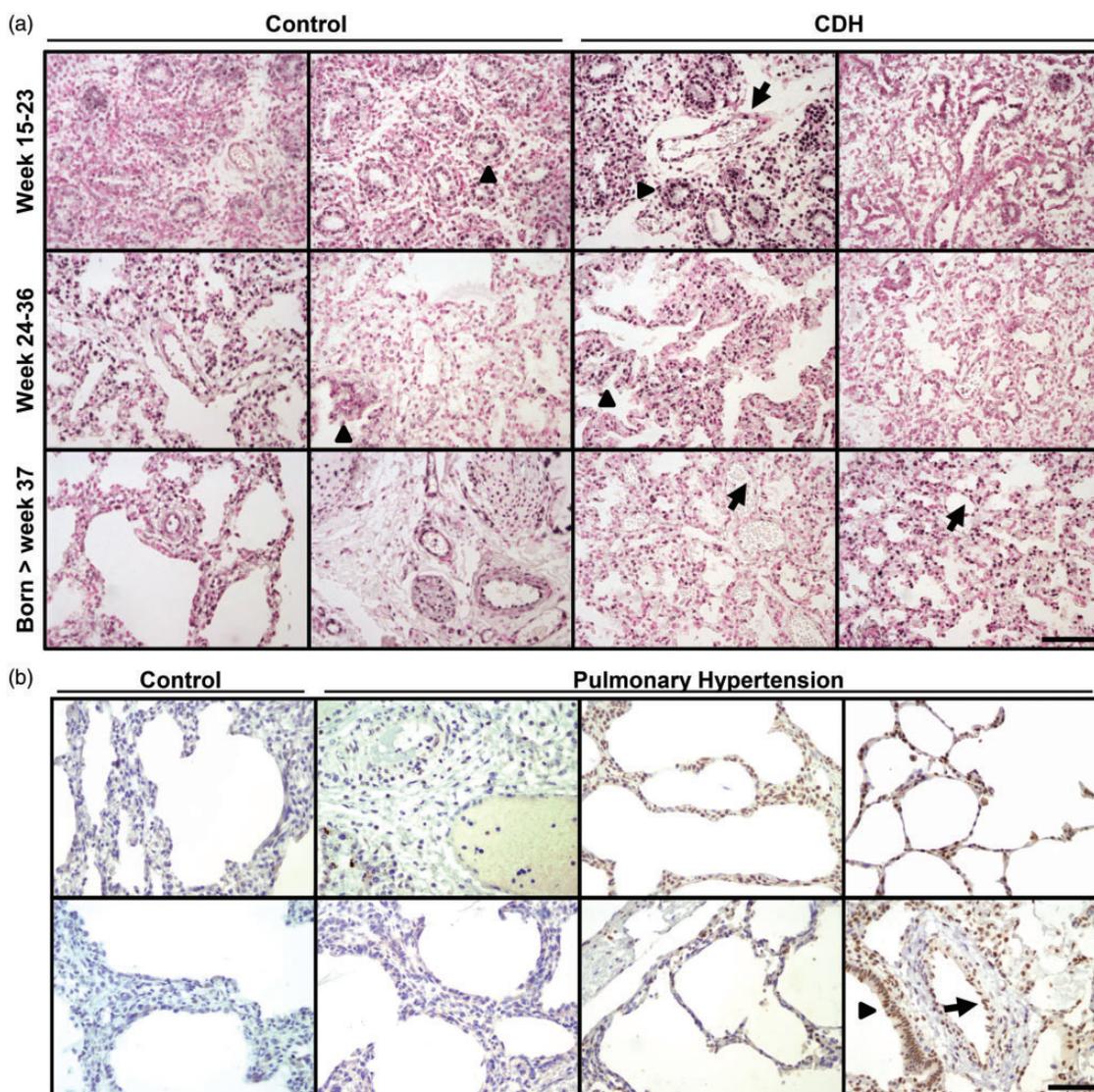


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**Fig. 1.** (a) Representative images of HIF2 $\alpha$  protein expression in lungs of two independent control (left) and two CDH patients (right) at three different gestational ages (weeks 15–23, weeks 24–36, and born after week 37). HIF2 $\alpha$  is expressed in half of the control lungs tested, although some expression is observed in the epithelial cells of the airways (arrowheads). In contrast, HIF2 $\alpha$  is expressed in all examined lungs of the CDH patients, in both epithelial cells (arrowheads) and endothelial cells (arrows). (b) HIF2 $\alpha$  is absent in age-matched control lungs, but prominently expressed in the lungs of some cases of PHN. Two representative images are shown for the control lungs and six images of PHN cases. HIF2 $\alpha$  is expressed in alveolar type II cells (arrowheads) and in endothelial cells (arrows). Scale bar: 100  $\mu$ m.

tissue was obtained from the archives of the Department of Pathology, Erasmus MC (Rotterdam).

### Immunohistochemistry

Tissue micro arrays were constructed as described.<sup>13</sup> Paraffin embedded lung material was sectioned, blocked, and incubated overnight at 4°C (HIF2 $\alpha$ , Genetex). The Envision kit (Dako) was used and images were taken with a charge-coupled device camera attached to an Olympus BX41 microscope.

### Results

HIF2 $\alpha$  was expressed in half of the fetal lungs that were analyzed at 15–36 weeks of gestation. In addition, lungs of neonates born after week 37 were also positive for HIF2 $\alpha$  (Fig. 1a). HIF2 $\alpha$  was localized in the nucleus of proximal and distal airway epithelial cells and alveolar epithelial cells (Fig. 1a, arrowheads). Hif2 $\alpha$  is also highly expressed in alveolar type II cells in lungs as we and others have shown before in mouse lungs.<sup>9,14</sup>

Next, we analyzed the expression of HIF2 $\alpha$  in lungs from a cohort of CDH patients of different gestational ages until

**Table 1.** CDH patient characteristics and the age-matched control lungs.

	Developmental stage	Gestational age (weeks)	Postnatal age	Birth weight (g)	HIF2 $\alpha$ -positive (n/total n)	VEGF-positive (n/total n)
Control	Premature	31.5 (15–36)	1 h (0–24 h)	2000 (57–2700)	5/9	5/9
	Term	39.5 (38–41)	36 h (1 h–1 week)	3220 (2490–3950)	4/8	9/11
CDH	Premature	31.5 (15–36)	1 h (0–48 h)	1032 (30–2515)	7/7	6/6
	Term	39 (37–40)	7 h (1 h–3 days)	2835 (2000–3800)	6/6	9/11

Indicated are the average age in weeks, the average postnatal age, birth weight, and the number of HIF2 $\alpha$ -positive and VEGF-positive samples per total samples tested.

birth. HIF2 $\alpha$  is prominently present in all CDH cases analyzed, contrasting the expression of HIF2 $\alpha$  in normal, unaffected lungs (Fig. 1a, Table 1). The sites of HIF2 $\alpha$  expression were in both alveolar epithelial cells (Fig. 1a, arrowheads) and all endothelial cells of the blood vessels (Fig. 1a, arrows). Moreover, in some CDH lungs, the level of HIF2 $\alpha$  appeared very high as indicated by the intense staining (Fig. 1).

We also analyzed 11 lungs of neonates with idiopathic PH and age-matched controls (Fig. 1b). In the neonatal control human lung, only few cells are positive for HIF2 $\alpha$  (Fig. 1b, control). Clear staining patterns were observed in the endothelial cells of the vessels of patients with PHN (Fig. 1b, arrows) and in alveolar epithelial cells (Fig. 1b, arrowheads), although not all PHN cases expressed HIF2 $\alpha$  (Fig. 1b). Our data suggest that expression of HIF2 $\alpha$  is maintained in some of the clinical cases of PH, supporting the relative hypoxia of these patients. Finally, we also analyzed the expression of one of the HIF2 $\alpha$  targets, VEGF, and found comparable numbers of positive samples as for the HIF2 $\alpha$  staining (Table 1).

## Discussion

Previously, we showed a gradual increase of HIF2 $\alpha$  messenger RNA (mRNA) during gestation, but no differences were detected in expression levels between CDH patients and controls.<sup>15,16</sup> Here, we report an increased expression of HIF2 $\alpha$  protein in CDH patients compared to controls. Even before birth, we found significantly higher expression of HIF2 $\alpha$  in CDH patients. Our data suggest that high levels of HIF2 $\alpha$  correlate with PHN and CDH-associated PH. Since prolonged exposure to hypoxia results in PH,<sup>5</sup> and the lungs of patients with PH are under constant hypoxic conditions, it may result in elevated levels of HIF2 $\alpha$ . Although all CDH cases tested were clearly positive for HIF2 $\alpha$ , not all cases of PHN expressed high levels of HIF2 $\alpha$ . This most likely reflects the patient variability and the heterogeneity of PHN.

In all fetal CDH cases, HIF2 $\alpha$  was expressed in the lung and at much higher levels than controls. Moreover, HIF2 $\alpha$  is also highly expressed in the lungs of patients suffering from PHN. This suggests that early in gestation, the lungs of CDH patients are already intrinsically different from

control lungs, and high levels of HIF2 $\alpha$  may contribute to postnatal PH, which develops in a significant number of patients with CDH after birth.

Prolonged hypoxia, which is also observed in PHN patients, results in structural changes of the pulmonary vasculature characterized by a thickening of the vascular wall of small pulmonary arteries leading to an increased vascular resistance and a worsening of gas exchange.<sup>2</sup> We recently showed that the vascular smooth muscle cells in the developing CDH lung had a different expression pattern of contractile components compared to control lungs, suggesting that these cells prematurely differentiate.<sup>17</sup> Furthermore, the expression of *VEGF-A* mRNA was increased in the lungs from CDH patients at the canalicular stage, while a significant decrease in the expression of *VEGF-A* mRNA was observed in the alveolar stage of lung development in CDH patients. However, the spatial distribution of VEGF-A was not different between control and CDH lungs.<sup>16,18</sup> Here, we report that HIF2 $\alpha$  is expressed in more CDH lungs than in control lungs, and that the expression of VEGF appears to correlate with HIF2 $\alpha$  expression. The site of expression does not differ between control and CDH lungs, as previously described. Additionally, we previously showed that a significant increase in Hif2 $\alpha$  in epithelial alveolar type II cells did not induce an increased expression of Vegf-A.<sup>14</sup>

Although the mechanisms of HIF2 $\alpha$  activity and its role in the development of PH are still incompletely understood, it may be a putative target for future therapies. In this respect, it is interesting to investigate the potential of the Hif2 $\alpha$ -specific competitor, FM19G11.<sup>19</sup>

In summary, we showed high expression levels of HIF2 $\alpha$  in the lungs of all CDH patients at different gestational ages and after birth, while only half of the age-matched controls showed expression of HIF2 $\alpha$ . From these results, we suggest that HIF2 $\alpha$  is associated with the development of PH in CDH, and possibly also with PHN.

## Declaration of conflicting interests

The author(s) declare that there is no conflict of interest.

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