

# Increased pulmonary vascular expression of Krüppel-like factor 5 and activated survivin in experimental congenital diaphragmatic hernia

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## Abstract

**Aim of the study** The high morbidity and mortality in congenital diaphragmatic hernia (CDH) is attributed to pulmonary hypoplasia and persistent pulmonary hypertension (PH). PH is characterized by increased pulmonary artery smooth muscle cell (SMC) proliferation, suppressed apoptosis as well as endothelial dysfunction. Krüppel-like factor 5 (KLF5) belongs to a family of transcription factors that has diverse functions during cell differentiation and embryonic development. KLF5 is preferentially expressed in proliferating SMCs but reduced in differentiated cells. KLF5 induces the expression of Survivin, a 16.5 kDa protein overexpressed in almost all malignancies but hardly detected in normal differentiated tissues. Survivin has been shown to inhibit apoptosis, promote cell proliferation, and enhance angiogenesis. Recent studies have implicated activation of KLF5 and Survivin in the pathogenesis of human and experimental PH. We designed this study to investigate the hypothesis that KLF5 and Survivin expression are increased in nitrofen-induced CDH.

**Methods** Pregnant rats were exposed to nitrofen or vehicle on D9. Fetuses were sacrificed on D21 and divided into nitrofen ( $n = 16$ ) and control group ( $n = 16$ ). Quantitative real-time PCR, western blotting, and confocal immunofluorescence were performed to determine pulmonary gene

expression levels and protein expression of KLF5, Survivin, and phosphorylated Survivin (p-Survivin).

**Main results** Confocal microscopy revealed markedly increased pulmonary vascular KLF5 and p-Survivin expression in lungs of nitrofen-exposed fetuses compared to controls. These results were confirmed by western blotting, showing increased pulmonary expression of KLF5 and p-Survivin. Furthermore, the relative pulmonary gene expressions of *KLF5* and *Survivin* were significantly increased in the CDH group compared to controls ( $p < 0.005$  resp.  $p < 0.01$ ).

**Conclusion** This study provides striking evidence of increased gene and protein expression of KLF5 and activated Survivin in the pulmonary vasculature of nitrofen-induced CDH, suggesting that increased expression of KLF5 may activate p-Survivin expression and play an important role in the pathogenesis of PH in nitrofen-induced CDH.

**Keywords** Congenital diaphragmatic hernia · Nitrofen · Pulmonary hypertension · KLF5 · Survivin

## Introduction

The high morbidity and mortality in congenital diaphragmatic hernia (CDH) is attributed to pulmonary hypoplasia and persistent pulmonary hypertension (PH) [1, 2]. PH is characterized by profound structural remodeling of pulmonary arteries and arterioles caused by increased pulmonary smooth muscle cell (SMC) proliferation and suppressed apoptosis. These pathological changes seen in the pulmonary microvasculature result in medial and adventitial thickening of the vessel wall causing a progressive increase in pulmonary arterial resistance [3].

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Krüppel-like factor 5 (KLF5) belongs to a family of transcription factors that has diverse functions during cell differentiation and embryonic development [4]. KLF5 is a zinc finger transcription factor implicated in the regulation of cell differentiation, proliferation, migration, and apoptosis, which is regulated by multiple signaling pathways at the transcriptional and post-translational level. Recent studies have implicated KLF5 to be involved in tissue remodeling in human PH and cardiovascular diseases [4–6]. At present, the best known function of KLF5 is its stimulatory role in the proliferation of different types of cells, including fibroblasts, epithelial cells, and SMCs [7]. Furthermore, KLF5 is well known to possess an anti-apoptotic function that was shown to induce the expression of Survivin, a survival factor making cells resistant to apoptosis in leukemia [8]. Previously, Survivin was thought to be expressed primarily in cancer cells, but now newer studies also have linked Survivin to the pathogenesis of PH showing that Survivin is expressed in the pulmonary vasculature of experimental PH and in lungs of patients with idiopathic PH [9]. In experimental PH, pulmonary Survivin expression was decreased after downregulation of KLF5 by siRNA [5]. Moreover, in vivo inhibition of Survivin reversed the pro-proliferative and anti-apoptotic phenotype of SMC and resulted in prolonged survival in experimental PH [9].

We hypothesized that expression of KLF5 and Survivin is increased in nitrofen-induced CDH and that activated and phosphorylated Survivin (p-Survivin) may lead to pathological changes in the pulmonary vasculature. We, therefore, designed this study to determine KLF5 and Survivin gene and protein expression as well as the activation of the transcription factor Survivin.

## Materials and methods

### Animals and drugs

Timed-pregnant, adult *Sprague–Dawley* rats (purchased at Harlan Laboratories, Sharnlow, UK, weight range 250–300 g) were randomly divided into two experimental groups (“CDH” and “Control”). The presence of spermatozooids in the vaginal smear was considered as proof of pregnancy; the day of observation was determined as day 0 (D0). On D9, animals in the CDH group received 100 mg of nitrofen (2,4-dichloro-p-nitrophenyl ether, WAKO Chemicals, Osaka, Japan) intragastrically dissolved in 1 ml of olive oil. Animals in the control group received only vehicle. On D21, dams were anesthetized with 2 % volatile isofluran (Piramal Healthcare UK, Morpeth, UK), followed by delivery of the fetuses via caesarean section. The fetuses were sacrificed by decapitation and divided according to

the two experimental groups ( $n = 16$  per group). In the CDH group, laparotomy was performed for inspection of CDH. Left lungs with a diaphragmatic defect and controls were dissected via thoracotomy and stored native either at  $-80$  °C, in formalin or in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) at  $-20$  °C. All animal experiments were carried out according to the current guidelines for management and welfare of laboratory animals. The experimental protocol was approved by the Department of Health and Children (Ref. B100/4360) under the Cruelty to Animals act 1876 (as amended by European Communities Regulations 2002 and 2005).

### RNA isolation from left lungs (D21)

TRIzol reagent (Invitrogen) was used for the acid guanidinium thiocyanate–phenol–chloroform extraction method to isolate total RNA from D21 lungs ( $n = 8$  per group) according to the manufacturer’s protocol. Spectrophotometrical quantification of total RNA was performed using a NanoDrop ND-1000 UV–Vis spectrophotometer (Thermo Scientific Fisher, Wilmington, USA). The RNA solution was stored at  $-20$  °C until further use.

### cDNA synthesis and quantitative polymerase chain reaction

Reverse transcription of total RNA was carried out at  $85$  °C for 3 min (denaturation), at  $44$  °C for 60 min (annealing) and at  $92$  °C for 10 min (reverse transcriptase inactivation) using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, UK) according to the manufacturer’s instruction. The resulting cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) using a LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) in a total reaction mix of 20  $\mu$ l per well. Following gene-specific primer were used (Eurofins, Ebersberg, Germany): Rat *KLF5* sense primer 5’GCTTGCTGTCATGCTCACTACTAG-3’ and rat *KLF5* anti-sense primer 5’CCCATAC TGAGATGCGACTGC-3’ as well as rat *Survivin* sense primer 5’ACTTGTCCCAGCTTTCC-3’ and rat *Survivin* anti-sense primer 5’GTCACAATAGAGCAAAGCCACA-3’. For normalization purposes, real-time RT-PCR was performed for GAPDH as described previously [10]. After 5 min of initial denaturation at  $95$  °C, 55 cycles of amplification for each primer were carried out. Each cycle included denaturation at  $95$  °C for 10 s, annealing at  $60$  °C for 15 s, and elongation at  $72$  °C for 10 s. Relative mRNA levels of gene expression were determined using a LightCycler 480 System (Roche Diagnostics) and the relative changes in gene expression levels of *KLF5* and *Survivin* were normalized against the level of *GAPDH* gene

expression in each sample ( $\Delta\Delta C_T$ -method). Experiments were carried out in duplicate for each sample and primer and results for relative mRNA expression are stated as average value of both samples.

#### Western blot

Western blots were performed as two sets of experiments for each antibody. Fresh frozen left lungs ( $n = 4$  for each group) were thawed, sonicated, and proteins were isolated in lysis buffer containing 25 mM Tris-HCl, 50 mM NaCl, 5 mM  $MgCl_2$ , 1 mM EDTA, 1 % NP-40, 10 % glycerol, and 1 % protease inhibitor cocktail (Sigma-Aldrich Ireland, Wicklow, Ireland). Protein concentrations were determined by Bradford assay (Sigma-Aldrich Ireland). Protein concentrations were equalized by dilution with distilled water. For gel electrophoresis, a concentration of 30  $\mu$ g of total protein was used for every specimen and denatured in Laemmli sample buffer (Sigma-Aldrich Ireland). Gel electrophoresis for protein separation was performed using precast 10 % SDS polyacrylamide gels (NuPAGE Novex Bis-Tris gels, Invitrogen) in NuPAGE MES SDS running buffer (Invitrogen). Proteins were then transferred to 0.45  $\mu$ m nitrocellulose membranes (Millipore Corporation, Billerica, USA) by Western blotting. Following Western blotting, the membranes were blocked in 3 % BSA + 0.05 % Tween for 30 min before antibody detection. Primary antibodies against KLF5 (goat polyclonal, sc-31393, dilution 1:300, Santa Cruz Biotechnology, Santa Cruz, USA) and p-Survivin (rabbit polyclonal, sc-23758R dilution 1:500, Santa Cruz Biotechnology) were incubated overnight at 4 °C. On the next day, followed by extensive washing (4 h), the membranes were incubated with the secondary antibodies in a dilution of 1:10000 (donkey anti-goat, sc-2020, Santa Cruz; anti-rabbit-#7074 S, Cell Signalling Technology, USA) followed again by extensive washing. Detection was performed with a PIERCE chemiluminescence kit (Thermo, Fisher Scientific, Dublin, Ireland). Loading controls were performed with  $\beta$ -actin (ab-8227, Abcam) and GAPDH (sc-32233, Santa Cruz) after membrane incubation with stripping buffer (# 46430, Thermo Scientific, Rockford, IL USA) for 15 min, three 5 min washing steps and blocking of the membrane in 3 % BSA + 0.005 % Tween for 30 min.

#### Immunofluorescence staining and confocal microscopy

Fetal left lungs ( $n = 4$  for each group) were fixed with 10 % buffered formalin (Santa Cruz Biotechnology) overnight. Whole organs were washed overnight in phosphate buffered saline (PBS), embedded in O.C.T. Mounting Compound (VWR International, Leuven, Belgium) and frozen at  $-80$  °C. Frozen blocks were sectioned

transversely at a thickness of 15  $\mu$ m and mounted on SuperFrost Plus slides (VWR International). After washing with PBS, the sections for KLF5 and p-Survivin underwent cell membrane permeabilization with 1 % Triton X-100 for 20 min at room temperature. All sections were then washed and subsequently blocked with 3 % BSA for 30 min to avoid non-specific absorption of immunoglobulin. Blocking solution was rinsed off and sections were incubated with primary antibodies against KLF5 (rabbit polyclonal, ab137676, dilution 1:100, Abcam, UK) and p-Survivin (rabbit polyclonal, sc-23758R dilution 1:500, Santa Cruz) and alpha smooth muscle actin ( $\alpha$ -SMA, mouse monoclonal, M0851, 1:400 dilution in PBST, DAKO Diagnostics Ireland, Dublin, Ireland) overnight at 4 °C. After washing, KLF5 as well as p-Survivin sections were incubated with corresponding secondary antibodies (anti-mouse Alexa 488—ab150105 and anti-rabbit Alexa 594—ab150080; resp. anti-mouse Alexa 488—ab150105, anti-rabbit Alexa 647—ab150075, Abcam) for 1 h at room temperature. After washing, sections were counterstained with DAPI antibody (1:1000 in PBST, Roche), washed again and mounted using Sigma Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA). Sections were scanned with a ZEISS LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) and evaluated independently by two investigators.

#### Statistical analysis

All numerical data are presented as mean  $\pm$  standard error of the mean. Student's *t* test was used for evaluation of differences between two normal distributed groups on D21. The confidence interval was set at 95 %.

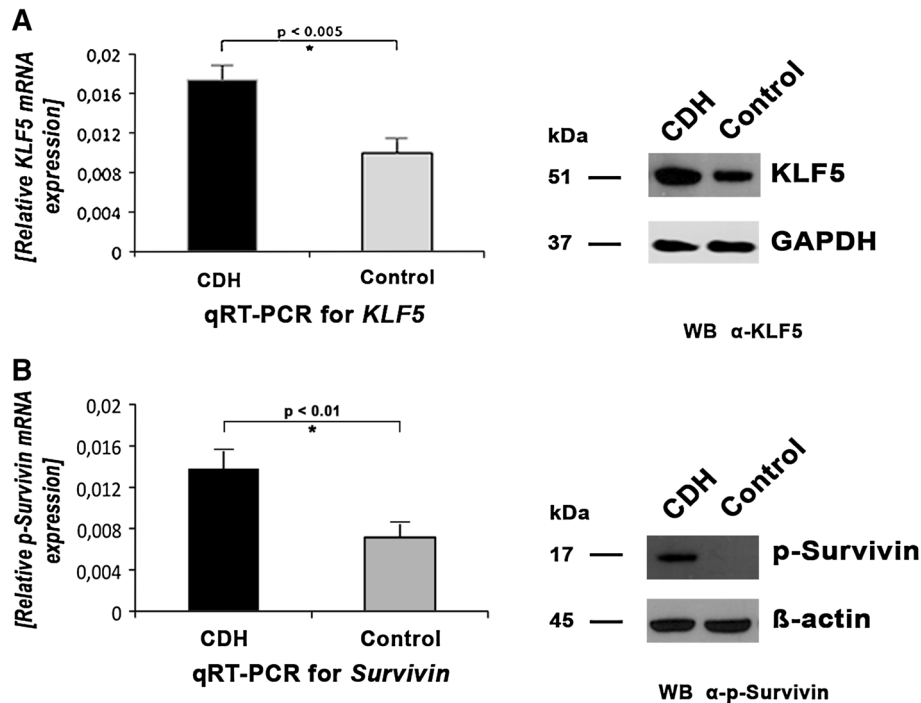
## Results

#### Relative mRNA expression levels of *KLF5* and *Survivin* in lungs

To determine the effects of nitrofen-induced pulmonary hypoplasia and hypertension on *KLF5* and *Survivin* gene expression, we performed qRT-PCR. The relative mRNA expression levels of *KLF5* and *Survivin* were significantly increased ( $p < 0.005$  resp.  $p < 0.01$ ) in lungs (D21) of the CDH group (Fig. 1), compared to lungs of control animals.

#### Western blot for KLF5 and p-Survivin

To show phosphorylation and, therefore, activation of Survivin in CDH lungs and to confirm that the quantitative increase of *KLF5* and *Survivin* mRNA transcripts in CDH lungs were translated to the protein level, we performed



**Fig. 1** qRT-PCR revealed significantly increased relative mRNA expression levels of **a** *KLF5* ( $p < 0.005$ ) and **b** *Survivin* ( $p < 0.01$ ) in nitrofen-exposed lungs (D21) compared to normal lung tissue. Western blot results show that the increased *KLF5* (**a**) and *Survivin* (**b**) mRNA transcripts in nitrofen-exposed lungs compared to controls resulted in increased amounts of KLF5 (**a**) and Survivin (**b**) protein expression. Western blot results show increased protein expression of

*KLF5* **a** in CDH lungs compared to control lungs. Furthermore, western blot revealed that increased *Survivin* transcripts in nitrofen-exposed lungs compared to controls resulted in increased amounts of activated p-Survivin **b** protein expression. Equal loading of electrophoresis gels was controlled by Bradford assay and confirmed by beta-actin and GAPDH staining of the stripped membranes (**a,b**)

western blotting to analyze the protein expression of KLF5 and p-Survivin. Our western blot results showed increased pulmonary protein expression for KLF5 and p-Survivin in the nitrofen-treated group when compared to controls (Fig. 1). Equal loading of electrophoresis gels was confirmed by beta-actin loading control of the stripped membranes (Fig. 1).

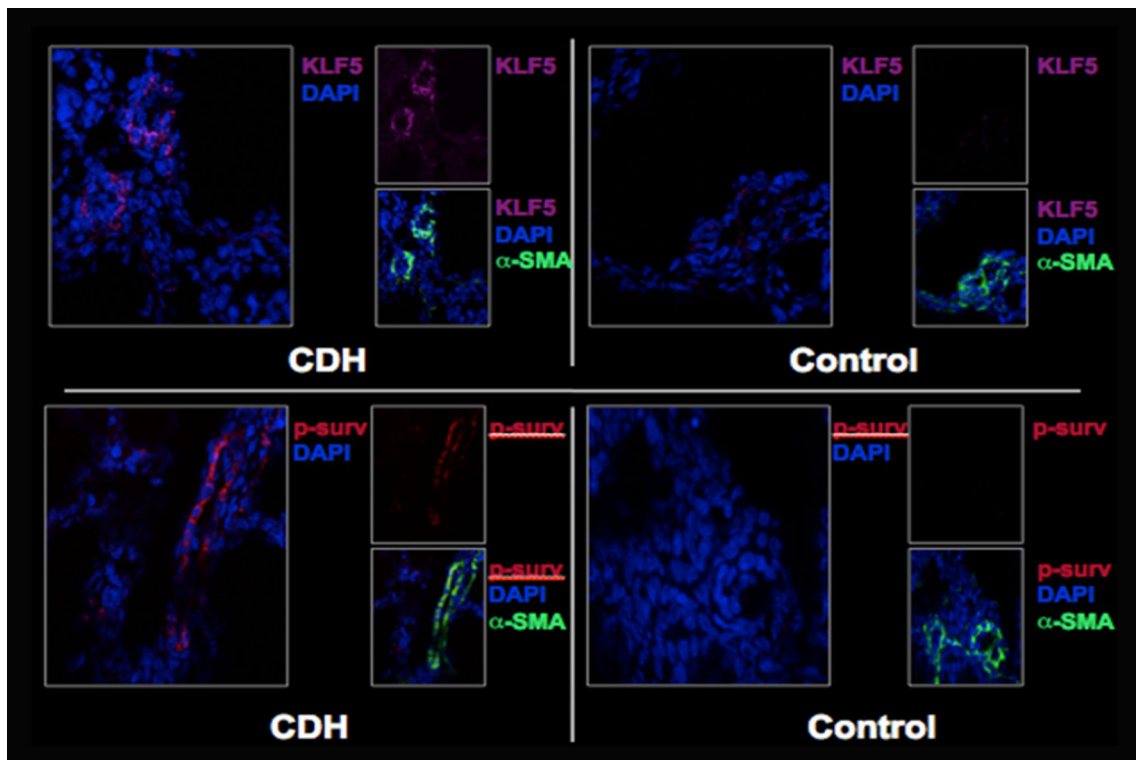
Immunofluorescence evaluation of KLF5, p-Survivin, and  $\alpha$ -SMA

Immunofluorescence evaluation of pulmonary tissue was performed to show lung morphology, protein expression, and localization of KLF5 and p-Survivin. We found noticeable increased medial and adventitial thickness of pulmonary arteries in the CDH group. Confocal microscopy confirmed qRT-PCR results as well as the western blot findings for KLF5 showing increased vascular KLF5 expression in SMCs of nitrofen-exposed lungs compared to control lung tissue (Fig. 2). Furthermore, we observed increased expression of phosphorylated p-Survivin of the pulmonary vasculature in comparison to control lungs, corroborating the western blot results.

## Discussion

To our knowledge, this is the first study investigating the expression of KLF5 and its, by phosphorylation activated, downstream target transcription factor p-Survivin in the pulmonary vasculature of nitrofen-induced CDH in rats. Significant upregulation of *KLF5* and *Survivin* gene expression was accompanied by increased protein expression of KLF5 and p-Survivin. Confocal microscopy confirmed these results of increased KLF5 and p-Survivin expression in the pulmonary vasculature of hypoplastic lungs.

The pathophysiology of PH involves multiple and complex mechanisms [11]. The role of endothelial cell dysfunction, impaired angiogenesis and vasculogenesis as well as the imbalance of proliferation and apoptosis of pulmonary vascular SMCs in the pathogenesis of PH has been recently outlined by several authors [2, 12, 13]. Impaired homeostasis of SMC proliferation and apoptosis of arterial SMCs leads concurrently to structural changes and vascular remodeling, such as increased adventitial and medial thickness of the vessel wall and extension of smooth muscle into previous non-muscularized arteries



**Fig. 2** Immunofluorescence evaluation of pulmonary tissue for KLF5 (a) and p-Survivin (b) as well as α-SMA (a,b). There was marked increase in medial and adventitial thickness in pulmonary arteries of all sizes in the CDH group compared to controls. α-SMA was used to

identify pulmonary arteries. Confocal microscopy further revealed increased vascular KLF5 and p-Survivin expression in the pulmonary vasculature of nitrofen-exposed lungs compared to normal lung tissue (a, b)

resulting in increased pulmonary vascular resistance and a rise in pulmonary arterial pressure [14, 15]. These pathological changes of the pulmonary microvasculature seen in infants with CDH and associated PH are strikingly similar to those seen in adult idiopathic PH, where endothelial cell dysfunction, SMC proliferation, and vasoconstriction occur as results of pathological vascular remodeling [2, 16]. Existing models of experimental PH, such as the hypoxia-induced PH model or the monocrotaline model, mainly focus on adult or post-natal PH [17, 18]. However, the underlying structural and molecular mechanisms of the pathogenesis of PH in CDH that lead to pulmonary vascular remodeling in utero are not fully understood. In this study, we used the well-established nitrofen-induced CDH model, which has been used since over 40 years and has provided some important new insights into the pathogenesis of CDH [1]. Maternal exposure of nitrofen in both mouse and rat models during a specific time in gestation results in a high rate of CDH with associated pulmonary hypoplasia and pulmonary vasculature abnormalities, strikingly similar to the human situation. Of all the surgical, transgenic, and toxicological models of CDH, the nitrofen model appears to be the best model available to date [1, 2].

Krüppel-like factor 5 is a zinc finger transcription factor that is implicated in important biological functions including cell proliferation, apoptosis, and developmental processes [7, 19]. As a basic transcription factor, KLF5 regulates a number of target genes, such as *cyclines*, *PDGFα*, and *Survivin* [5, 7]. It is well accepted that KLF5 is accountable for vascular remodeling in cardiovascular disease and is preferentially expressed in proliferating SMCs, but reduced in differentiated cells under physiological and pathological conditions [4, 6]. Recently, KLF5 has also been suggested to play a role in the pathogenesis of both human and experimental PH [5]. Courboulin et al. found increased levels of KLF5 in human and rodent pulmonary hypertensive lung tissue compared to normotensive lung samples. Pulmonary KLF5 levels correlated directly with severity of PH, in both human and experimental PH. Moreover, in vivo inhibition of KLF5 in monocrotaline-treated rats improved PH by reducing right ventricular hypertrophy and the mean pulmonary arterial pressure [5].

In the present study, western blotting and immunofluorescence staining confirmed that increased amounts of *KLF5* mRNA transcripts in lungs of nitrofen-induced CDH compared to controls were translated to the protein level, as observed in the pulmonary vasculature of hypoplastic



lungs. These results suggest that increased KLF5 protein expression may lead to SMC proliferation and consecutive pulmonary vascular remodeling in nitrofen-induced CDH. Additionally, KLF5 has been shown to have an anti-apoptotic function. In vitro studies investigating pulmonary arterial SMCs from patients with PH revealed that inhibition of KLF5 by siRNA decreased the expression of Survivin, a survival factor thus making the cells prone to apoptosis [5].

Survivin has been reported to essentially promote cell proliferation under pathological conditions [20]. It is expressed in pulmonary arteries of patients with PH as well as in the pulmonary vasculature of experimentally induced PH in the monocrotaline model of PH. Similar to human tissue, Survivin was not expressed in control tissues. To determine the role of Survivin in the pathogenesis of PH rats were treated with an inhaled adenoviral gene therapy. In this experimental model of PH, the inhibition of endogenous Survivin in vivo improved survival as well as pulmonary hemodynamics and resulted in a significant reduction of medial thickness in small- and medium-sized pulmonary arteries [9]. In our study, we found increased *Survivin* gene expression in CDH lungs compared to controls. Furthermore, we did not see any protein expression of activated p-Survivin in control lung tissue. On the contrary, western blotting and confocal immunofluorescence demonstrated protein expression of the activated form of Survivin in hypoplastic CDH lungs. These results imply that *Survivin* transcripts in lungs of nitrofen-induced CDH were translated to the protein level and that increased *Survivin* expression is followed by post-translational modification of Survivin phosphorylation suggesting that the activation of pulmonary Survivin is biologically relevant in the nitrofen-induced CDH model.

One of the major challenges in finding a therapeutic target for PH is that the pulmonary circulation is selectively diseased and that the majority of drugs targeting the vasculature are given systemically and, therefore, may have an affect on healthy circulation and thereby limit the efficacy or have adverse systemic cardiovascular effects [21, 22]. However, the approach of an inhaled gene therapy that selectively inhibits Survivin expression and consecutively leads to reversal or inhibition of SMC proliferation in the pulmonary vasculature may be a promising new therapeutic approach for PH in CDH [9].

Taken together, this study provides striking evidence of the markedly increased gene and protein expression of KLF5 and its downstream transcription factor Survivin in the pulmonary vasculature of nitrofen-induced CDH. Our results suggest that the activation of p-Survivin induced by increased expression of KLF5 may be linked to the development of PH in the nitrofen-induced CDH.

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