The Cellular Origin of

Congenital Diaphragmatic Hernia and Potential Translational Approaches

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The Cellular Origin of

Congenital Diaphragmatic Hernia and Potential Translational Approaches

De cellulaire grondslag van congenitale hernia diafragmatica en de potentiele translationele aanpak

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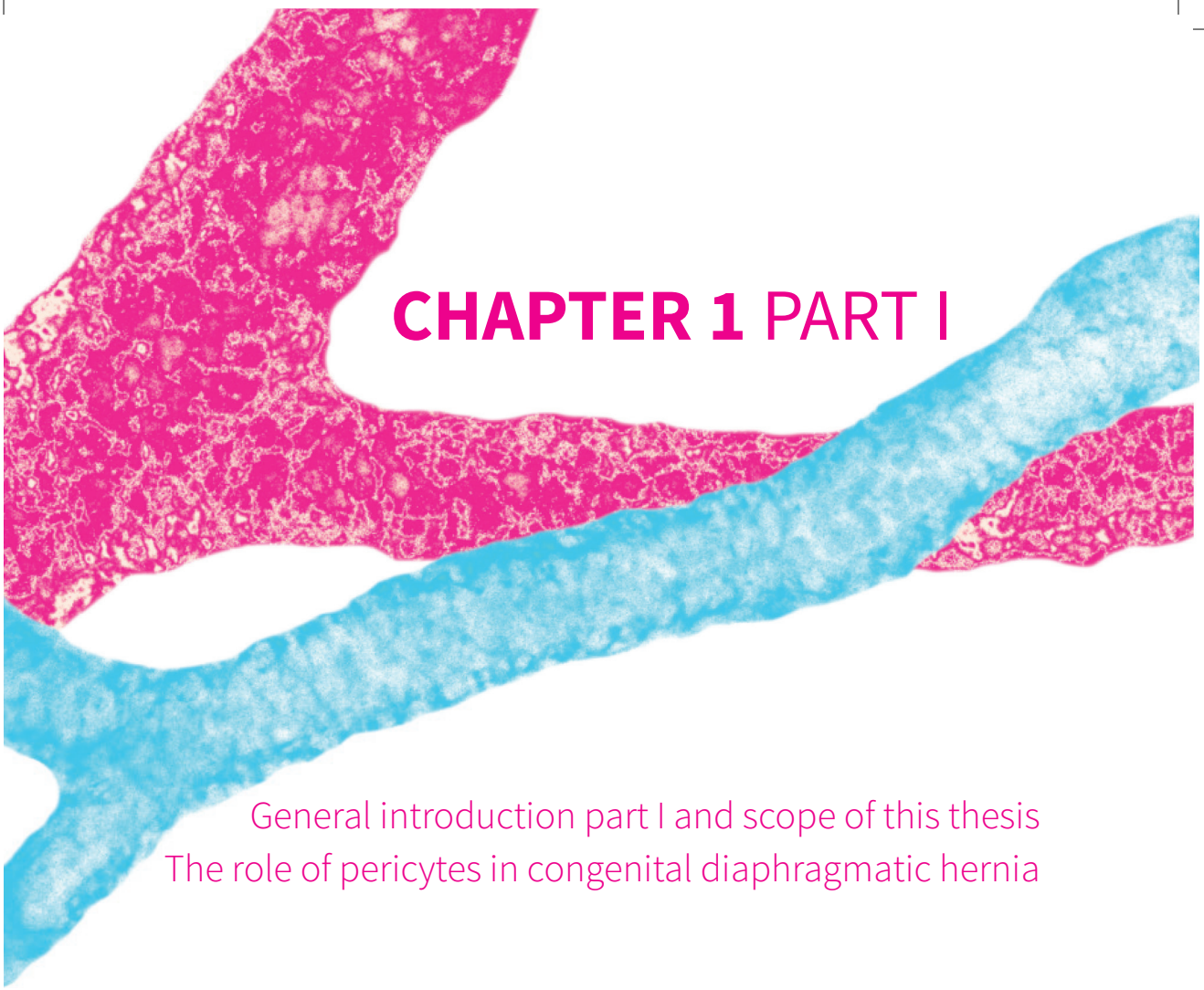
Paranimfen
Evelien Eenjes
Daphne Mous

Voor Maarten
Home is wherever I'm with you

Contents



CHAPTER 1 PART I

General introduction part I and scope of this thesis
The role of pericytes in congenital diaphragmatic hernia

Pulmonary hypertension associated with CDH is characterized by extensive muscularization of the vessels, which is already noticeable early in gestation. This indicates that the structural abnormalities start to develop when the lung is very immature. Previously, we have shown that the pulmonary vasculature mainly develops through angiogenesis[1]. The process of angiogenesis is described as a mechanism where endothelial cells sprout from pre-existing vessels to form new tubules [2]. Newly formed tubes need to be stabilized by pericytes and this happens in a PDGFβ depended manner[3 5]. Many studies have studied angiogenesis in the vasculature of the systemic circulation and in vascular tumor growth [2, 4]. To better understand the onset of the pathological features of pulmonary hypertension (PH) associated with congenital diaphragmatic hernia (CDH), a detailed analysis of the vascular development and organization under normal conditions and under specific pathological conditions is required. Understanding the development and organization of pulmonary vascular development in the normal condition could help to elucidate the pathological features of PH. The pathology of PH is characterized by hypermuscularization of the midsized and large vessels and neomuscularization of the small capillaries. Pericytes are prime candidates to underlie and eventually modulate the structural changes observed in PH associated with CDH [5, 6]. However, little is known about the pericyte population during lung development. Pericytes in the proximal end of the lung have been shown to originate from a multipotent cardiacpulmonary progenitor pool of cells [7].This suggests that pericytes in the proximal end of the lung originate from a different progenitor pool than pericytes in the distal end of the lung. The different origins of pericytes within the lung indicate the heterogeneous nature of the pericyte population.

Differences in pericyte coverage have been linked to multiple diseases such as diabetic retinopathy, cancer and adult pulmonary arterial hypertension[8 9 10]. Therefore, we hypothesized that alterations in pericyte coverage in CDH is the first pathological event in the development of hypermuscularisation of the pulmonary vascular wall and neomuscularization of the capillaries (Figure 1). Furthermore, alterations in pericyte coverage in combination with aberrant expression of the contractile marker ACTA2 indicate the start of aberrant pericyte muscularization (Figure1). Muscularization of pericytes in CDH may hamper their function in angiogenesis resulting in reduced growth of the pulmonary vasculature, in particular the capillary bed. Thus, a cascade of events during the different phases of lung development can eventually lead the pathological characteristics of PH (Figure 1).

Further identification of differences in pulmonary vascular cell populations may help to understand how PH associated with CDH arises. Whole transcriptome analysis has been proven to be effective in revealing molecular pathways in developmental processes [11]. This method provides an excellent opportunity to reveal new differentially expressed markers and thereby revealing new molecular mechanisms in the CDH cell populations. Once executed these kinds of studies can be very valuable in characterizing not only pathological mechanisms but also understanding normal developmental mechanisms.

Regarding treatment possibilities for CDH, current therapeutic opportunities and treatment, which are beneficial for the development of the vasculature should be carefully considered. Sildenafil treatment has been effective in pulmonary hypertension associated with congenital heart disease [12]. It is of high importance to identify the narrow window of opportunity for the administration of sildenafil since CDH is usually diagnosed during the 20-week ultra sound examination of all pregnancies in the Netherlands

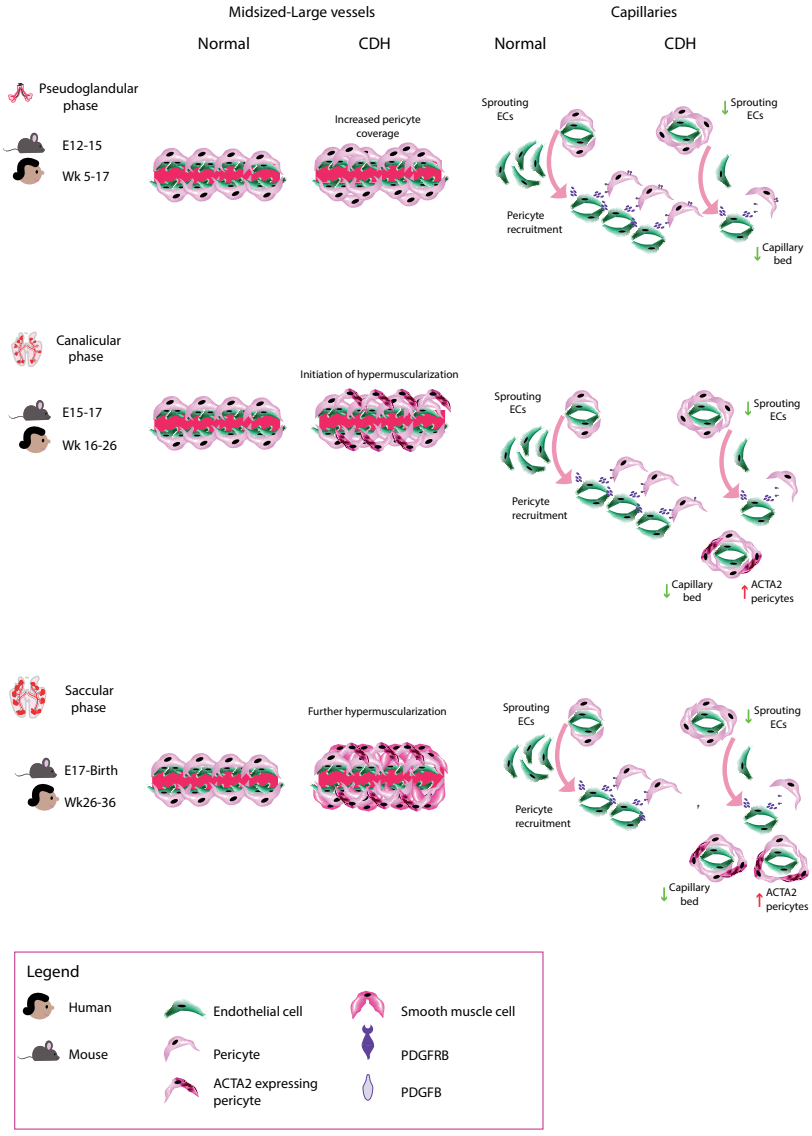


Figure 1

Multiple events during the pulmonary vascular development in congenital diaphragmatic hernia lead to fewer capillaries and extensive muscularization of the mid-sized vessels and neo-muscularization of the small capillaries.

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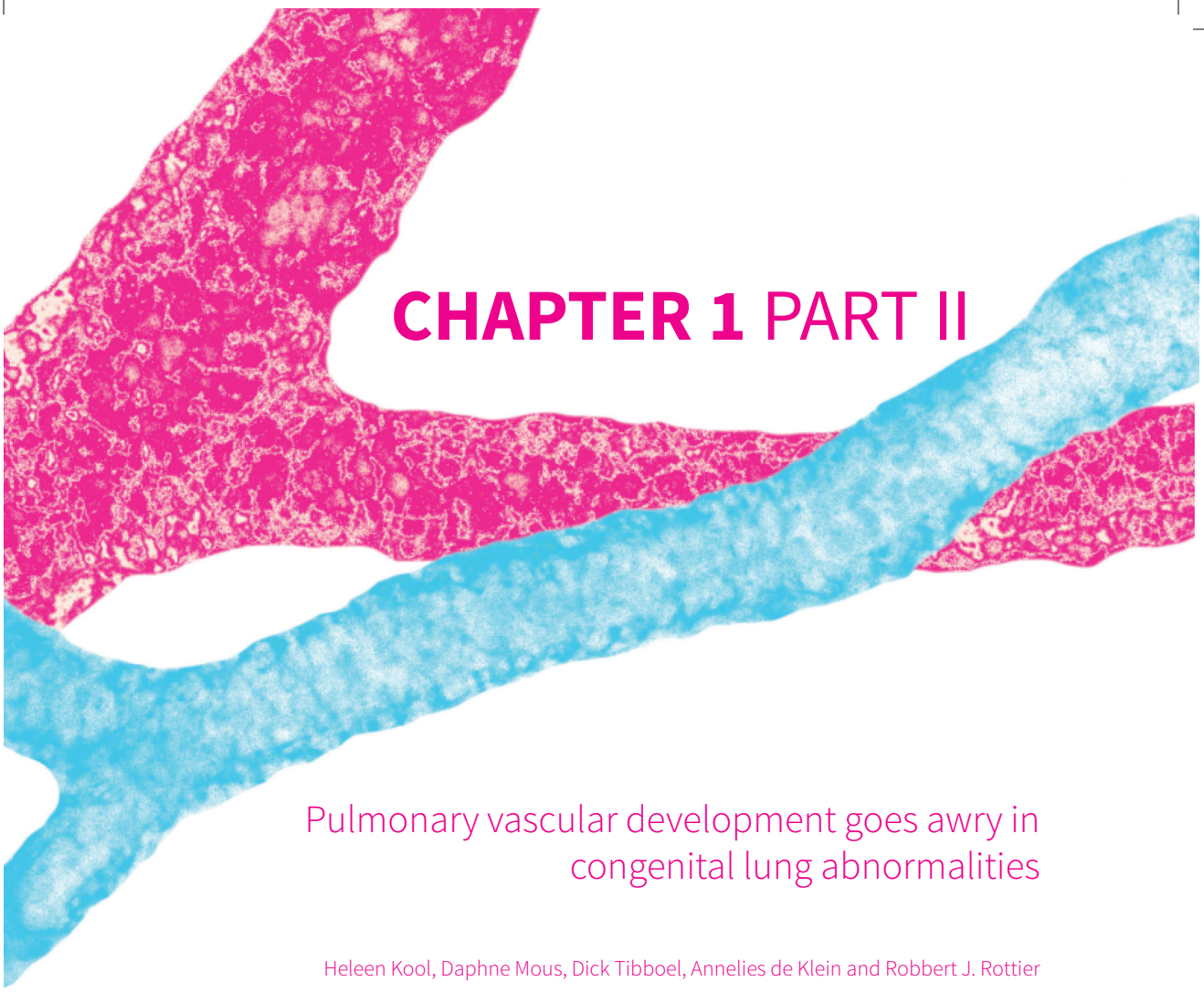
Scope of the thesis

The aim of this thesis is to identify early structural changes and the associated molecular mechanism associated with these changes. Together these results should provide new insights into the development of PH associated with CDH.

In the first chapter I give an overview of the current state of research towards pulmonary vascular abnormalities associated with congenital diseases. Furthermore, I suggest that multiple congenital lung diseases show similar changes in the vasculature, which implies that these diseases may have some overlap in origin and cause.
In the second chapter I investigate the effect of retinoic acid inhibition on the development of the pulmonary vasculature. Delicate immunohistological analysis in combination with FACS experiments of pulmonary vascular development could be a first step in understanding the vascular changes in CDH. NG2 was identified as a specific pericyte marker during lung development. Furthermore immunofluorescent whole mount analysis together with FACS analysis showed increased pericyte coverage from the late pseudoglandular phase in the CDH mouse model. In addition, alterations in proliferation, migration and differentiation were observed after the inhibition of the retinoic acid pathway.

The third chapter describes the whole transcriptome analysis of four different cell populations isolated from embryonic lungs of E13 normal and CDH mice. This analysis facilitates to find the connection between genes, which are known to be involved in CDH to specific cell populations. Additionally, further analysis of RNA sequence data revealed downregulation of KLF4 in the endothelial cell population in CDH. KLF4 acts as a upstream regulator of NOTCH signaling, which is required for activation of the tip cell and thereby initiates the sprouting of endothelial cells to form new tubules. The downregulation of KLF4 therefore underlies the simplification of the capillary bed observed in CDH. The downregulation of KLF4 was further confirmed with whole mount immunofluorescent analysis.
The fourth chapter describes the effects of treatment with the PDE5 antagonist sildenafil. Moreover, time pregnant rats were treated with nitrofen and during the canalicular phase treated with sildenafil. This resulted in beneficial effects for the pups with CDH. The body weight improved, the lung/kidney ratio improved and the alveolar airspaces increased in diameter.
In the fifth chapter, the general discussion, I summarize our own findings of the different studies and describe future possibilities to study CDH.



CHAPTER 1 PART II

Pulmonary vascular development goes awry in congenital lung abnormalities

Heleen Kool, Daphne Mous, Dick Tibboel, Annelies de Klein and Robbert J. Rottier

Abstract

Pulmonary vascular diseases of the newborn comprise a wide range of pathological conditions with developmental abnormalities in the pulmonary vasculature. Clinically, pulmonary arterial hypertension (PH) is characterized by persistent increased resistance of the vasculature and abnormal vascular response. The classification of PH is primarily based on clinical parameters instead of morphology and distinguishes five groups of PH. Congenital lung anomalies such as alveolar capillary dysplasia (ACD) and PH associated with congenital diaphragmatic hernia (CDH), but also bronchopulmonary dysplasia (BPD), are classified in group three.

Clearly, tight and correct regulation of pulmonary vascular development is crucial for normal lung development. Human and animal model systems have increased our knowledge and make it possible to identify and characterize affected pathways and study pivotal genes. Understanding of the normal development of the pulmonary vasculature will give new insights in the origin of the spectrum of rare diseases such as CDH, ACD and BPD, which render a significant clinical problem in neonatal intensive care units around the world.

In this review we will describe the normal pulmonary vascular development and we will focus on four diseases of the newborn in which abnormal pulmonary vascular development play a critical role in the morbidity and mortality. In the future perspective we indicate the lines of research that seems to be very promising for elucidating the molecular pathways involved in the origin of congenital pulmonary vascular disease.

The morphology of the pulmonary vasculature

In mammals, blood is transported through the cardiovascular system that can be divided in the systemic and the pulmonary circulation. These two types of circulations have histological similarities but differ in their physiological function and anatomic position to the heart. Oxygenated blood is transported and distributed throughout the body by the systemic circulation, whereas oxygen depleted blood is transported to the lungs by the pulmonary circulation. The blood supply in the lung can be divided into the bronchial circulation and the pulmonary circulation. The bronchial circulation is mainly separated from the pulmonary circulation, although some overlap exists in the pre capillary region. The bronchial circulation comprises arteries, which align with the bronchial tree. A third of the blood in the bronchial circulation returns to the right atrium through the bronchial vein. The pulmonary circulation transports oxygen deprived blood to the gas exchange areas and oxygen-rich blood back to the left atrium. The bronchial circulation is part of the systemic circulation and delivers oxygen rich blood to the cells of the lung at high systemic pressure.

The pulmonary vasculature comprises anatomically and functionally different compartments: the arterial tree, the capillary bed and the venular tree. The pulmonary arteries also support the intrapulmonary structure and ultimately regulate gas exchange via the capillary bed. Prenatally, the pulmonary circulation is characterized by high pulmonary vascular resistance (PVR) and low blood flow (compared to the ventricular output). The thick wall and high vasomotor tone contribute to the high PVR. The majority of the blood flow of the cardiac output is diverted to other organs than the lung through the foramen ovale and the ductus arteriosus. This process is facilitated by the relative high resistance in the pulmonary circulation compared to the systemic circulation. After birth, there is a large transition from relative hypoxic conditions to normoxic condition. This transition induces dramatic changes in the PVR leading to physiological adaptations in the lung. This adaption of the lung is required to exerts its important function exchange gas and oxygenate the blood.

The cellular composition of the pulmonary vascular wall varies depending on the functionality of the vessel. The outer layer of the pulmonary arteries, the adventitia, is a loosely organized structure consisting of an extracellular matrix with fibroblasts, vasa vasorum and a neuronal network [1, 2]. There is gradual change in structure from the proximal to distal end of the lung, which corresponds with the maturation of the developing airways. The large pulmonary arteries at the proximal end of the lung have a media consisting of a layer of smooth muscle cells in between the lamina elastic interna and externa. Towards the distal area of the lung, the arteries have a smaller lumen with a thinner smooth muscle cell layer and no lamina elastica. The smooth muscle cells in the tunica media form a heterogeneous population, ranging from cuboidal, synthetic cells to the characteristic elongated contractile cells. The contractile smooth muscle cells have more contractile fibers, have less proliferation and

less migration activity compared to the synthetic phenotype [3, 4]. The pulmonary capillaries are the most distal compartment of the pulmonary vasculature and are the site where gas exchange takes place. Capillaries exist of a monolayer of endothelial cells, which are in direct contact with perivascular cells. The structure of the pulmonary veins is comparable to the structure of small arteries. Pulmonary veins consist of a thin intima, smooth muscle cell containing media in the larger veins and an adventitia containing a vaso vasorum, nerves and bundles of collagen and elastin fibers [2].

The development of the pulmonary vasculature

Understanding the process of normal pulmonary vascular development is a prerequisite to comprehend the origin of pulmonary hypertension and its associated diseases of the newborn. The pulmonary vasculature develops in close relation with the airways and has extensively been studied in rodent models. In mice, the first molecular sign of lung development is around embryonic day 8 when the expression of Nkx2-1 starts in the ventral wall of the anterior foregut (see table 1 for lung developmental stages of human and mouse). At embryonic day 9.5 (E9.5) in the mouse, a primitive bud evaginates from the ventral side of the foregut and invades the surrounding mesenchyme [5]. This bud splits into two buds, which will form the right and left lung, but this embryonic phase is very short and rapidly turns into the pseudoglandular phase when the primary buds expand into the mesenchyme and start budding and branching until E16.5. After E16.5, when the bronchial tree is formed, development of the lung goes into a new stage, the canalicular phase. In mice it is very short (E16.5-E17.5) and during this period the terminal buds narrows. From E17.5 until postnatal day 5 (P5) lung development goes into the saccular stage and the precursors of the alveoli are formed. And finally from postnatal life onwards alveolarization starts and ends around P14. In humans, lung development follows a similar sequence of stages, but with a different timetable. Budding starts at four weeks of gestation, the pseudoglandular stage ends around week 6, followed by the canalicular (week 16-26), saccular (week 26-36) and alveolarisation (postnatal until 3 years of age) stages (Table 1).

Table 1 Overview of stages in lung development in mouse and human

Stage	I Embryonic	II Pseudoglandular	III Canalicular	IV Saccular	V Alveolar
Mouse	E9-12	E12-15	E15-17	E17-Birth	Birth-P20
Human	Wk 3-7	Wk5-17	Wk16-26	Wk26-36	Wk36-3Years

The lung endoderm and mesoderm are interacting during all these developmental stages via multiple molecular pathways. These molecular pathways controlling these stages have been discussed in extensively in two recent reviews [6 5]. In this review we focus on congenital

diseases associated with pulmonary abnormalities and only describe the molecular players that have been associated with these diseases.

The past two decades new insights into the development of the pulmonary vasculature have been obtained. It was suggested that pulmonary vasculature in mice developed through two main mechanisms: the central vasculature through angiogenesis and the distal vasculature through vasculogenesis and either angioblasts from the mesenchyme or blood lakes would provide endothelial cells for vessel development [7]. These two structures would fuse around embryonic day E13/E14 through a lytic process and circulation would start [8]. A histological and morphological study seemed to confirm this hypothesis and the same processes would underlie pulmonary vascular development in human [9]. The results from these studies were mainly obtained by histological analysis. However, fixation artifacts have led to inappropriate conclusion and analysis of lung development using transgenic mice expressing a lacZ reporter gene under the control of an early marker for endothelial cells (fetal liver kinase 1 (Flk1)) [10], showed that the proximal and distal pulmonary vasculature was already connected at embryonic day 10.5 [11]. In addition, detailed analysis of lung samples of transgenic mice expressing the lacZ reporter under the control of the endothelium specific Tie2 promoter showed that already at day E9.5 the presence of a vascular network surrounded the primitive lung bud connected to the systemic circulation. This network mainly expands as the lung develops through angiogenesis, a process called distal angiogenesis [12]. It is still not completely understood how the pulmonary vasculature develops and where progenitor cells involved in angiogenesis in the lung come from. Lineage trace experiments, instrumental in deciphering the origin and the fate of early precursor cells in the lung, indicate that specific, cardiopulmonary, progenitor cells differentiate into both cardiac and pulmonary mesenchymal cells. Moreover, these progenitor cells can differentiate into vascular smooth muscle cells and pericyte-like cells, but were only observed in the proximal end of the lung [13]. It remains unclear what the progenitors are for the perivascular cells and endothelial cells in the distal end of the lung. Proper lineage trace studies throughout pulmonary vascular development could serve to answer these questions.

Important molecular players in pulmonary vascular development

Normal pulmonary vascular development requires tight regulation of cell migration, proliferation and differentiation. The family of Vascular endothelial growth factors (Vegf), and their receptors Fetal liver kinase1 (Kdl1 or Vegfr1) and Kinase domain receptor (Kdr or Vegfr2), are one of the most potent angiogenic factor signaling cascades and are required for vascular growth and endothelial cell proliferation [14, 15]. Early during lung development, Vegf is expressed by the epithelium and mesenchyme, but later its expression is restricted to the epithelium [16] where it is required for epithelial branching and morphogenesis [17].

In response to hypoxic conditions, as in the prenatal lung, Vegf expression is induced by Hypoxia-inducible transcription factor-1 and 2 (Hif1/Hif2). Hif1 and Hif2 are heterodimers existing of an oxygen sensitive subunit, Hif1α or Hif2α, and a constitutive Arnt/Hif1β subunit. At normoxic conditions, specific prolyl hydroxylases (Phd) hydroxylate the Hifα subunit, which is subsequently ubiquitinated and targeted for degradation via the Von-Hippel-Lindau tumour suppressor protein pathway [18-20]. Under hypoxic conditions, the Hifα subunit is not hydroxylated and the Hifα/Hifβ complex translocates to the nucleus where it binds to hypoxic responsive elements in the regulatory unit of target genes to induce the transcription of these genes. Among the genes that are activated under hypoxic conditions are several angiogenic genes, such as Vegf, which results in the growth and expansion of the vasculature.

Vascular development is consists of vasculogenesis and angiogenesis: vasculogenesis is the process where the vascular plexus is formed de novo from mesodermal progenitor cells [21], and angiogenesis is the process where endothelial cells sprout from preexisting vessels to form new tubes. There is constant competition between the leading cell, the tip cell, and the trailing cell, the stalk cell, to become or to stay on the tip of the sprout. Endothelial cells of the newly formed tubes recruit pericytes in a Platelet-derived growth factorβ (Pdgfβ) depended manner (Figure 1). Pericytes wrap around the newly formed endothelial tubes and induce stabilization and maturation [22 23] and the interaction between these two cells is crucial for normal vascular development. This interaction is regulated by different growth factors and their receptors, such as Pdgf(r) and Tgfb(r) [24]. Tight regulation of this interaction is required for normal vascular development and disruption of this process may lead to pathological conditions. However, pericytes comprise a very heterogenic population in the lung and therefore they are rather difficult to identify. New, specific markers are required to better understand the interaction of pericytes and endothelial cells in both health and disease.

The specification of arteries and veins is one of the first events that take place in the development of the circulatory system. Arteries and veins can be distinguished from each other by the expression of members of a tyrosine kinase family Ephrin2 and Eph4 [25]. However, the specification of the pulmonary network occurs relatively late and the expression of Ephrin2 and Eph4 is not restricted to artery endothelial or vein endothelial cells, respectively until late in the pseudoglandular stage (Figure 1). In mice, at E13.5 endothelial cells still express both Ephrin2 and Eph4, but from E15.5 onwards the endothelial cells express either Ephrin2 or Eph4 when they become committed to arteries or veins, respectively. Furthermore, Ephrin expression in the lung is not restricted to endothelial cells but is also highly expressed by mural cells [26]. Modulation of the Notch pathway results in arterial defects and can lead, depending on which member of the pathway is affected, to early prenatal death,.. For example, heterozygous *Dll4* embryos suffer from remodeling defects in the yolk sac and have a smaller dorsal aorta [27] while the full *Dll4*-deficient embryos die due

to early lethal loss of arterial identity at E9.5[28]. To study the lung developmental phenotype, tissue specific inhibition of the Notch pathway is necessary and may give new insights in the specification of pulmonary arteries and veins. Specification of venous endothelium includes expression of the nuclear receptor Chicken ovalbumin upstream transcription factor II (CouptfII), which is expressed in venous and lymphatic endothelium (Figure 1). CouptfII is highly expressed in the foregut mesenchyme at the site where later in development the lung will be formed. CouptfII knock-out mice die at E10 from heart defects and loss of venous identity in the vasculature [29]. Lung specific deficient CouptfII mice show a Bochdalek-type congenital diaphragmatic hernia (CDH) [30], lung hypoplasia associated with CDH indicates the importance of CouptfII in normal lung development.

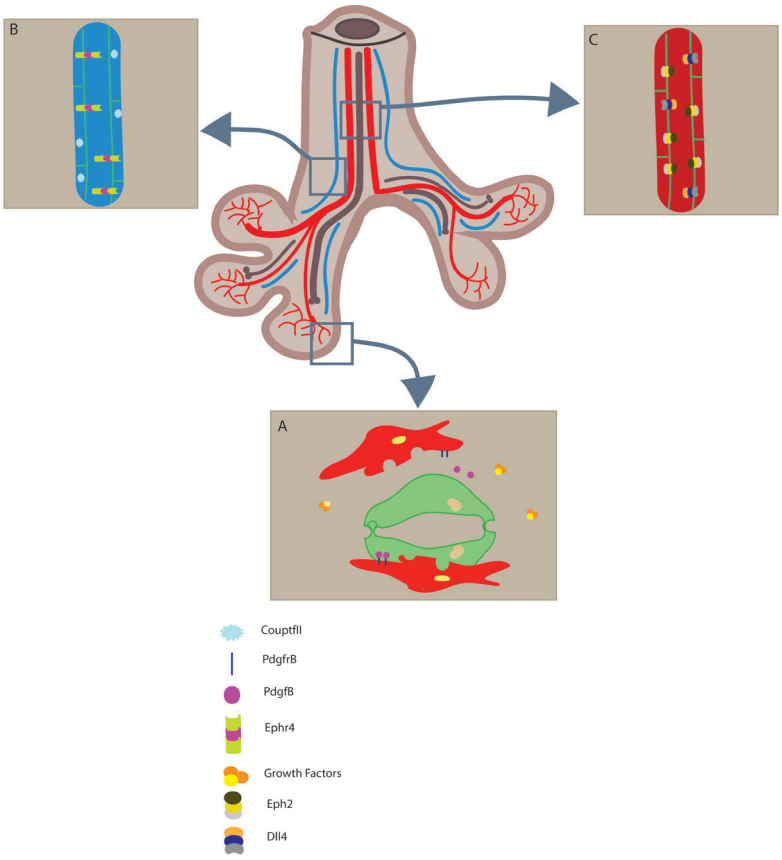


Figure 1: Simplified scheme of pulmonary vascular compartments

Schematic overview of pulmonary vasculature, with veins (A), arteries (B) and capillaries (C). Endothelial cells recruit pericytes in a Pdgfβ dependent manner in the distal end of the lung(C). The pulmonary arteries are characterized by the expression of Eph2 and Notch family member Dll4 (A). Specification of the pulmonary veins includes expression of Ephrin4 and CouptfII (B).

Fibroblast growth factors (Fgf) belong to a family of mitogens that are identified as regulators of lung development [31]. Early during lung development Fgf10 is expressed in the mesoderm around the budding lung endoderm, which expresses its receptor Fgfr2. Knockout mice of Fgf10 [32] or Fgfr2 [33] resulted in mice without lungs, indicating the crucial role for this signaling pathway in the development of the lung. [34]. However, recently it was shown that Fgf10 is not just inducing budding and branching of the lung during development, but that expression of Fgf10 is also important for the maintenance of epithelial progenitor cells by preventing these cells to differentiate [35]. Another member of the fibroblast growth family, Fgf9, is important for lung mesenchyme growth and proliferation [36]. More specific, Fgf9 stimulates proliferation of mesenchymal cells and regulates mesenchymal Sonic hedgehog signaling (Shh). [37]. Furthermore, it is also shown that Fgf9 and Shh regulate Vegfa expression what is required for capillary development in the distal end of the lung [17].

Retinoic acid (RA) signaling has been shown to be of high importance for lung development [38]. Vitamin A in the blood plasma is transported by Retinol binding protein 4 (Rbp4), it binds to the extracellular receptor Stimulated by retinoic acid 6 (Stra6) and then through several enzymatic reactions it is converted into its active form RA. Active retinoic acid is secreted and taken up by retinoic acid responsive cells. In the cytoplasm RA binds to one of the three Retinoic acid receptor (RAR), Rarα, Rarβ or Rarγ [39]. These complexes bind to a Retinoic Acid active Responsive Element (RARE) in the regulatory elements of their target genes and modulate transcription of these genes [40, 41]. Targeted deletions of members of Rar and Rxr family have different effects. Double knockouts of Rarα and Rarβ result in failure to separate the esophagus and trachea and hypoplasia of the left and right lung. However, deletion of other members of the RAR and RXR family did not result in an obvious lung phenotype [42]. Binding of retinoic acid to its receptor directly affects the target genes either by inducing or repressing gene expression. Many genes regulated by the RA pathway are involved in embryogenesis [39]. However, it is possible that still many target genes have yet to be discovered. Tracing the activity of RARE's in embryonic development revealed high activity of the RA pathway in multiple developing organs, for example in heart, hindbrain and diaphragm [43] [44]. Activity of the retinoic acid receptors is important for proper lung development and at E9 in mice, when the first lung buds start to develop from the foregut, RA signaling is highly active [38]. Furthermore, in absence of retinoic acid, levels of Fgf10 decrease and levels of Tgfβ increase, and there is reduced budding and branching of the lung [45]. More specific, molecular processes required for formation of the lung primordium from the foregut are controlled by RA receptor activity. RA is a major regulator of Wnt signaling and the Tgfβ pathway and thereby controls Fgf10 expression, early in lung development [46]. The role of RA signaling in vascular development has so far only been shown in the development of the systemic blood circulation. In RA deficient embryos endothelial cell growth and proliferation is uncontrolled, indicating a role for RA in suppression of endothelial cells during vasculogenesis [47]. Although there is no direct evidence yet that the RA pathway is

involved in the development of the pulmonary vasculature, it may be that this pathway is involved based on the intimate relation between the airways and the vasculature.

Abnormal pulmonary vascular development

Perturbations of the described molecular pathways in the pulmonary vascular development may cause congenital anomalies, like pulmonary hypertension (PH), in newborns, infants and children [48]. PH is characterized by persistent increased resistance of the vasculature and abnormal vascular tone, which is regulated by the contraction of smooth muscle cells. Five groups of PH can be distinguished: pulmonary arterial hypertension, pulmonary hypertension due to left heart disease, pulmonary hypertension due to lung diseases and/or hypoxia, chronic thromboembolic pulmonary hypertension and pulmonary hypertension with unclear multifactorial mechanisms [49, 50](Table 2). Normally the PVR is high antenatally and decreases immediately after birth, reaching levels that are comparable to adult values within 2 months after birth. PH has an incidence of approximately 63.7 per million children [51] and can be idiopathic or associated with other diseases. It can cause significant morbidity and mortality. In children, idiopathic pulmonary arterial hypertension (iPAH) and PH due to congenital heart disease comprise the majority of cases. Other important causes include persistent pulmonary hypertension of the newborn (PPHN), bronchopulmonary dysplasia (BPD) and developmental lung diseases, like congenital diaphragmatic hernia (CDH), alveolar capillary dysplasia (ACD) and lung hypoplasia and surfactant protein abnormalities [48, 49]. Mutations in specific genes have been reported (Table 3), but PH in children can also be associated with genetic syndromes, like Down syndrome, DiGeorge syndrome, VACTERL syndrome, CHARGE syndrome and Noonan syndrome [52]. Perinatal care and prognosis in pediatric PH has improved over the last years, but despite the fact that there are significant differences in pulmonary vascularity between adults and children, most treatment is based on experimental research or trials in adults [53]. We will focus on iPAH, CDH, ACD and BPD which are all characterized by an abnormal pulmonary vascular development and in which PH plays an important role in the mortality and morbidity.

Idiopathic pulmonary arterial hypertension

iPAH is characterized by restricted blood flow through the pulmonary arterial circulation, elevated pulmonary vascular resistance and progressive right heart failure [54]. iPAH, previously known as primary pulmonary hypertension, has an incidence of approximately 0.7 per million [49] with hypertensive vasculopathy exclusively in the pulmonary circulation without a demonstrable cause. Young children have a reduction in arterial number and a failure of the vasculature to relax, whereas in older children intimal hyperplasia, occlusive changes and plexiform lesions are found (Figure 2). In contrast to adults, children with iPAH have more pulmonary vascular medial hypertrophy and less intimal fibrosis and fewer plexiform lesions [55, 56]. Younger children have a more reactive pulmonary vascular bed with an increased prevalence of acute pulmonary

Table 2 Classification of pulmonary hypertension*

Pulmonary arterial hypertension
Idiopathic PAH (iPAH)
Heritable PAH
BMPR2
ALK-1, ENG, SMAD9, CAV1, KCNK3
Unknown
Drug and toxin induced
Associated with other diseases
Connective tissue disease
HIV infection
Portal hypertension
Congenital heart diseases
Schistosomiasis
Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis
Persistent pulmonary hypertension of the newborn (PPHN)
Pulmonary hypertension due to left heart disease
Left ventricular systolic dysfunction
Left ventricular diastolic dysfunction
Valvular disease
Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies
Pulmonary hypertension due to lung diseases and/or hypoxia
Chronic obstructive pulmonary disease
Interstitial lung disease
Other pulmonary diseases with mixed restrictive and obstructive pattern
Sleep-disordered breathing
Alveolar hypoventilation disorders
Chronic exposure to high altitude
Developmental lung diseases
Congenital diaphragmatic hernia (CDH)
Bronchopulmonary dysplasia (BPD)
Alveolar capillary disease (ACD)
Lung hypoplasia
Surfactant protein abnormalities
Pulmonary interstitial glycogenosis
Pulmonary alveolar proteinosis
Pulmonary lymphangiectasia
Chronic thromboembolic pulmonary hypertension (CTEPH)
Pulmonary hypertension with unclear multifactorial mechanisms
Hematologic disorders: chronic hemolytic anemia, myeloproliferative disorders, splenectomy
Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphangioleiomyomatosis
Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

*Adapted from the updated Dana point classification [50]

hypertensive crises [55, 57]. Possible mechanisms that play a role in PAH development are endothelial cell dysfunction, smooth muscle cell migration and dysfunction, and abnormal apoptosis. In adult iPAH, in-vitro studies showed increased expression of endogenous vasoconstrictors and decreased expression of vasodilators [58-61]. The same vasoactive factors could play a role in pediatric iPAH. An increased expression of thromboxane and endothelin-1 (ET-1) , both vasoconstrictive and proliferative mediators, are elevated in both adults and children [57, 62, 63]. However, besides these two factors, this might also be the case for other vasoactive factors.

Heritable forms of pulmonary hypertension are caused by mutations in several genes. Point mutations and deletions in the bone morphogenetic protein receptor 2 (*BMPR2*) have been identified in approximately 10-40% of all patients with iPAH and are the major cause of heritable PAH [64]. Both pediatric and adult patients with *BMPR2* mutations appeared to have more severe disease compared to those without this mutation [57]. Pfarr et al. found mutations in *BMPR2* and two receptors of the TGFβ/BMP pathway, activin receptor-like kinase 1 (*ACVRL1*) and endoglin (*ENG*), in 8/29 (27.6%) of the pediatric iPAH patients [65]. A genetic polymorphism detected in the serotonin 5-hydroxy tryptamine transporter (*5HTT*) gene is associated with iPAH in adults and might also play a role in iPAH in children. This polymorphism leads to elevated levels of 5HT and results in increased smooth muscle cell proliferation [66]. Most of the genetic mutations in iPAH are only studied in adults and in contrast to adults, PAH in children is often associated with genetic syndromes. However, not all patients with a mutation in the same gene will develop severe PAH, suggesting that modifiers and or epigenetic regulation of expression could also play a role.

Congenital diaphragmatic hernia

Congenital diaphragmatic hernia (CDH) has an incidence of approximately 1 in 2500-3000 live births. Beside a diaphragmatic defect, CDH is characterized by pulmonary hypoplasia and pulmonary hypertension, which may be due to an altered development of the pulmonary vasculature and a disordered process of pulmonary vascular remodeling [67, 68]. Previous studies showed excessive muscularization of the pulmonary arteries and maladaptive pulmonary vascular remodeling in CDH patients [4, 67-70] (Figure 2). In contrast to the positive effect of inhaled NO in preterms with PH, the effectiveness of this treatment is only around 30-40 % of patients with CDH.

Over the last years several factors involved in the abnormal pulmonary vascular development in CDH have been identified. Expression levels of these factors have been analyzed both in lung tissue of CDH patients and experimental animal models. We studied the role of the Von Hippel-Lindau protein (pVHL) and HIF1α and found a decrease of pVHL and HIF1α expression in the arterial endothelium and an elevated expression of pVHL in the pulmonary arterial media of human CDH cases compared to age matched controls [71]. Shehata et al. showed increased VEGF expression in the bronchial epithelium and medial smooth muscle

cells and positive VEGF staining in endothelial cells, which were negative in age-matched controls [72]. However, we have found lower expression of VEGF mRNA in the alveolar stage in CDH patients [73]. In the process of normal remodeling of the pulmonary vasculature, extracellular matrix membrane proteins (MMPs) are of fundamental importance. Altered expression of certain MMPs and tissue inhibitors of MMPs (TIMPs) was found in human CDH lungs compared to control [74]. Decreased expression of VEGF and its receptors is also seen in the nitrofen rat model of CDH [75, 76]. In summary, an increase in pVHL may downregulate HIFα, leading to decreased expression of VEGF and a disturbance of vascular growth and endothelial cell proliferation during development. It would be interesting to investigate the oxygen concentration during the development of the (CDH) lung, to evaluate whether this may contribute, through HIFα, to the structural changes that contribute to the hypertension.

Abnormal RA signaling contributes to the etiology of CDH, and the first evidence of its involvement in CDH came from observations of pups born to rat dams with vitamin A deficient diets. In 25-40% of these pups a diaphragmatic hernia was present [77]. This finding is supported by the development of a diaphragmatic defect, pulmonary hypoplasia and pulmonary vascular abnormalities after disruption of the retinoid signaling pathway by nitrofen [78]. Furthermore, retinoic acid receptor (RAR) α/β double knock-out mice were found to have offspring with a diaphragmatic hernia [79]. In addition to the animal models, measurements of the levels of retinol and retinol-binding protein (RBP) in de first hours after birth in human CDH newborns showed a significant reduction compared to matched controls, independent of maternal retinol status [80, 81]. As described above, Chen et al. showed that lower levels of RA could cause an increase in TGFβ and a decrease in Fgf10 [45]. Increased expression of TGFβ1 with immunostaining at the midpseudoglandular, late pseudoglandular and saccular stage of lung development is detected in the nitrofen rat model of congenital diaphragmatic hernia [82]. Also increased mRNA levels of TGFβ and TGFβRII are observed in the same model [83]. Teramoto et al. described a decrease in gene expression of Fgf10 in the nitrofen rat model [84]. Since TGFβ plays a role in the airway branching and muscularisation of the pulmonary vasculature and Fgf10 was thought to regulate lung budding and branching, this might implicate that the neomuscularization and reduced branching in CDH may be caused by disturbances in the RA-TGFβ-Fgf10 interactions.

Over 450 chromosomal aberrations have been reported in CDH [85] Some of the recurrent genetic changes are found in retinoid related genes. In autosomal recessive conditions as Matthew-Wood syndrome (Microophthalmia syndromic 9 (MCOPS9) or Donnai-Barrow syndrome; OMIM #222448 mutations in the *STRA6* and *LRP2* genes have been reported. STRA6 is the membrane receptor for retinol binding protein (RBP1) and mutations of the LRP2 gene leads to proteinuria with spillage of retinol-binding proteins. Deletions of *COUP-TFII* on chromosome 15q26.1-26.2 [86], and of *FOG2* (ZFPM2; chromosome 8q23.1) or SOX7 (8p23.1) lead to an autosomal dominant form of CDH with variable penetrance [87, 88]. Beck et

al. showed that a deletion of the FRAS1-related extracellular matrix 1 (*FREM1*) gene, which encodes an extracellular matrix protein, can cause CDH in both human and mice [89].

Several CDH animal models have been developed, such as the surgical models in lambs and rabbits, several knockout models in mice and teratogenic models in rats [78, 90]. Surgical animal models are useful for the investigation of interventional therapies, but are less informative in studying the etiology and pathogenesis of CDH [90]. The nitrofen model is the most commonly used teratogenic model for CDH. When administered to pregnant rat dams at gestational day 9.5, the herbicide nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl ether) causes diaphragmatic defects, lung hypoplasia and pulmonary hypertension in pups, strikingly similar to the human condition [90, 91].

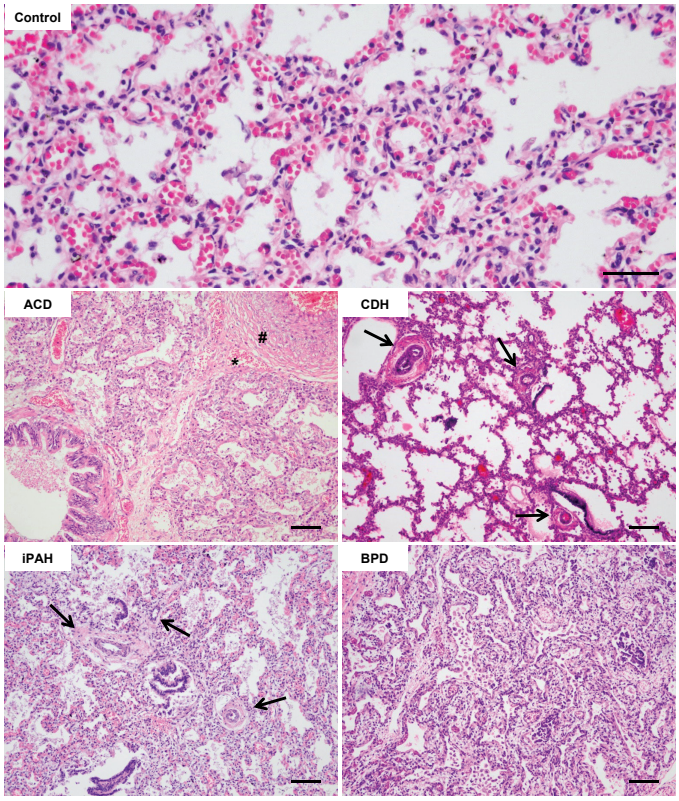


Figure 2: Characteristic histology of four pulmonary vascular disease samples

Hematoxylin and eosin staining of human lungs: control, idiopathic pulmonary hypertension (iPAH), congenital diaphragmatic hernia (CDH), alveolar capillary dysplasia (ACD) and bronchopulmonary dysplasia (BPD). Scale bars 100µm.
iPAH: thickening of the arteries (arrows), CDH: excessive muscularisation of the arteries (arrows), ACD: medial hypertrophy and muscularisation (#), malpositioning of the pulmonary veins (*) and central positioning of the capillaries in the alveolar septa ,BPD: fibrosis with widening of the alveolar septa.

Alveolar capillary dysplasia

Alveolar capillary dysplasia (ACD) is a rare lethal developmental lung disorder with failure of alveolar capillary formation, often accompanied by misalignment of the pulmonary veins. This results in abnormal gas exchange, severe hypoxemia and pulmonary hypertension. The prevalence and incidence is not known, but the mortality rate approaches 100%. ACD is characterized by premature growth arrest with immature lobular development, reduced capillary density, thickened alveolar septa, medial hypertrophy and muscularization of small pulmonary arteries and distal arterioles and malposition of pulmonary veins (Figure 2). In 50-80% of patients, ACD is associated with other congenital anomalies. Although at the moment a definitive diagnose can only be obtained by histological examination of lung tissue [92], the detection of genetic changes of the Forkhead Box F1(*FOXF1)* locus on chromosome 16q24 can aid the diagnosis.

Mutations of *FOXF1* and deletions of the 5' regulatory region of this transcription factor gene have been reported in most patients with ACD [93, 94]. FOXF1 deficiency is associated with reduced numbers of pulmonary capillaries in patients with ACD and similar observations have been made studying Foxf1 heterozygous knockout mice. Conditional deficient Foxf1 mouse models showed that loss of Foxf1 in the endothelial lineages resulted in an impaired angiogenesis, endothelial proliferation and VEGF signaling [95]. Involvement of the FOXF1 protein in SHH signaling has been shown both in vitro and in vivo in human and mice [92, 93, 95]. Mahlapuu et al. showed that SHH induces the transcriptional activation of Foxf1[96]. This may imply that other genes from this pathway are involved in the etiology of ACD. In addition to the large phenotypic overlap between human ACD and the mouse Foxf1mutant mice, overlapping expression profiles of lung specimens indicate that the Foxf1 mouse model is an excellent animal model for ACD.

Table 3 Genes involved in pulmonary vascular disease in newborns

	Gene	Chromosome	Reference
iPAH	*BMPR2*	2q33	65, 97, 98
	ACVRL1	12q13	65, 97
	ENG	9q34.11	65
	5HTT	17q11.2	66
	BMPR1B	4q22.3	99
CDH	*FOG2*	8q22.3-23.1	100
	COUP-TFII	15q26.1-26.2	86
	STRA6	15q23-25.1	101
	FREM1	9p22.3	89
	WT1	11p12-15.1	102
ACD	*FOXF1*	16q24.1	93, 94

In addition to the Foxf1 ACD mouse model other knock-out models show similarities to ACD and may potentially be used to study ACD. For example, the pulmonary phenotype and associated congenital defects observed in endothelial nitric oxide synthase (eNOS)-deficient mice are strikingly similar to the pathological features seen in ACD [103]. NO plays a role in the downstream signaling of angiogenic factors and the regulation of angiogenic gene expression in the developing lung. Furthermore, mice lacking the phosphatase and tensin homologue deleted from chromosome 10 (Pten) showed defects in the pulmonary microvasculature similar to those seen in ACD. Pten inactivation caused increased expression of Fgf9, Fgf10 and Fgf7 and decreased expression of Shh, Ptch1 and Gli1. They also found a decreased expression of FOXF1 in these mice [104], which might indicate a role for Pten in the regulation of FOXF1.

As described above, Fgf9 signaling, SHH signaling and Vegfa expression in lung mesenchyme are required for the pulmonary capillary formation. In an in vitro study in mice it was observed that Fgf9 and SHH regulate each other and the expression of angiogenic factors such as Vegfa [17]. Fgf9 and SHH might play a possible role in the development of ACD.

It is important to improve our knowledge of the pathology of ACD. The discovery of mutations in the FOXF1 gene locus has been a great improvement in the research on ACD. However not in all patients with ACD a mutation in this gene locus can be found, indicating that there might be other genetic or etiological factors involved in the genesis of this disease. Since the HIF1 and HIF2 complexes are involved in vascular expansion during development of the lung, alterations in HIF1/HIF2 may play a role in the premature growth arrest and vascular abnormalities in ACD. However, no altered expression of HIF1α in lungs of human ACD patients has been observed [105], but other genes in this pathway like HIF2α could play a role.

Bronchopulmonary dysplasia

Bronchopulmonary dysplasia (BPD) is a chronic lung disease associated with preterm newborns that weigh <1000g and receive respiratory support with mechanical ventilation and/or prolonged oxygenation [106]. More than 30% of preterm infants born before 30 weeks of gestation develop BPD and the incidence is still rising [107]. It is characterized by decreased or arrested alveolarization and pulmonary microvascular development (Figure 2). The definition of BPD changed over the past 50 years. It was last redefined in 2000 by the National Institute of Child Health and Human Development (NICHD). [108] The current definition is graduated by the severity of the disease, where mild BPD is defined as the need for supplemental oxygen at ≥28 days but not at 36 weeks of gestation, moderate BPD as the need for supplemental oxygen at 28 days in addition to supplemental oxygen at ≤30% at 36 weeks of gestation, and severe BPD as the need for supplemental oxygen at 28 days and the need for mechanical ventilation and/or oxygen >30% at 36 weeks of gestation [109]. Since the alveolar and distal vascular development in premature born infants are still in

a crucial state, BPD results from the need for the lung to develop while continued injury and repair are occurring [109, 110]. The vascular pathology in BPD shows immature vessels with a dysmorphic structural configuration of the distal microvasculature and an abnormal distribution of alveolar capillaries with more distance from the air surface [111]. Just like in CDH, intrapulmonary shunting through precapillary arteriovenous anastomotic vessels was found in the lungs of patients with severe BPD [112]. This dysmorphic growth and impaired function of the pulmonary vasculature can be caused by various prenatal and postnatal factors and can result in pulmonary hypertension [113].

Mechanical ventilation and oxygen therapy in preterm infants can result in impaired angiogenic signaling with an increased expression of antiangiogenic genes and a decreased expression of proangiogenic genes [113]. After short periods of ventilation fewer arteries and endothelial cells are seen, whereas longer periods of ventilation can cause decreased vessel branches and increased endothelial cell proliferation [114]. Changes in VEGF expression are observed in lungs of human BPD patients and in an experimental animal model. Where most of the in vitro studies in humans and animals showed a decrease in VEGF expression [115-117], one in vitro study in a baboon model of BPD showed an increase in VEGF protein [118]. Levels of soluble VEGFR1 (sVEGFR1), an endogenous antagonist of VEGF, were found to be elevated in amniotic fluid and maternal blood in preeclampsia and intra-amniotic administration of sVEGFR1 to pregnant rats resulted in pups with blunted alveolarization and reduced lung vessel density [119, 120]. This implicates a role for preeclampsia by perturbations in VEGF levels in the development of BPD. During fetal lung development, levels of HIF1α are high and are important for the expression of VEGF and other angiogenic factors. In premature born children, levels of HIFα decline rapidly [118], possibly because of the absence of a hypoxic environment or even because of the use of oxygen therapy. This may cause a decrease in angiogenic factors resulting in less vascular expansion. Also HIF2α is a regulator of VEGF and is critical for fetal lung maturation. However, it plays a more important role in the alveolar epithelial cells than in the vascular cells [121]. We showed earlier that Hif2α is a key regulator in the maturation of type II pneumocytes and that ectopic expression of an oxygen insensitive, constitutive active form of Hif2α leads to a severe surfactant deficiency in the newborn [122], which is also seen in BPD patients. In contrast to the downregulated angiogenic factors found by others, Paepe et al. found an upregulation of endoglin mRNA and protein levels in ventilated preterm infants. Endoglin is a hypoxia-inducible TGFβ coreceptor and is an important regulator of angiogenesis. They speculated that there might be a shift in angiogenic regulators which contributes to the dysangiogenesis in BPD. Furthermore, the upregulated endoglin possibly modulates vascular permeability resulting in interstitial edema, which is a morphological feature of early BPD [123]. As such BPD forms an interesting model of postnatal injury and repair showing similarities in expression profiles of a number of transcription factors involved in normal development. This disease can thus be used to gain knowledge on these processes and can be implemented in our developmental studies.

Besides the angiogenic factors, there may be a possible role for the retinoid signaling pathway in the development of BPD. As already shown in CDH, a shortage in vitamin A can disrupt the retinoid signaling pathway. Preterm infants have low vitamin A levels at birth and supplementing very low birth weight infants with vitamin A was found to be associated with a reduction in incidence of BPD [124]. The shortage in vitamin A could possibly be a cause of impaired pulmonary vascular development and pulmonary hypertension in BPD.

Many genes with a putative role in the development of BPD have been investigated in genotype association studies. These genes have been described in a recent review [125]. Many of these studies have tested polymorphisms in potential candidate genes such as surfactant proteins or cytokines but only weak associations implicating susceptibility to the disease have been reported [126].

Over the last decades many animal models have been developed to study the impairments in lung development in BPD. These models are based on hyperoxia, mechanical ventilation and inflammation. Since newborn rodents are born during the saccular stage of lung development, they are well suited to model BPD. The hyperoxia animal model is most commonly used and results in acute lung injury, disrupted lung structure and impaired alveolarization and vascularization, resembling the pathology seen in BPD. However, in contrast to the used animal models, preterm infants normally receive lower concentrations of oxygen with a lot of fluctuations, possibly resulting in differences in molecular signaling. Over the last years animal models gave us a better insight in the pathogenesis of BPD and resulted in the development of new therapies [107]. Since HIF1α and its expression of angiogenic factors seem to play an important role in the development of BPD, this may be a good target for the treatment of BPD.

Conclusion and future perspectives

Over the past decades, human studies focusing on abnormal pulmonary vascular development have primarily been descriptive and molecular players have been investigated in archival and resection material. Human cell cultures have been instrumental in describing molecular pathways that may contribute to specific aspects of these congenital anomalies. Although these studies have been very valuable for generating hypotheses about the origin of congenital pulmonary diseases, the majority of the studies fail to identify the underlying mechanisms. Human studies linking molecular mechanisms to diseases remain rare, because the limited number and quality of human material prevents the initiation of large-scale studies. The combination of human studies with animal models facilitates the analysis of molecular mechanisms and pathways, although the different animal models only partly reflect and phenocopy the human pathology. For instance, the mouse model for

BPD is induced by exposing mice to much higher levels of oxygen than the levels that are used in the clinical situation. The surgical CDH rabbit model is sufficient to explore surgical techniques, but cannot be used to study the etiology and pathogenesis of the disease.

The –omics era has opened new ways to generate and analyze large data sets, which facilitated discovery and characterization of specific chromosomal locations, SNPs, associated with specific diseases by Genome Wide Association Studies (GWAS). However, it remains unclear in the majority of cases how the identified loci or SNP are involved in the origin of diseases. In the near future, it will be interesting to investigate whether these SNPs harbor specific binding sites for transcription factors or other DNA associating proteins, like DNA methylases. Alterations in binding efficiency may have a huge impact on downstream processes, such as transcription, leading to changes in developmental processes. It may also be that these loci SNPs are involved in spatial and or temporal long-range chromosomal interactions, which may be investigated with specific techniques, such as 3C-Seq [127].

Another putative approach is to investigate the interaction network between proteins, which may identify specific partners that are involved in developmental processes. Searching for Sox2 binding partners in neural stem cells, we recently showed that SOX2 interacts with CHD7. Mutations in SOX2 cause Anophthalmia-Esophageal-Genital (AEG) syndrome and mutations in CDH7 are associated with CHARGE syndrome (Coloboma of the eye, Heart defects, Atresia of the nasal choanae, Retardation of growth and/or development, Genital and/or urinary abnormalities, and Ear abnormalities and deafness). AEG and CHARGE have overlapping clinical features, and disturbing the interaction between SOX2 and CHD7, or other members of this cascade, may cause a variety of clinical symptoms [128]. Moreover, several genes that are implicated in related syndromes, like JAG1 and GLI3), were shown to be activated by SOX2/CHD7. In addition, we showed that the HMG domain of SOX2 and SRY contains a binding site for the nuclear-cytoplasmic shuttling protein Exportin4. Several mutations have been described in the human SRY gene, which were shown to be involved in XY sex reversal. These mutations prevented SRY from associating with EXP4, leading to a block in its translocation to the nucleus and thus its transcriptional activity [129]. So, the study of protein-protein interactions may provide mechanistic insights in specific disease.

Aside from (familial) genetic studies, epigenetics has become a major field of interest, and encompasses three classes: chromatin modifications (DNA methylation), histone modifications (methylation, acetylation, phosphorylation) and noncoding RNA molecules (lncRNA, miRNA). Recently, microRNA-206 (miR-206) was found as a possible triggering factor of early stage hypoxia-induced PH by targeting the Hif-1α/Fhl-1 pathway [130]. Others have identified epigenetic changes in adult patients suffering from COPD, Asthma and interstitial lung disease (reviewed by [131]), and it would be interesting to analyze pulmonary vascular diseases with these whole genome epigenetics techniques to establish the full

methyl-Cap-RNA Sequence, miRNA or lncRNA profiles of the congenital pulmonary vascular diseases.

Fetal lung explants have been studied for a long time, and have generated ample evidence for branching morphogenesis in the developing lung. Human lung explants have been used, but these cultures also suffer from technical limitations [132]. As human samples are very scarcely available, and mostly derived from end-stage disease, it is mandatory to investigate alternative ways of setting up culture systems beyond the classical cell culture. Currently, several emerging 3-D culture systems, such as tracheospheres [133], alveolar spheres [134], lung organoids [135], decellularized lungs [136], bioartificial lung [137] and lung on a chip [138, 139], are being employed to address specific developmental mechanisms or to optimize systems for regenerative medicine (for reviews, see [140-142]). Moreover, the generation of hiPS cells has become a standard technique in most institutes, and the use of patient specific cells in combination with protocols to differentiate these cells into cells representing the three germ layers has provided new ways to explore human (pulmonary vascular) diseases [143-148]. Especially the development and employment of bioartificial lungs, such as the lung on a chip and related cultures, with patient derived hiPS cells will contribute significantly to the understanding of how different cell layers interact during development and disease. We believe that the use of these systems in combination with patient specific hiPS cells will also benefit the testing of putative therapeutic agents.

In summary, understanding lung development and the molecular pathways leading to the mature gas exchanging organ is necessary to decipher the underlying causes of congenital pulmonary vascular diseases. It is obvious from the above perspectives that the interaction between different scientific disciplines, such as development, cell science, genetics, bioengineering, bioinformatics, will be a prerequisite to take the next steps in this process.

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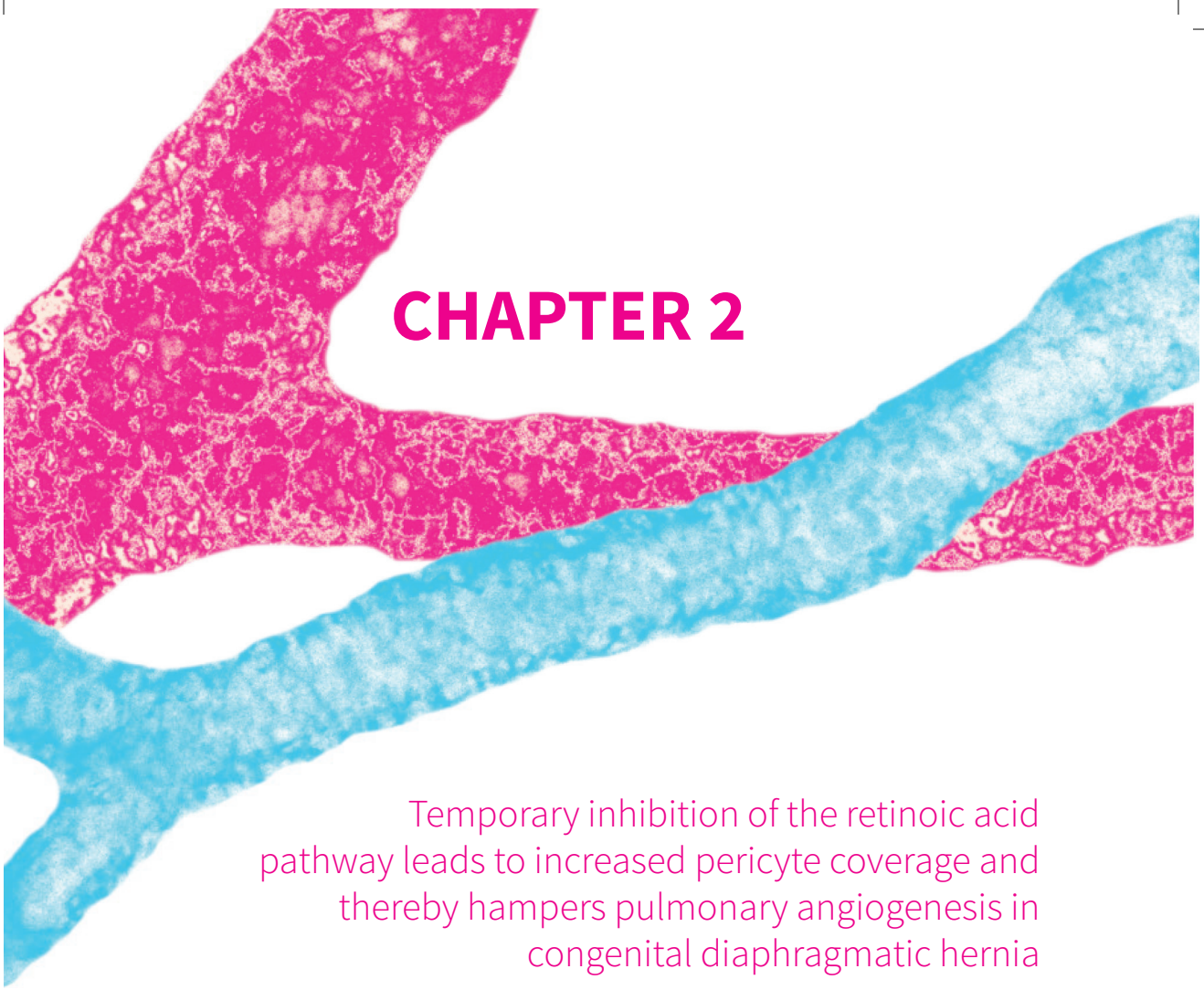

CHAPTER 2

Temporary inhibition of the retinoic acid pathway leads to increased pericyte coverage and thereby hampers pulmonary angiogenesis in congenital diaphragmatic hernia

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Abstract

Rationale

The mortality and morbidity of patients with congenital diaphragmatic hernia (CDH) is primarily caused by a treatment-resistant, persistent pulmonary hypertension. Structural vascular changes, exemplified by extensive muscularization, are already present early in gestation, but the origin of these abnormalities is unknown.

Objective–the purpose of the study

Understanding the origin of the vascular defects of CDH patients is important to improve treatment modalities. We therefore aimed to delineate the origin of this extensive muscularization by focusing on the pericytes.

Methods and Results

Using a mouse model of CDH by inhibiting retinoic acid (RA) signaling, we showed by immunohistochemistry and FACS analysis the early origin and expansion of the pericyte population in normal and abnormal development. Pericytes revealed a reduced migratory potential after inhibition of RA signaling in vitro. Analysis of immunofluorescently labeled embryonic lungs showed reduced expansion of the capillary bed at gestational age 15 (E15) and E18 in CDH, and a significant increase of the pericyte coverage. Furthermore, both pericytes and endothelial cells showed less proliferation and elevated expression of a-smooth muscle actin (ACTA2) in CDH-pericytes. In addition, CDH samples had reduced collagen IV expression from E15 onwards, which was also observed in human CDH patients.

Conclusions

One of the earliest pathogenic signs leading to the structural vascular changes in CDH is the increased pericyte coverage, resulting in reduced angiogenic activity and fewer capillaries. Furthermore, the reduced proliferation and migration activity of pericytes observed in CDH, as well as the increased expression of ACTA2 indicates that pericytes in CDH lungs are well differentiated. Reduced collagen IV expression in CDH indicates a loss of basal membrane integrity between pericytes and endothelial cells. Taken together, the pericyte population in CDH lungs starts to differentiate much earlier in gestation as a result of reduced RA signaling, leading to aberrant vascular development.

Introduction

Persistent pulmonary hypertension in the newborn is characterized by high pulmonary vascular resistance after birth with structural changes in the cardio-pulmonary circulation [1]. Pulmonary hypertension in the newborn may occur as a primary disease or in association with a congenital disease such as congenital diaphragmatic hernia (CDH) [2]. CDH is a severe congenital anomaly characterized by a variable defect of the diaphragm, different gradations of pulmonary hypoplasia and pulmonary hypertension [3 4]. Previously, we showed that CDH patients have already pathological changes in the vascular smooth muscle cell layer in the tunica media from early stages onwards, indicating that these changes are result of disturbed development of the pulmonary vasculature [5].

The first step of lung development is the patterning and separation of the foregut. Shortly after this separation, the first lung buds appear and start to grow and expand, this is in the pseudoglandular phase (in human week 4-17 and mice E9.5-E16.5). A capillary network that is connected to the central circulation surrounds the newly formed lung buds [6]. The development of the airways and pulmonary vasculature remain closely connected during the canalicular stage (in human week 16-26 and in mice E16.5-E17.5) and the saccular stage (in human week 26-36 and in mice E18.5-P5) [7].We and others revealed that the expansion of the pulmonary vasculature is required for normal lung development, since disturbances in normal growth of the vasculature leads to improper development of the airways and the lungs remain hypoplastic [8 9, 10]. The pulmonary vasculature develops predominantly by the expansion of the existing vascular network, a process we described as distal angiogenesis [6]. Angiogenesis is characterized by the sprouting of endothelial cells from pre-existing vessels to form new, unstable tubules [11]. These primitive tubules are stabilized by cells from the surrounding mesenchyme, the perivascular cells or pericytes. The endothelial cells of the newly formed tubule release Platelet derived growth factor β (PDGFβ) which binds to the PDGF receptor β (PDGFRβ) expressed by pericytes [12-14]. As a result, the pericytes are recruited to the neo-vessel and interact with the endothelial cells via direct contact or paracrine cross-stimulation, thereby stabilizing the primitive tubule and causing maturation of the vessel [12 15]. As a consequence of the interaction between pericytes and endothelial cells, the pericytes start to release collagen IV (COLIV), a key component of the basement membrane (BM). Through the BM the endothelial cells and pericytes interact and allow stabilization and growth of the micro vascular bed [16].

Altered pericyte function is related to multiple pathological conditions such as fibrosis, diabetic renopathy and cancer biology [17]. Pericyte coverage differs per organ and fluctuation in this ratio indicates malignancies.[17] Furthermore, in pulmonary arterial hypertension (PAH) increased pericyte coverage was found in both PAH mouse and human PAH samples[18],

indicating that increased pericyte coverage may be associated with structural changes observed in adult pulmonary hypertension.

As mentioned above, in a previous study we observed already early in lung development structural changes in the vasculature of CDH patients [5]. Based on our data and other studies, pericytes are prime candidates to underlie these vascular changes. However, characterization of pericytes remains difficult and fundamental knowledge about pericytes during lung development is still missing. Although a range of markers for pericytes have been described, each marker depends on the type of tissue, developmental age and location[17, 19-21]. Therefore, we first analyzed the expression of the different perivascular markers during normal and abnormal lung development in mice to identify which of the known markers of pericytes would serve as an appropriate marker for pericytes during pulmonary development. Furthermore, we analyzed the pericyte coverage at different gestational ages with Fluorescence-activated cell sorting (FACS) and by immunofluorescently labeling complete embryonic lungs. Lastly, we linked a decreased proliferation and a higher expression of alpha smooth muscle actin (ACTA2) of pericytes in CDH to be the source of the hyper-muscularization in CDH, and we translated our findings to human patient material.

Taken together, we found that increased pericyte coverage is an important factor in the pathogenesis of CDH and a lack of Collagen IV in the BM leads to reduced stabilization of the vessel bed and therefore insufficient development of the pulmonary vasculature.

Methods

Mouse CDH model

Different compounds to inhibit the retinoic acid pathway have been tested in different doses based on literature [22]. This pilot showed that the combination of nitrofen (2,4-dichlorophenyl-p-nitrophenylether) and Biphenyl carboxylic acid (BPCA, Sigma) resulted in the most consistent way of inducing CDH in the offspring of pregnant mice (Table 1). Thus, timed-mated pregnant mice received 20 mg nitrofen and 5 mg BPCA in 500 µl olive oil (Sigma) per 25 grams of body weight at day 8.5 of gestation. At different gestational ages pregnant mice were sacrificed, fetal lungs were isolated and their morphology was used to divide the phenotypic appearance of the fetuses into severely affected and mildly affected. Subsequently, both samples were analyzed with Immunohistochemistry (IHC) and showed comparable pathology.

Table 1: Compounds tested to induce CDH in mice

Concentration	Compound	Pups (N)	CDH (N)	Deaths (N)	(N)
5 mg / kg	BMS493	72	4	1	8
25 mg / 0,5 ml	BPCA	74	19	13	23
25 mg / 0,5 ml	Nitrofen	72	7	1	12
25 mg / 0,5 ml	Bisdiamine	26	4	1	4
25 mg / 0,5 ml	BPCA/Nitrofen	20	9	0	7
25 mg / 0,5 ml	Nitrofen/Bisdiamine	42	21	0	8

Immunohistochemistry and Immunofluorescence

IHC was performed on 5 µm thick paraffin sections of lungs according to standard protocols, using the DAKO Envision™ amplification kit. Primary and secondary antibodies used for IHC and for Immunoflourescence (IF) are listed in supplementary table 1. Antigen retrieval was performed with Tris-EDTA buffer (pH 9.0) on the first day and on the second day for signal detection and amplification the Envision™ detection system (DakoCytomatic) [23] was used. The protocol used for IF staining was similar to the IHC protocol, with small adjustments on the second day since Alexa Fluor secondary anti-bodies were used (Table 2). Negative controls were performed by omitting the primary antibody. Analysis of the proliferating cells was performed by counting all the endothelial cells and the $NG2^+$ cells around the endothelial cells. The percentages of Ki67 positive cells from all the counted cells were statistically analyzed using two tailed T-test in Graphpad.

Table 2: Primary and secondary antibodies used for IHC and IF

Antibody name	Dilution	Company	Specie
NG2	1:500	Merck KGaA	Rabbit
NG2	1:500	Prof. W.Stallcup	Guinea pig
αSMA	1:1200	Thermo Fisher	Mouse
αSMA-FITC	1:250	Sigma	Mouse
SMMHCII	1:800	Abcam	Mouse
PDGFRβ	1:250	Cell Signal	Rabbit
KI67	1:500	Thermo Fisher	Rat
COLIV	1:500	Millipore	Rabbit
COLIV	1:100	AbD Serotec	Rabbit
CD31	1:50	Thermo Fisher	Biothynilated
Alexa488	1:500	Jackson	Donkey
Alexa594	1:500	Jackson	Donkey
Alexa647	1:500	Jackson	Streptavidin

Whole mount immunofluorescence and analysis

Whole mount immunofluorescence staining of total fetal lungs was based on Yokomizo et al., 2012 [24]. Shortly, the left lung lobe was dissected of freshly isolated lungs and fixed in 4% paraformaldehyde (PFA) overnight. The next day, the lungs were gradually dehydrated with methanol and could be stored in pure methanol at -20°C for up to 3 months. The next day, the lungs were re-hydrated and blocked in PBS, 0.4% triton X-100 and 1% skimmed milk powder for 4 hours, blocking buffer was refreshed every hour. Subsequently, the lungs were incubated with primary anti-body diluted in blocking buffer (CD31 R&D 1:100, NG2 generously provided by Prof. W. Stallcup 1:500, SMA-FITC labeled, Sigma 1:250) for at least 4 days at 4^{o}C. The samples were washed with PBS-0.4% Triton for at least 48 hours, washing solution was refreshed every 4 hours and at the end of the day. Secondary anti-bodies conjugated with Alexa Fluor 594 or biotinylated Alexa Fluor 647 appropriate for the choice of primary antibody were diluted in blocking buffer and incubated for at least 48 hours. After this incubation, the lungs were washed, cleared with benzyl alcohol/benzyl benzoate (BABB). Samples were imaged using a Tile Scan option in the Leica application Suit (LAS) on the Leica SP5 confocal microscope. 3D reconstructions were made using AMIRA 6.3 software.

Lung explant culture and confocal time- lapse imaging

Embryonic lung explants were grown based on previous published studies [25, 26]. Briefly, time pregnant NG2DsRedBAC transgenic [27] mice were sacrificed at E10.5 and the embryonic lungs were isolated and placed on a floating, porous membrane inserts (0.4 µm pore size) on DMEM/F12, 10% FCS. Lung explant cultures were kept in a 37 ^{O}C incubator until imaging.

Imaging and analysis of explant cultures

Explants cultures were placed on the stage of the climate controlled Leica SP5 microscope. The reflection of the 633 laser (625-640 nm; 0,5% laser power, 650V) by the culture filters was used to autofocus with the matrix screening option of the microscope software provided in the Leica Application Suite software (LAS). DsRed fluorescent signal was detected with the 561 laser (520-558 nm; 8% laser power, 950V) using the HCX PL Fluotar 5 X dry objective. Image capturing was done using the 1,5x zoom factor. Images were analyzed with Fiji software by importing data of the complete imaging period to make hyperstacks with multiple variables (Z for depth of tissue, T for time, C for fluorescent channels). First, a Z-projection of the Z-stacks were made and the different channels were split. The background noise in the red channel was reduced using the Gaussian blur filter. Next, the size of a positive, red cell was measured (“particle”) and subsequently used to automatically count the cells in the multiple stacks of each time point using the function “analyze particles”. This number of particles was plotted with Microsoft Excel.

Imaging and analysis of the immunofluorescent images

Images were made using a Leica SP5 microscope. The lasers that were used for all pictures were 405 (415-480nm), 488 (500-550), 561 (571-630 nm) and the 633 (643-700 nm). Laser power was kept under 20% and the gain between 650V-750V for all images. The 20X HCX PL APO CS was used to image the whole mount stained lung samples and the 40X HCX PL APO CS oil objective for sections. Analysis of images of both whole mount and sections started of with setting the threshold using the auto threshold "moments". A region of interest (ROI) was selected for each section and the threshold was set for the NG2 and ACTA2 channels. Colocalization of NG2 and ACTA2 was calculated using the AND function in the image calculator in Fiji.

Human lung samples

Human lung samples were retrieved from the archives of the Department of Pathology of the Erasmus MC, Rotterdam, following approval by the Erasmus MC medical Ethical Committee. Lung samples were selected of CDH patients by different gestational ages and which did not show severe hemorrhage or necrosis. To circumvent the problem of secondary changes due to exposure to high oxygen concentrations and shear forces in all specimens the survival was less than 12 hours thus representing the most severe cases.

FACS experiment and analysis

Lungs were isolated at different gestational ages, and collected in 1 ml DMEM (Lonza) in Eppendorf tubes. Collagenase I, Collagenase II and Collagenase IV were (10 µg/ml) added and incubated at 37 ^{o} C for 10 minutes in an Eppendorf shaker at 1000 rpm. Then, the mixture was mixed vigorously to completely disrupt the tissue and the cell suspension was put through a 40 µm cell strainer. The filter was rinsed with 1ml DMEM 10% FCS and spun down at 4^{o} C for 10 minutes at 400 *g.* Supernatant was removed and the pellet was washed with PBS 10% FCS and resuspended in 300 µl PBS 10% FCS, of which 10 µl was taken to count the number of cells and to check for doublets and cell aggregates. Samples were centrifuged at 4^{o} C for 10 minutes at 400 *g* and cells were resuspended in 100 µl of CD31-PE-Cy7 (1:100,Thermo Fisher), CD45-PE-Texas Red (1:100, Thermo Fisher), NG2-488 (1:100, Merck KGaA) PDGFRβ-APC (1:100, Thermo Fisher) in PBS-10%FCS, for 40 minutes at 4^{o} C. Additionally, a part of the sample was only incubated with a single antibody to set the laser voltages. Samples were then washed by adding 150 µl PBS-10%FCS and gently mixed and centrifuged. Pellets were re-suspended in live/dead mix (Thermo fisher Scientific) in 100 µl PBS and incubated for 20 minutes at 4 ^{o} C. Samples were then washed by adding 150 µl PBS 10% FCS, mixed gently and centrifuged. Pellets were resuspended in 400 µl and kept in darkness at 4^{o} C until analyzed on the LSR Fortessa II FACS machine (B&D). Analysis of the data was done using FlowJo.

Cell culture and Migration assay

Human Brain Vascular Pericytes (ScienCell) were cultured on gelatin coated plates in DMEM (supplemented with 100U/ml penicillin/streptomycin; Lonza, and 10% FCS; Lonza) , in 5% CO_2 at 37° C. Pericytes were plated at a density of 0.5 x 10^5 cells/well in an OrisTM Universal Cell migration Assembly Kit (Platypus Technologies) derived 96 well plate with cell seeding stoppers. Twenty-four hours post sub culturing, the cell stoppers were removed and cells were allowed to migrate into the cell free region for 16 hours in 5% CO2 at 37° C. Subsequently, the cells were washed in PBS and stained by Calcein-AM followed by visualization using fluorescence microscopy. Wells in which cell seeding stoppers were not removed, were used as a negative control. Fluorescent colors were adjusted using ADOBE Photoshop and data was analyzed by Clemex Software.

Statistical analysis

Statistical analysis was done using Graphpad Prism V7 and for all measurements two-tailed T-test was performed.

Results

Perivascular cells during normal and abnormal development

Pregnant female mice were treated at gestational age E 8.5 with a single dose of different combinations of teratogens to test which retinoic acid inhibitors induced a consistent CDH-like phenotype (Table 1). Morphological analysis of the fetal lungs isolated at E18.5 revealed that the combinatory treatment with nitrofen and BPCA resulted in a similar lung and diaphragm phenotype as the well-known nitrofen-induced rat model [28, 29]. Moreover, at all of the examined gestational ages the pups of the mothers treated with the combination of nitrofen/BPCA showed lung hypoplasia (Fig. 1A). Subsequent histological analysis showed an increased staining of ACTA2 in the small capillaries and a prominent expression of Smooth muscle myosin heavy chain II (SMMHCII) in the CDH samples, comparable to the CDH rat model and human CDH [4, 29] (Fig. 1B).

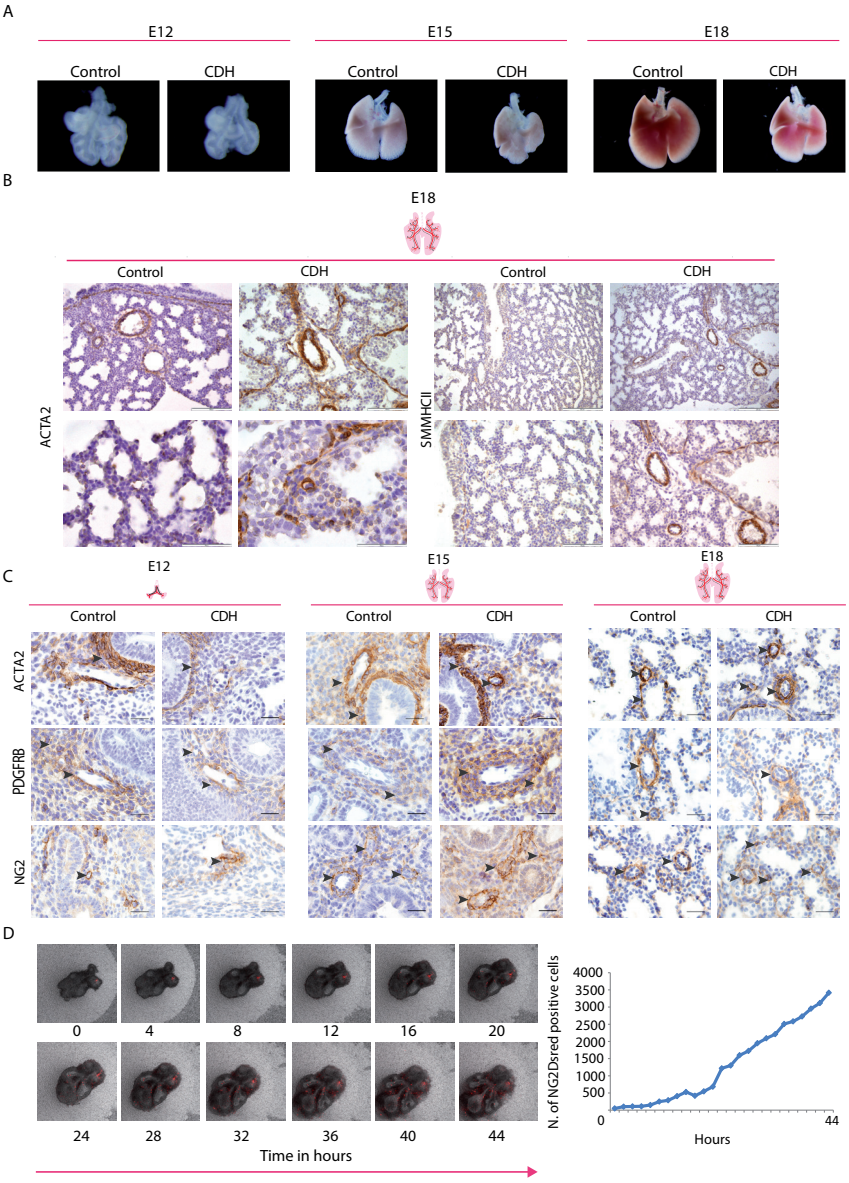


Figure 1

Pathology of the mouse congenital diaphragmatic hernia (CDH) model and distribution of perivascular markers during lung development. A Representative images of lungs from mothers who retrieved the combination of retinoic acid inhibitors Nitrofen/BPCA at E8.5. The complete litter of the treated mothers showed lung hypoplasia, lungs were isolated at E12, E15 and E18. B Lungs of CDH mice show the pathological characteristics for CDH. Increased expression ACTA2 in the small capillaries and expression of SMMHCII, which is barely expressed in the pulmonary vessels of control pups. Scale bar is 200 μM. C Distribution of pulmonary vascular markers PDGFRβ, NG2 and ACTA2 analyzed at E12, E15 and E18. PDGFRβ is expressed throughout the mesenchyme at E12 and E15, the expression becomes more restricted to the vasculature at E18. NG2 expression is observed around the small and mid-sized vessels throughout development (E12, E15 and E18). Scale bar is 20 μM. D At E10 lungs from NG2DsRedBAC mice were put on a filter. After 12 hours the first NG2 positive cells were observed. The number of cells expanded rapidly.

We showed that vascular abnormalities associated with CDH are already present early during gestation in patient samples [5], which are here mimicked by the CDH mouse model (Fig 1A, B). As perivascular cells are associated with angiogenesis and may contribute to the smooth muscle cell layer, we next investigated the emergence and distribution of perivascular cells, which are not well described during lung development. Therefore, analysis of the temporal and spatial expression patterns of NG2, PDGFRβ and ACTA2 at embryonic days E12, E15 and E18 in lungs of control and CDH pups was performed. At E12 the expression of ACTA2 is found in cells around the large airways, and at E15 and E18 in cells around the mid-sized and large vessels and airways. However, significant more ACTA2 positive cells were detected around the capillaries in CDH samples at E18 (arrow heads in Fig. 1C). In the early pseudoglandular phase of lung development, at E12, the expression of PDGFRβ is observed in cells surrounding the larger vessels (>50 μm) and distributed throughout the mesenchyme (Fig 1C). This pattern is consistent at E15 but diminishes at E18 in the mesenchyme and becomes more restricted to vasculature (arrowheads in Fig 1C).

At E12, the cells which surround the small capillaries are NG2 positive and this pattern continues at E15 and E18, at which stage of development the mid-sized (40-50 μm) and larger vessels (>50 μm) also become positive (arrowheads in Fig. 1C). To explore the emergence, invasion and distribution of perivascular cells in the lung during the initial phases of lung development, time-lapse imaging was performed using explants of fetal lungs from NG2DsRedBAC transgenic mice. This transgenic mouse line harbors a bacterial artificial chromosome that specifically expresses DsRed in NG2 positive cells [27]. Therefore, time-lapse experiments were performed with lungs isolated at E10 and images showed that 12 hours after isolation the first NG2 positive cells appeared and the number of NG2 positive cells expanded rapidly in time (Fig. 1 D). The early presence in the development of the lung and the swift expansion of the number of cells indicates the potential importance of NG2 positive cells. The rapid emergence of the NG2 positive cells in the early stage of lung development coincides with the expansion of the capillary network at the same time.

Increased pericyte coverage in the pulmonary vasculature CDH in mouse results in an impaired capillary bed

The cellular composition of the pulmonary vascular wall varies depending on the size and functionality of the vessel. The large pulmonary arteries exist of an adventitia (the outer layer) where fibroblast are loosely organized and a vasa vasorum where smooth muscle cells are present [30]. The smaller arteries, the arterioles do not have a distinguished vasa vasorum as an outer layer but discrimination can be made between the outer perivascular cell layer and the layer which is in direct contact with the endothelial cells, the more inner perivascular cell layer. The capillaries which are present in the distal end of the lung consist of a layer endothelial cells with a monolayer of perivascular cells around them. Immunofluorescent

double labeling with PDGFRβ and ACTA2 specific antibodies at different gestational ages revealed that at E12 and E15 PDGFRβ (Fig 2A) is expressed throughout the mesenchyme and outer layer of the perivascular wall of the arterioles. No difference in expression of PDGFRβ and ACTA2 was observed between control and CDH at E12. The expression pattern of PDGFRβ becomes more restricted to the vasculature at E18, however only the cells in the outer layer of the arteries and arterioles are PDGFRβ positive (indicated by white arrow heads in Fig 2A). CDH samples showed a decrease in PDGFRβ expression in the mesenchyme at E15, which could indicate that there are fewer PDGFβ responsive cells in CDH and thus possibly explaining the reduced expansion of the pulmonary vasculature. Double labeling of fetal lung samples with NG2 and ACTA2 specific antibodies revealed that the pericytes which are in direct contact with the endothelial cells are NG2 positive in the arterioles (indicated by white arrowheads; Fig 2A). Furthermore the expression of NG2 is consistent during pulmonary development and based on this expression and the location of the positive cells close to the endothelial cells we concluded that NG2 more specifically marks perivascular cells in the developing lung than PDGFRβ.

To further understand the structural differences in the pulmonary vasculature in CDH, whole mount immunofluorescence staining was performed. The left lobe of lungs isolated at E15 and E18 were stained with antibodies against CD31, NG2 and ACTA2 to get a better insight of the entire three dimensional (3D) structure of the pulmonary vessels and the perivascular cells. Besides the evident lung hypoplasia, the pulmonary vasculature of lungs of CDH pups was highly affected. Although the number of large vessels and their branches seemed comparable to the control, a major difference was observed in the capillary beds. The capillary network in the CDH samples appeared to be less dense and the number of capillaries was also reduced at E15 and E18 (Fig. 2B). CDH samples showed a simplification in the structure because of a decreased number of capillaries. The decreased numbers of capillaries observed at E15 was consistent throughout development and was also clearly visible at E18 (Fig 2B). The 3D images revealed a simplification of the capillary bed from E15 onwards and this raised the question whether we could detect more structural differences in lungs of CDH pups. Therefore, the NG2 positive area was measured of the second large branch of the left lung lobe was performed in control and CDH at E15 (Fig 2C). This revealed a significant increase of pericyte coverage in the CDH samples in the second large (>70 μm) branch of the pulmonary vasculature tree. So, besides structural differences in the capillary bed, lungs of CDH pups showed also increased pericyte coverage in the large pulmonary vessels. The observed increase in pericyte coverage may be the first cellular change in CDH eventually leading to the hyper-muscularization. To further understand the differences in the capillary bed, high resolution imaging was performed on the whole mount samples (Fig 2D). The high magnification images further confirmed the increase of NG2 expression in the capillary bed at E15 and E18 (Fig. 2D). The increase in the number of pericytes resulted in an incorrect development of the capillary bed at E15 and became even more pronounced at E18 (Fig. 2D). The capillaries in the CDH samples at

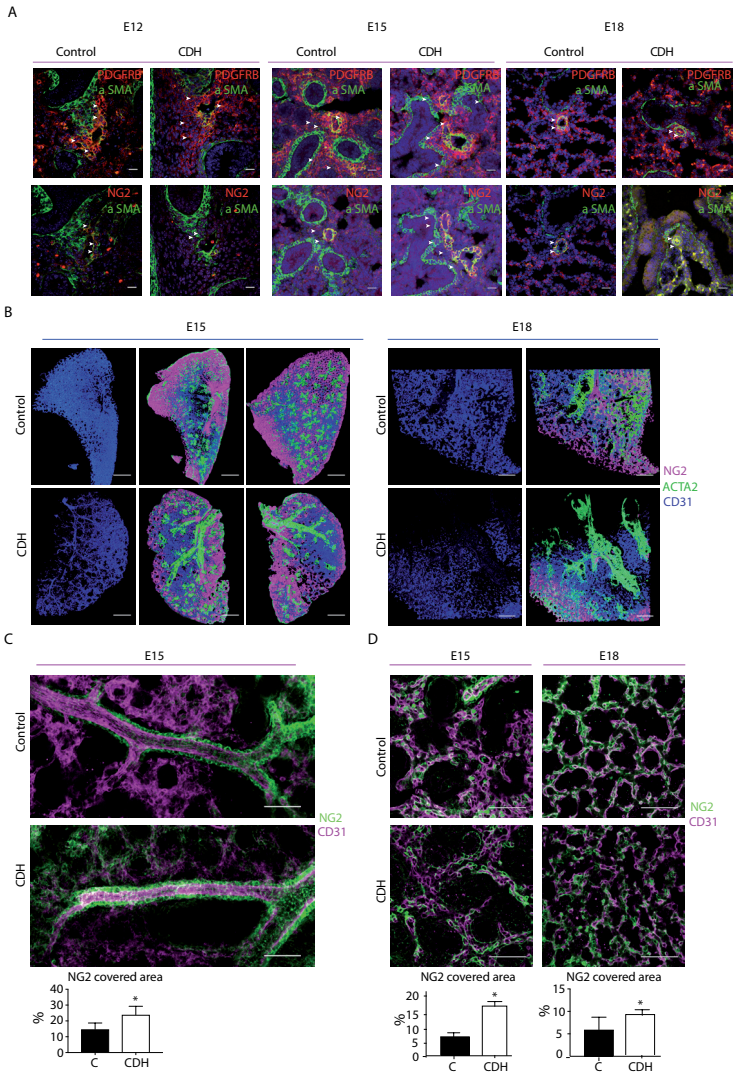


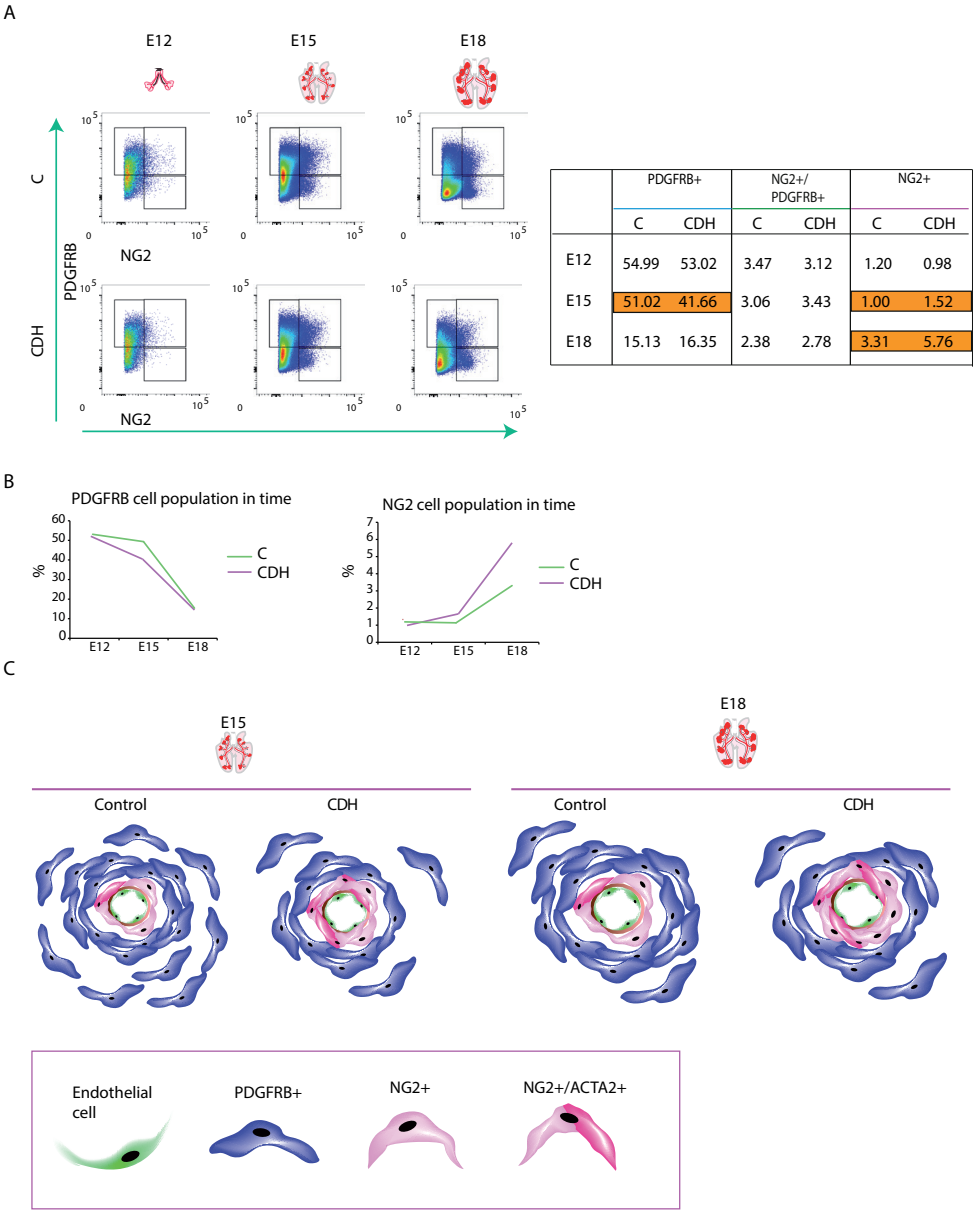
Figure 2

Distribution of perivascular cells during pulmonary development in control and CDH. A Double labeling of PDGFRβ and ACTA2 at E12, E15 and E18. PDGFRB is expressed throughout the mesenchyme and αSMA is expressed around the large airways and in the inside layer of the perivascular wall. Double labeling of NG2 and ACTA2 at E12, E15 and E18. The inside of the perivascular wall is mainly NG2 positive and some cells are NG2 and ACTA2 positive from E15 onwards. More double positive cells were observed in the CDH samples. Scale bar is 20 µM. B. The lungs of CDH pups show an under developed capillary bed at E15 and E18 (CD31 in blue, NG2 in Magenta and ACTA2 in green). Scale bar is 100 µM. C. Analysis of the second large branch of the left lung lobe revealed significant increase in NG2 positive area in CDH at E15. Scale bar is 50 µM. D. High magnification images of the capillary bed at E15 and E18 showed significant increase in the NG2 positive area and a simplified structure of the capillaries in CDH. Scale bar is 50 µM. Two sided T-test was performed.

E18 appear to be loosened from each other resulting in an impaired capillary bed (Fig. 2D). Taken together, we show here for the first time increased pericyte coverage in pulmonary hypertension associated with CDH as early as from E15 onwards in both the large pulmonary vessels and the capillaries. The simplified network of capillaries observed in CDH may the results of this increased pericyte coverage and the shift in the number of endothelial and perivascular cells may be the basis for the impaired angiogenesis.

Fluctuation of Ng2 positive pericytes in the lung during normal and abnormal development

The whole mount analysis of the pulmonary vasculature at E15 and E18 showed a highly affected capillary bed in CDH and significant increase in pericyte coverage. These results gave rise to the hypothesis that the angiogenic process is affected in CDH, or more precisely, that an increased number of pericytes is the potential source of these angiogenic defects observed in CDH. To further confirm this increase and in order to assess and more precisely quantify this putative difference at E12, E15 and E18, we performed a FACS analysis of three different cell populations: $CD45^-$/ $CD31^-$/ $PDGFR\beta^+$ (hereafter $PDGFR\beta^+$), $CD45^-$/ $CD31^-$/ $NG2^+$ (here after $NG2^+$) and $CD45^-$/ $CD31^-$/ $NG2^+$/$PDGFR\beta^+$ (hereafter $PDGFR\beta^+$ /$NG2^+$) (Fig 3A). At E12 there were no significant differences between control and CDH in the different cell populations. However at E15 we observed a significant decrease in $PDGFR\beta^+$ and a significant increase in $NG2^+$ cell populations in lungs of CDH pups (Fig. 3A). The difference in the $PDGFR\beta^+$ population diminished at E18, but the significant increase in $NG2^+$ population continued (Fig. 3A). At all time points examined we analyzed the $NG2^+$/$PDGFR\beta^+$ population, but we did not detect any differences in this population between control and CDH (Fig. 3A). Plotting the percentages of $NG2^+$ and $PDGFR\beta^+$ populations at different time points revealed an antagonistic behavior of these populations (Fig 3B). Thus, the $NG2^+$ cell population expanded during development, while the $PDGFR\beta^+$ cell population decreased, indicating that NG2 possibly marks more established perivascular cells and that PDGFRβ marks more uncommitted perivascular cells. Taken together, no significant differences were observed at E12 between control and CDH samples in any of the investigated populations. In contrast, from E15 onwards a decrease in the number of $PDGFR\beta^+$ is observed. The FACS data further confirmed that the significant increase in the number of $NG2^+$ cells, indicating increased pericyte coverage in CDH.



	PDGFRB+		NG2+/ PDGFRB+		NG2+	
	C	CDH	C	CDH	C	CDH
E12	54.99	53.02	3.47	3.12	1.20	0.98
E15	51.02	41.66	3.06	3.43	1.00	1.52
E18	15.13	16.35	2.38	2.78	3.31	5.76

Figure 3

Perivascular cell populations during lung development in control and CDH A. Representative FACS plots of perivascular population at E12, E15 and E18. The PDGFRβ positive population is the largest population, then the PDGFRβ/NG2 positive population and the NG2 positive population is the smallest. Table with cell percentages, significant differences between control and CDH are indicated in orange. B Adverse behavior of PDGRβ and NG2 positive population during pulmonary vasculature development. Scale bar is 20μM in all sections. Two sided T-test was performed. C Schematic summary of perivascular cell distribution at E15 and E18 in control and CDH samples.

Increased NG2 positive pericytes coverage of the pulmonary vasculature in CDH

The observation that the number of pericytes is increased during pulmonary development in the CDH mouse model raised the question whether this may underlie the pathological changes observed in CDH. So far, it has remained unclear what precursor cells could lead to this extensive muscularization and whether there are differences in this cell population between CDH and control lungs. Pericytes are suitable candidates to underlie these perivascular changes given the timing of their appearance and their function in angiogenesis. Therefore, the ratio of the $NG2^+$ pericytes per $CD31^+$ endothelial cell was determined as an indication for pericytes coverage per vessel. The pulmonary vasculature of control and CDH lungs isolated at E15 and E18 was visualized and clearly showed difference in the complexity (Fig 2 and 4A). Although the gross appearance of the vessels in CDH appeared different from controls, we aimed to analyze the individual vessels to ascertain the cellular composition of the vascular wall and study their development. Therefore, a FACS analysis was performed at gestation day E12, E15 and E18 to select the $CD45^-$/ $CD31^+$ population (Fig 4 B). The percentage of $CD31^+$ cells was significantly decreased in the CDH samples compared to controls, corresponding with the whole mount analysis. The pulmonary vasculature of both the control and CDH lungs rapidly expands in time, although this expansion is more prominent in control lungs, as indicated by the steeper curve (Fig 4C).

Next, the vascular coverage was determined at E12, E15 and E18 by measuring the ratio of the $PDGFR\beta^+$, $NG2^+$ and $PDGFR\beta^+/NG2^+$ populations over the CD31+ population (Fig 4D). At E12 no overt differences in pericyte coverage between control and CDH was noted, but at E15 a significant increase in $NG2^+$ coverage in the CDH samples was detected. This difference is continuous and also observed at E18. The other cell population ratios did not show any significant differences. Thus, although CDH lungs have a simplified vascular tree, a significant increase of the inner perivascular layer was observed from E15 onwards in CDH lungs, which may trigger the pathological muscularization defects observed in pulmonary hypertension associated with CDH.

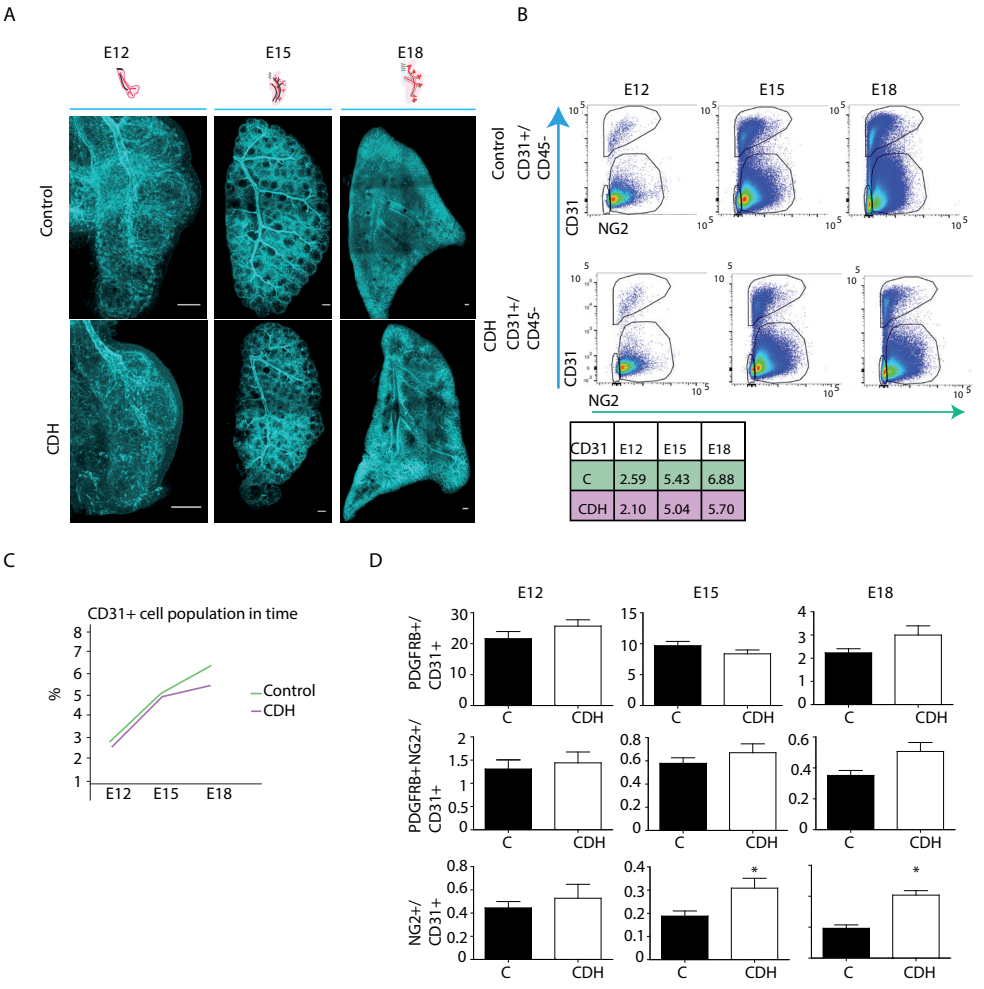


Figure 4

Pericyte coverage is increased during pulmonary vascular development in CDH. A The pulmonary vasculature at E12, E15 and E18. CDH samples show lung hypoplasia and simplification the pulmonary vasculature from E12 onwards. Scale bar is 50 µM B FACS analysis of CD31 population at E12, E15 and E18 of control and CDH samples. C The behavior of CD31 positive cell endothelial cells during pulmonary vascular development. The pulmonary vasculature of CDH pups expands slower than the control samples. D Ratio of different perivascular cells and endothelial cells. No significant differences were observed in the ratio of PDGFRβ/CD31 and PDGFRβ-NG2/CD31 positive cells during pulmonary vascular development. A significant increase was observed from E15 onwards in the ratio of NG2/CD31 positive cells. Two tailed T-test was performed.

Reduced proliferation of endothelial cells and pericytes during pulmonary vascular development in CDH

The apparent differences in the perivascular layer between control and CDH lungs could result from differences in the cell cycle. Thus, control and CDH lungs were stained with Ki67 and NG2 to analyze the proliferation and cell division of endothelial cells and pericytes. The vasculature was divided into small vessels (20-70 μm) (Fig. 5A) and large vessels (>70 μm) (Fig. 5A). At E15 we observed a non-significant decrease in number of proliferating endothelial cells in the small vessels, but a significant decrease in proliferating pericytes in the small vessels (Fig. 5B). No significant differences in proliferation of pericytes and endothelial cells were observed in the large vessels between control and CDH at E15 (Fig. 5B). At E18, both the small and large vessels showed a significant decrease in number of proliferating endothelial cells in CDH (Fig. 5B). No difference was observed in the number of proliferating pericytes in CDH in both the large and the small vessels at E18 (Fig. 5B) aside from differences in proliferation, the changes in the vascular wall in CDH lungs may also be related to migration of proliferating progenitor cells. Therefore, human brain pericytes were cultured with or without the retinoic acid inhibitor BMS493 (figure 5C), which significantly inhibited the migration potential of the pericytes (Fig 5C). This suggests that the pericytes in the lungs of CDH may have a reduced migratory potential.

These results imply that the balance of proliferating pericytes and endothelial cells in CDH is disrupted, which may lead to disordered angiogenesis and a simplification of the pulmonary capillary bed in CDH.

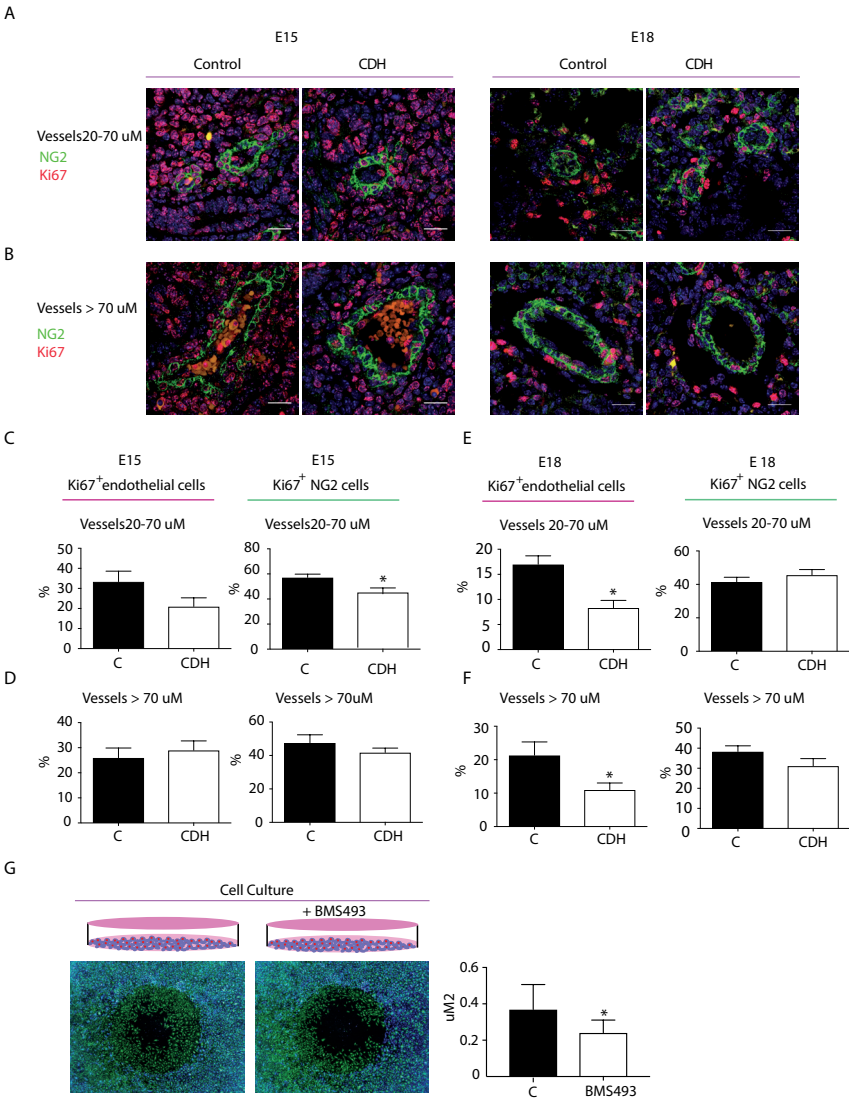


Figure 5

Proliferation of endothelial cells and proliferation and migration of pericytes in the retinoic acid inhibited mouse model and in cultured human brain pericytes. A Double staining for NG2 (green) and Ki67 (red) of the small vessels (20 µM -70 µM) at E15 and E18. . Scale bar is 20µM B Double staining for NG2 (green) and Ki67 (red) of the large vessels (>-70 µM) at E15 and E18. Scale bar is 20µM C A Significant decrease was observed in the number of NG2/ Ki67 double positive cells in the small vessels of the CDH mouse model at E15. The endothelial cells showed a trend in the quantity of Ki67 positive cells in the inner layer of the small vessels at E15. D No significant differences were observed between proliferating cells in the large vessel between CDH and control at E15. E –F Significant decreases were observed in the inner layer of the small and the large vessels of the lung of CDH pups at E18. No difference was observed between control and CDH in the proliferating NG2 population. G Human brain pericytes were cultured and migration capacity of these cells was tested in the presents of the retinoic acid inhibitor BMS493. The addition of BMS493 to the cultured pericytes resulted in significant lower migration capacity. Two-sided T-test was performed. 25 sections were analyzed for the proliferating assay. The migration assay was performed 3 times independently; each time at least 9 wells were analyzed per condition.

Decreased collagen IV production of pericytes leads to altered differentiation and simplification of the capillary bed in CDH

The increase of pericyte coverage and the simplification of the pulmonary capillary bed led to the conclusion that the interaction between endothelial cells and pericytes is affected in CDH. Therefore, we analyzed the presence of COLIV, which is the main constituent of the basement membrane forming between the endothelial cell and the pericytes. The production of COLIV is a parameter for the BM integrity is. Moreover, the BM between pericytes and endothelial cells is important in the communication between these two cell types.

At E12 we did not observe apparent differences in COLIV expression between control and CDH (Data not shown). However, at E15 a reduced level of collagen was observed throughout the mesenchyme in CDH and we noticed discontinued basement membranes around the midsized vessels indicated by the white arrow (Fig 6A). The reduced expression of COLIV in the BM was also detected at E18 throughout the mesenchyme and the basement membrane of the midsized vessels was also discontinued indicated by the white arrow (Fig 6B).

The reduced proliferation and migration (Fig 5) and an affected basement membrane (Fig 6A-6B) suggested that pericytes in CDH are different and that there may be changes in the expression of pericyte-specific genes. Therefore the double positive area for NG2 and ACTA2 at the proximal (large vessels >70 µm) and distal side (small vessels 20-70 µm) of the pulmonary vasculature was quantified (Fig. 6C). At E15 there was no significant difference in co-localization of NG2 and ACTA2 in CDH. However, at E18 there was a significant increase in co-localization of NG2 and ACTA2 in the large and in the small vessels in CDH. This indicates that pericytes in lungs in CDH contain more ACTA2 fibers, leading to more contractile pericytes. To test whether this was an effect of the inhibition of the retinoic acid pathway, pericytes were cultured in the presence or in the absence of the retinoic acid pathway inhibitor BMS493. Pericytes that were cultured in the presence of BMS493 clearly expressed more ACTA2 than the pericytes that were cultured in absence of BMS493 (Fig. 6D). This indicates that the increase of ACTA2 expression in CDH is an effect of the inhibition of retinoic acid pathway and may be the result of up regulation of other signaling pathways. Taken together, these data show that pericytes in cell culture and in the lungs of CDH pups are more contractile and that altered differentiation of pericytes in the distal end of the lung leads to neo-muscularization observed in pulmonary hypertension associated with CDH.

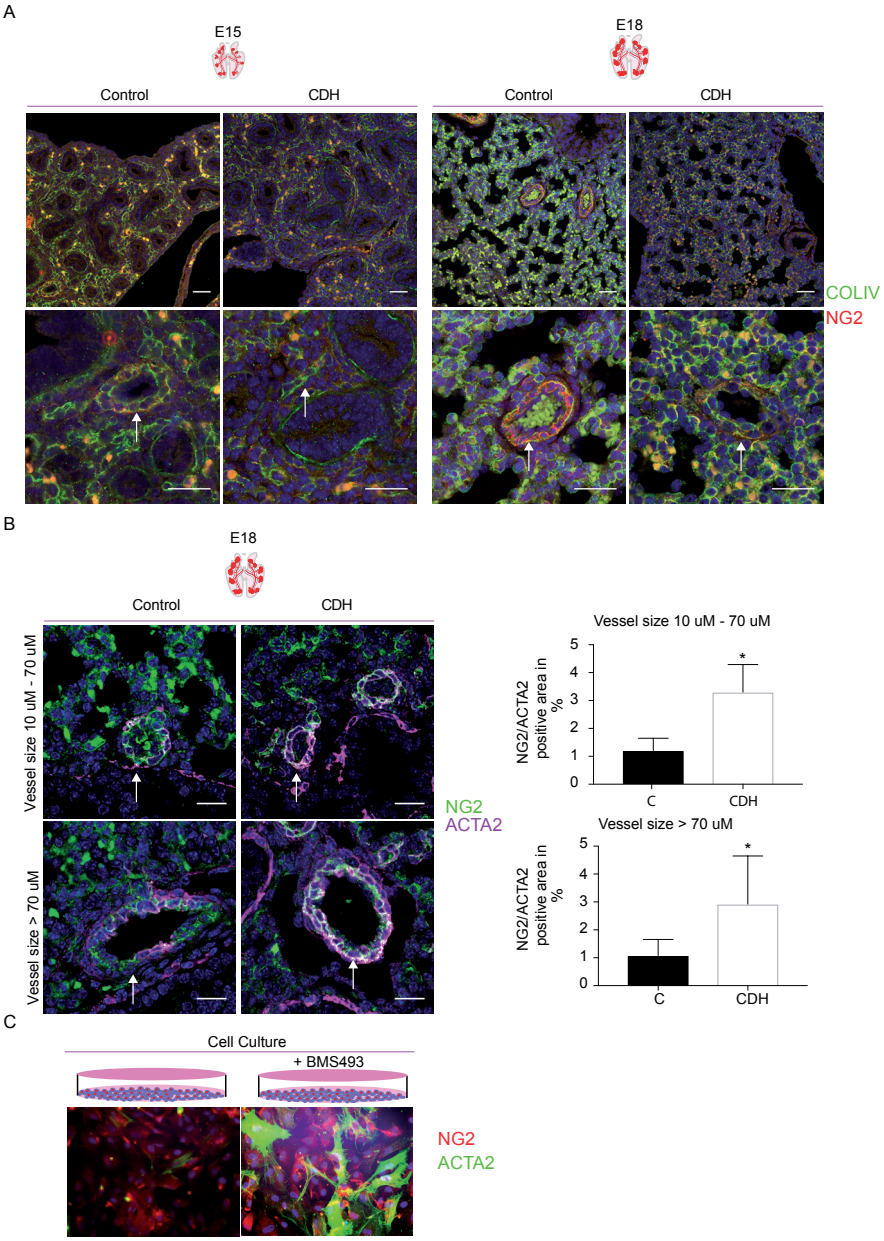


Figure 6

Reduced Collagen IV production in CDH and an increase of ACTA2 expressing pericytes. A COLIV is expressed throughout the mesenchyme at E15. Decreased expression the CDH samples was observed in the mesenchyme and around the vessels (indicated with white arrow). This effect hold true until E18. Scale bar is 20 µM. B The number of αSMA expressing pericytes in the small and in the large vessels was significant higher in CDH samples at E18. Scale bar is 20µM C Human brain pericytes were cultured in for 24 hours with the addition of the retinoic acid inhibitor BMS493. Pericytes cultured in the presents of the inhibitor expressed more αSMA than the pericytes in the control conditions. Two-sided T-test was performed.

Human CDH patients show imbalance in PDGFRβ and Collagen IV expression during late pseudo-glandular phase in lung development

To test whether the findings in the CDH mouse model could be extrapolated to human CDH patients, the expression of PDGFRβ and COLIV was analyzed. Since pericyte markers may be different per organ, gestational age and also per species, we first examined different pericyte markers in human tissue. This indicated that PDGFRβ is the most reliable and specific pericyte marker in the late pseudoglandular phase of human lung development. Since the first differences in COLIV and PDGFRβ appeared in the pseudoglandular phase of lung development in the CDH mouse model, lungs of CDH patients and age-matched controls at the pseudoglandular phase were analyzed. Increased expression of PDGFRβ was observed in CDH patients, which correlates with a lower COLIV expression (Fig. 7A and Fig. 7B). This result is in line with the findings in the CDH mouse model, so increased coverage of pericyte in the pulmonary vasculature leads to aberrant pericyte attachment and maturation. The BM showed reduced expression of COLIV in CDH, this could indicate that the attachment or cross-signaling between pericytes and endothelial cells is inadequate. This may lead to decreased angiogenesis and a simplification of the capillary bed in the distal end of the lung in CDH. The current findings together with our previous work where we have shown the importance of the interaction between the vasculature and the growth of the lung [8], the simplification of the capillary may be the cause of the hypoplastic lungs in CDH.

Discussion

Pulmonary hypertension associated with CDH results in high mortality and morbidity [31]. Studies concerning the structural vascular anomalies have mainly focused on the increased thickening of the tunica media of the vascular wall. In this study we dissected the events before this thickening occurs and what may be the source of the hyper-muscularization and neo-muscularization. Pericytes may be good candidates to cause these pathogenic defects.

However characterization of pericytes during lung development has not been studied intensively and therefore we firstly analyzed the expression of multiple pericyte markers. Based on their expression patterns we showed that NG2 and PDGFRβ are the most specific markers for mouse and human pericytes during lung development, respectively. The establishment of the most useful pericytes marker in mouse development allowed us to further analyze potential differences between control and CDH lung samples. Although the CDH lung displays a simplified vascular tree, we also demonstrate that the pericyte coverage was increased in CDH lungs already in the late pseudoglandular stage of lung development at the individual vessels. 4D analysis of pericytes and endothelial cells revealed that the pulmonary vasculature in the CDH mouse model is highly affected. Specifically, the micro vascular bed lacks an organized structure and exists of a reduced number of capillaries.

Additionally, less proliferating pericytes and endothelial cells were observed in CDH, which could direct into a deficiency of sufficient numbers of pericytes to support extensive vascular growth by angiogenesis. We found that the BM in CDH was affected due to decreased COLIV expression of pericytes in lungs of CDH pups. This indicates that the establishment of newly formed vessels is interrupted by reduced expression and thus deposition of COLIV by pericytes. As a result, endothelial cells do not receive inhibitory signals and therefore keep recruiting pericytes, which is supported by the observed increased pericyte coverage in CDH. To be more specific, there are less cells expressing PDGFRβ in the mesenchyme of E15 lungs in CDH, indicating that these cells do not respond to PDGF secreted by the endothelial cells. This may explain why endothelial cells continue to recruit pericytes, resulting in a reduced pulmonary vascular growth and a thickening of the perivascular wall. Moreover, there was not only an increase in pericyte coverage, but also pericytes in CDH expressed more ACTA2, which indicates that they may be more contractile and could therefore be the source of the neo-muscularization observed in CDH.

Fluctuations and alterations in pericytes coverage and interaction with their surrounding cells have been linked to multiple pathological conditions such as diabetic renopathy, cancer biology and pulmonary hypertension in the adult [16, 18, 32-34]. Especially the finding of increased pericyte coverage in pulmonary hypertension in the adult is in line with our findings [18]. We are the first to show during lung development in CDH an increase in pericyte coverage which has not been demonstrated before. We show by FACS analysis and extensive IF experiments that more pericytes are detected in the CDH mouse model, which was surprisingly linked to reduced expression of COLIV. Previously, increased pericyte coverage of the micro-vasculature has been linked to pulmonary hypertension in the hyperoxia rat model for pulmonary arterial hypertension [35]. However this has not been linked to altered COLIV production. Aberrant COLIV expression by pericytes has been described in literature before, but mainly cases of increased COLIV production such as in wound healing processes [36, 37]. The observed reduced expression of COLIV in CDH, however, explains the reduced capillary bed since a reduced expression of COLIV leads to less stabilization of the newly formed tubules and therefore could lead to inefficient tubule maturation what is required for further sprouting and thereby growing of the capillary network [38]. Additionally, increased pericyte coverage in the lungs of CDH pups was observed and these pericytes also significantly expressed more ACTA2. Increased pericyte coverage and increase of ACTA2 expressing pericytes is linked to pulmonary arterial hypertension in adults before [18]. Furthermore it has also been shown that resident fibroblasts and pericytes express more ACTA2 in idiopathic fibrosis [34]. These studies highlight the importance of pericytes in pathological conditions in the lung in addition, these alterations in pericyte coverage and pericyte contractility lead to inhibited angiogenesis and therefore less extensive capillary bed in CDH underlining the concept that CDH is a developmental disorder which occurs already early during lung development [39 40].

Furthermore, lung samples of human CDH patient show more expression of PDGFRβ indicating more pericytes and reduced COLIV expression early in lung development. This confirms that CDH is an early developmental disease and the focus of the treatment should shift from postnatal to prenatal. We have shown before that prenatal treatment of the nitrofen induced CDH rat model results in reduced muscularization of the pulmonary vasculature [29]. However, current results show that treatment of CDH should even occur earlier in lung development aiming at modulation of pulmonary vascular development primarily

CDH is a complex disease and is still difficult to treat while the underlying "natural history" of the disease is still not well understood. The pathology has been known for decades based on morphological descriptions only. This study confirms that CDH is an early developmental disease and that pericytes are the source of alterations in muscularization of the vasculature observed in pulmonary hypertension associated with CDH. The observation that CDH is an early developmental abnormality can be used as an argument for prenatal treatment. Moreover, early intervention could help to diminish vascular abnormalities and may result in reduced lung hypoplasia and less severe muscularization defects. Further understanding of the interaction of endothelial cells and pericytes may help to understand the aberrant development of the pulmonary vasculature in CDH.

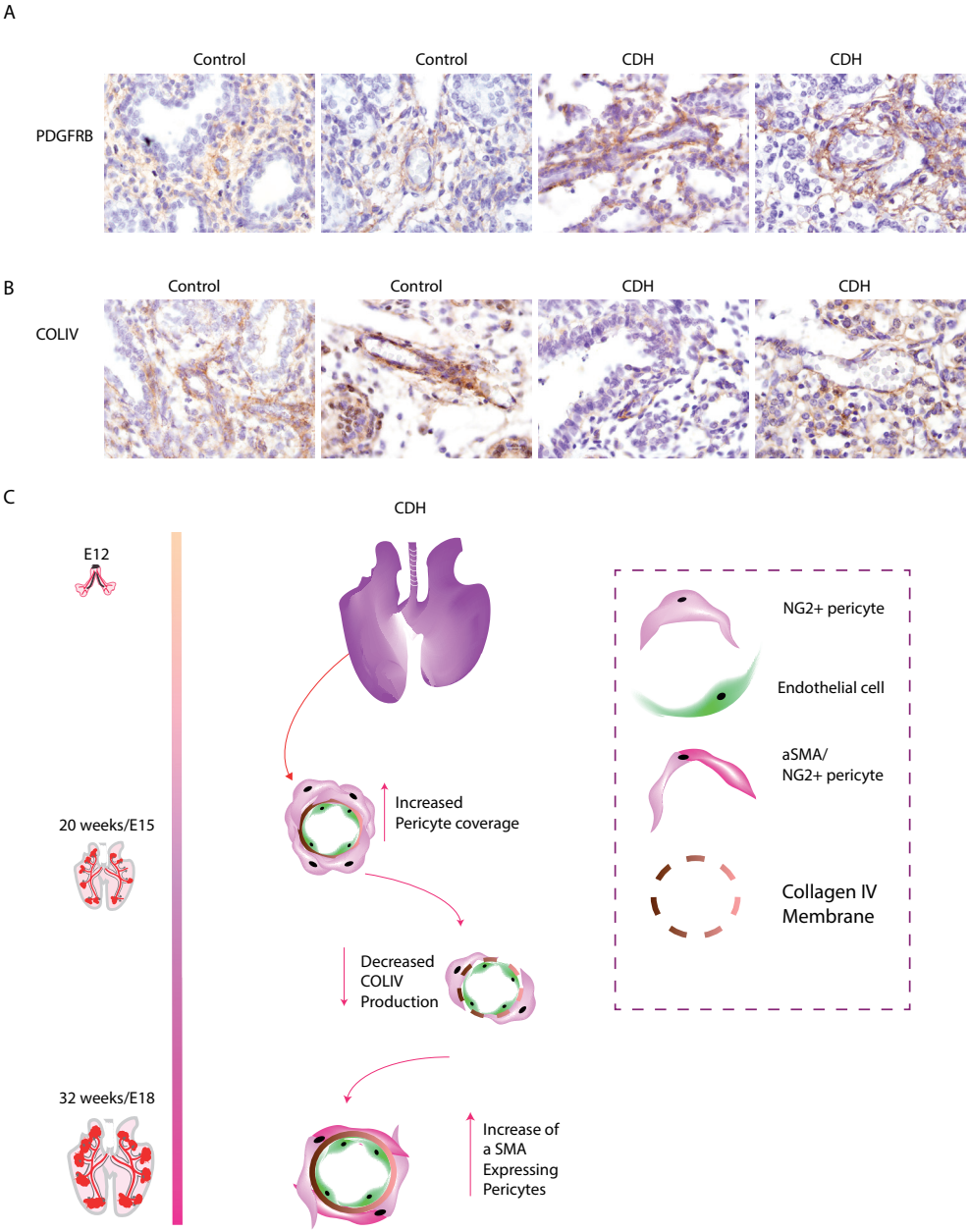


Figure 7

CDH patient samples show that imbalance of PDGFRβ expression is related to reduced COLIV expression. A PDGFRβ expression in aged matched control samples (18+0 weeks and 19+1 week) is reduced compared to the CDH patient samples (23+3 weeks and 17+6 weeks). B COLIV expression is decreased in the CDH patient samples. Indicating an incomplete basal membrane, resulting in dysfunction of pericytes. C Summary of vascular developmental errors that appear during pulmonary development in CDH and may underlie the muscularization defects observed later in development.

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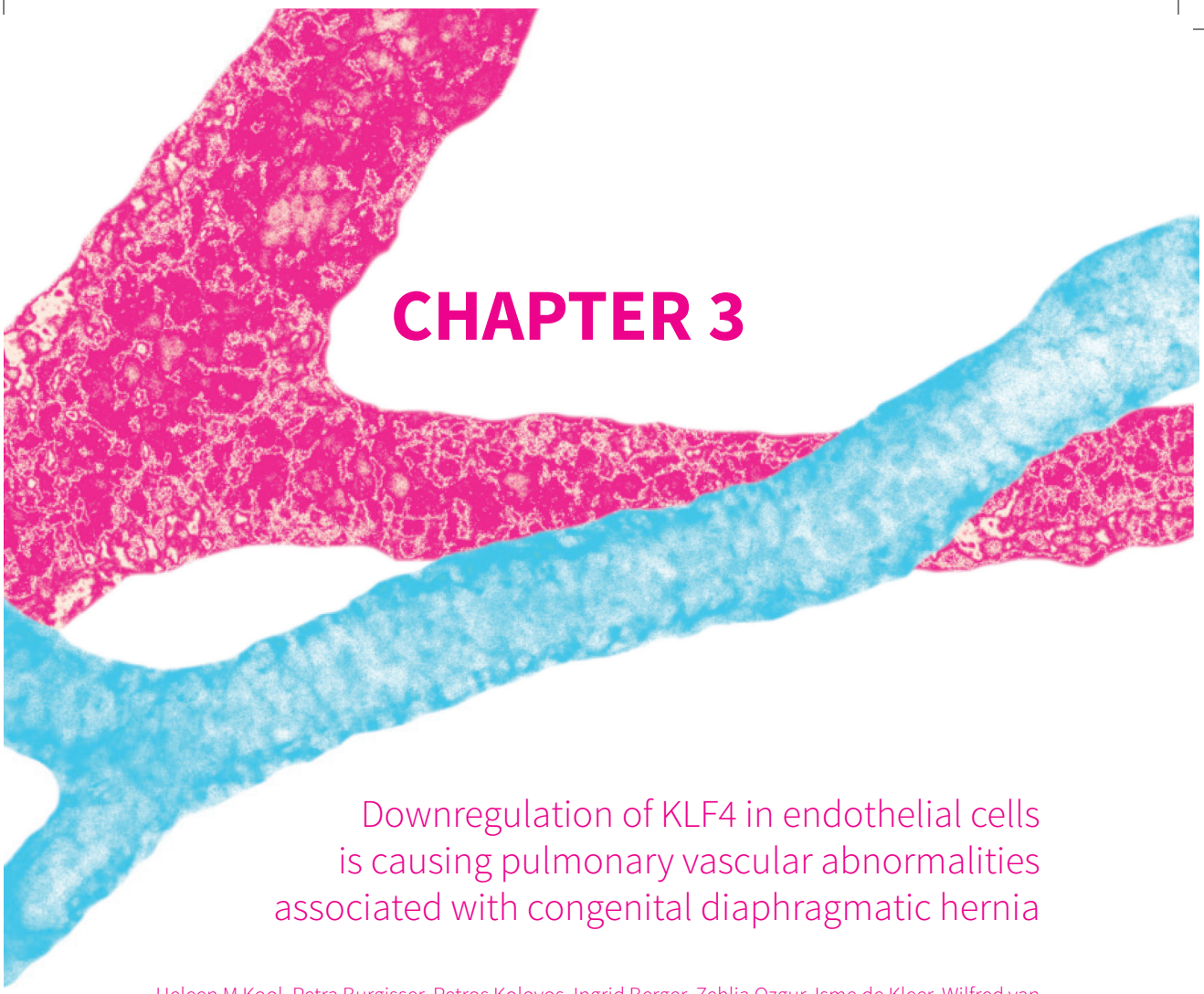
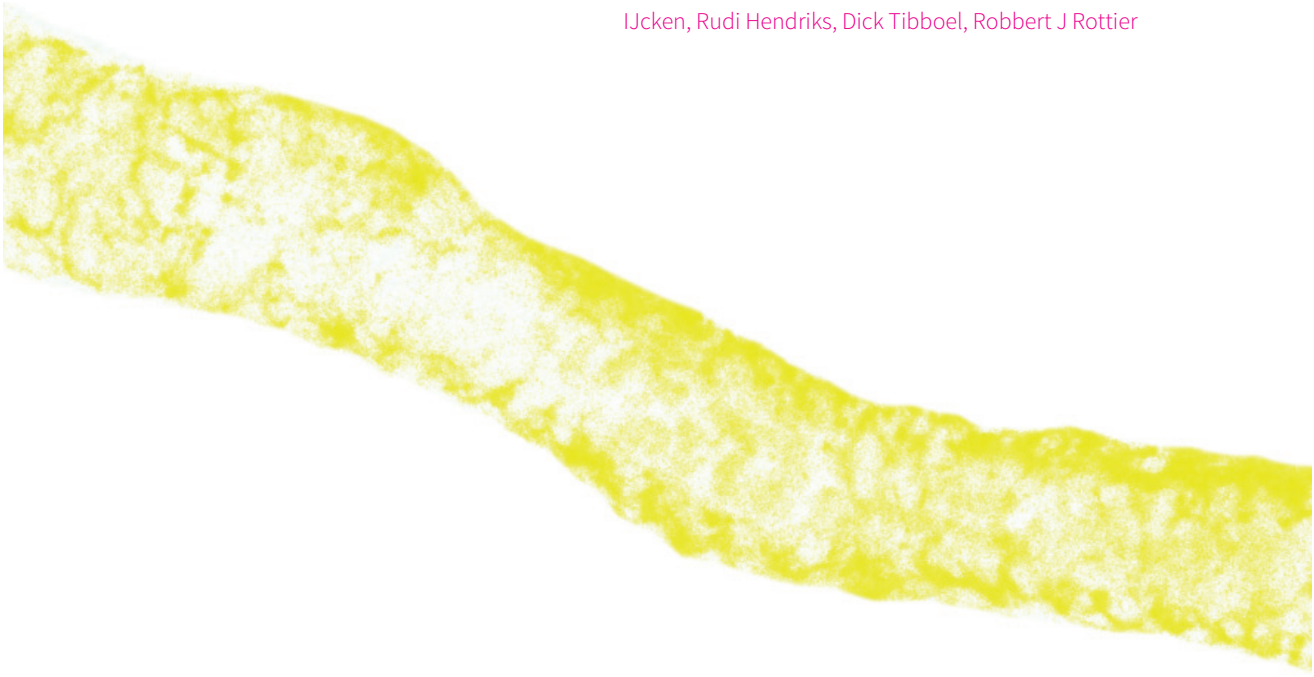


CHAPTER 3

Downregulation of KLF4 in endothelial cells is causing pulmonary vascular abnormalities associated with congenital diaphragmatic hernia

Heleen M Kool, Petra Burgisser, Petros Kolovos, Ingrid Berger, Zehlia Ozgur, Isme de Kleer, Wilfred van IJcken, Rudi Hendriks, Dick Tibboel, Robbert J Rottier



Introduction

Congenital diaphragmatic hernia (CDH) is a severe birth defect characterized by diaphragmatic structural defects, pulmonary hypoplasia and pulmonary hypertension (PH). The incidence of CDH is 1:2500 live births and the associated pulmonary hypertension is unpredictable regarding the response to current treatment modalities. PH is the major cause of death in a newborn with CDH, and the cause of long-term hospitalization and sometimes lifelong morbidity [1, 2]. The pathology of the vascular abnormalities of CDH are characterized by extensive thickening of the smooth muscle cells in the medial layer and neo-muscularization of the small capillaries. The understanding of how these pathological characteristics of CDH occur is very complex because of the heterogeneity of the disease [1]. In contrast to the well-described morphological changes in CDH lungs, little is known about the molecular pathways that are involved in the onset of CDH.

Several studies in rodents and humans have contributed to the identification of potential genes that could be involved in the pathogenesis of CDH. Numerous lines of evidence show that the retinoic acid (RA) pathway contributes to the occurrence of this disease [3-5]. Moreover, studies in the rat model where CDH is induced by Nitrofen, an inhibitor of the retinoic acid pathway, have strengthened the role of the RA pathway in the development of CDH [6, 7 8]. Furthermore, studies in neonates with CDH have shown significant lower levels of retinol and retinol-binding protein[9, 10]. In addition, whole genome sequencing studies of CDH patients have identified chromosomal changes in genes such as *COUPTFII, TCF7L2, FOXP4* and *GATA6* and although valuable, these studies did not analyze the molecular and cellular effects of these mutations with respect to the pathogenesis of CDH[11 12 13]. Thus, human and animal studies hint to a role of the retinoic acid pathway in the onset of CDH, but it remains unclear what the downstream signaling cascade is that ultimately leads to this severe disease.

Previously, we observed structural changes in the pulmonary vasculature of CDH patients and in the CDH mouse model already early in development [14] (Chapter 2). In order to get a better insight in the cellular origin of CDH and in the interactions between the different cell types, we analyzed the gene expression of different cell populations in the CDH mouse model. Therefore, the transcriptome of four different cell populations isolated by Fluorescent activated cell sorting (FACS) were analyzed to reveal new candidates involved in the early events of the development of CDH. Revealing genes involved in CDH could help to understand how interactions between cell types are affected and how this relates to the onset of the pathological conditions associated with CDH.

The analysis of the whole transcriptome data showed a high similarity in GO terms between the NG2 positive pericyte population and the CD31 endothelial cell population. This

suggests a disturbed interaction between endothelial cells and pericytes. The disturbed interaction is the basis for the aberrant development of the pulmonary vasculature in CDH at the cellular level. Detailed analysis of the RNA sequence data revealed that altered expression of the *Kruppel like factor 4* (*Klf4*) gene is involved in the abnormal developed pulmonary vasculature in CDH. The reduced expression of KLF4 was confirmed with whole mount fluorescent analysis. Previously, KLF4 was shown to be an up stream regulator of NOTCH signaling and thereby important for proper angiogenesis [15]. We further found that several members of the NOTCH signaling pathway are differentially expressed, indicating that NOTCH signaling is disturbed in CDH. Indicating that the aberrant vascular development in CDH is a result of decreased KLF4 expression what result in disturbed NOTCH signaling.

Material and Methods

Mouse CDH model

Timed-mated pregnant mice received 20 mg nitrofen and 5 mg BPCA in 500 µl olive oil (Sigma) per 25 grams of body weight at day 8.5 of gestation. At different gestational ages pregnant mice were sacrificed, fetal lungs and diaphragms, if possible given the gestational age, were isolated and their morphology was used to divide the phenotypic appearance of the fetuses into severely affected and mildly affected.

Immunohistochemistry and Immunofluorescence

IF with the FACS antibodies was performed on 5 µm thick cryo-sections. Tissue was isolated and immediately embedded in OCT. Five µm thick sections were fixed in ice-cold methanol for 10 minutes. Blocking was performed using 5% heat inactivated donkey serum in PBS-0.5% Tween incubated for 1 hour on room temperature. Slides were washed and incubated with the primary antibody overnight. The next day, slides were washed and incubated with a fluorescent secondary antibody for 2 hours on room temperature. IHC was performed on 5 µm thick paraffin sections of lungs according to standard protocols. Antigen retrieval was performed with either Tris-EDTA buffer (pH 9.0) or citric acid (pH 6.0) on the first day and on the second day the Envision™ detection system (DakoCytomatic) was used. Primary and secondary antibodies used for IHC and for IF are listed in supplementary table 1
Negative controls were performed by omitting the primary antibody**.**

FACS

Lungs were isolated at different gestational ages, and were collected in 1 ml DMEM (Lonza) in Eppendorf tubes. Collagenase I, Collagenase II and Collagenase IV were added and incubated at 37^{O}C for 10 minutes in an Eppendorf shaker at 1000 rpm. Then, the mixture was mixed vigorously to completely disrupt the tissue and the cell suspension was put through a 40 µM cell strainer. The filter was rinsed with 1ml DMEM 10% FCS and spin down

at 4° C for 10 minutes at 400 g Supernatant was removed and the pellet was washed with PBS-10% FCS and resuspended in 300 µl PBS 10% FCS, of which 10 µl was taken to count the number of cells and to check for doublets and cell aggregates. Samples centrifuged at 4° C for 10 minutes at 400 g and cells were re-suspended in 100 µl of staining mix (CD31, EPCAM, CD45 and NG2) or single stains, to set the laser voltages, for 40 minutes at 4° C. Samples were then washed by adding 150 µl PBS-FCS and gently mixed and centrifuged. Pellets were re-suspended in 200 µl PBS-FCS and kept in darkness at 4° C until sorted and just prior to sorting 0.1 µl DAPI solution was added and samples were measured on the ARIA FACS machine (B&D). A 100 micron nozzle was used and the gap was kept around 8. Cells were caught up in RNA later (Thermofisher) and kept in RNA later at 4° C until RNA isolation was performed. Analysis of the data was done using FlowJo

RNA isolation, sequencing, amplification and qpcr

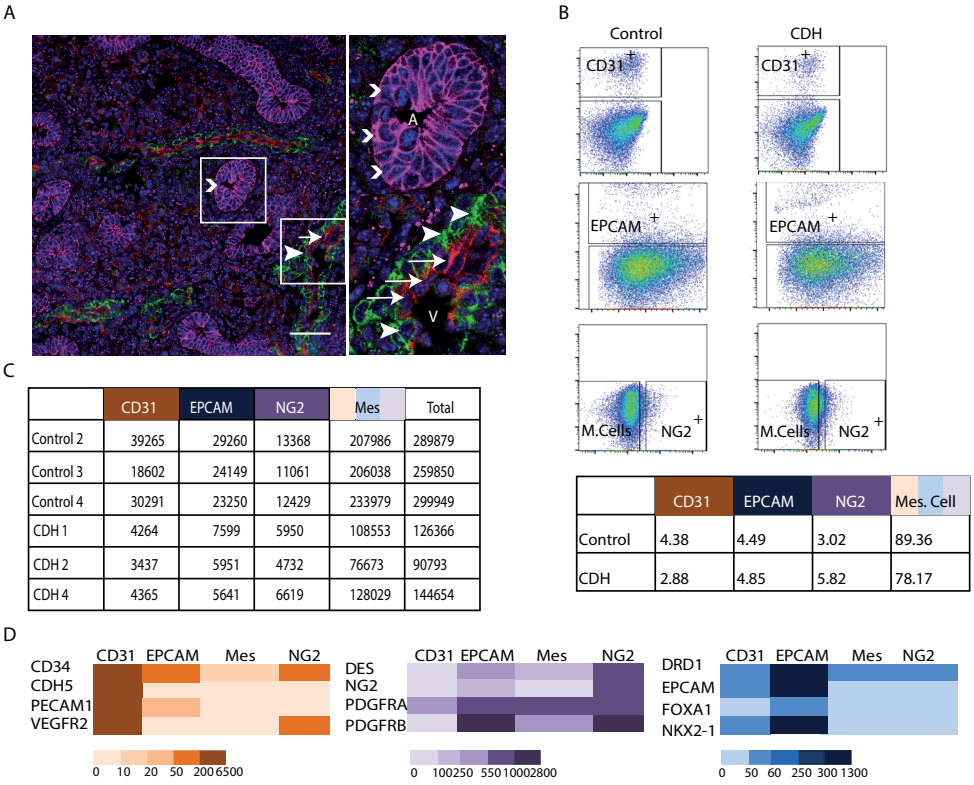
RNA was isolated using the mirVana Paris kit (Thermosfisher). Samples in RNA later were 1 on 1 diluted with ice-cold PBS and spin down on 15000 rpm. RNA sequencing was performed following the Smarter-2Seq protocol with small adjustments [16].

Analysis of RNA sequencing results

The RNA sequences (demultiplexed) were mapped using TopHat. The RNA profiles in FPKM values (Fragments Per Kilobase of transcript per Million mapped reads) were generated by Cufflinks. This output was further analyzed using R. A log2 fold change cut off of 0.6 was used for the upregulated genes and a -0.6 was used for the downregulated genes. Downstream pathway analysis was done using Inguinity pathway analysis (IPA).

Whole mount immunofluorescence

Whole mount immunofluorescence staining of total fetal lungs was based on Yokomizo et al., (2012). Shortly, freshly isolated lungs were fixed in 4% paraformaldehyde (PFA) overnight. The next day, the lungs were gradually dehydrated through washes in increasing methanol and could be stored in pure methanol at -20°C for up to 3 months. The next day, the lungs were re-hydrated by sequential steps of decreasing concentration of methanol and blocked in PBS, 0.4% triton X-100 and 1% skimmed milk powder for 4 hours, blocking buffer was refreshed every hour. Subsequently, the lungs were incubated with primary anti-body for at least 4 days at 4°C. Then samples were washed with PBS-0.4% Triton for at least 48 hours, washing solution was refreshed every 4 hours and at the end of the day. Secondary anti-bodies appropriate for the choice of primary antibody were conjugated with Alexa 488, Alexa 594, biotinylated 647 and were incubated for at least 48 hours. After this incubation, several washes were performed and the lungs were cleared with benzyl alcohol/benzyl benzoate (BABB). Samples were imaged using a Tile Scan option on the Leica Sp5 confocal microscope. Pictures were processed using FiJi, pictures were adjusted with brightness/ contrast option and background was reduced in Photoshop using Levels function



	CD31	EPCAM	NG2	Mes	Total
Control 2	39265	29260	13368	207986	289879
Control 3	18602	24149	11061	206038	259850
Control 4	30291	23250	12429	233979	299949
CDH 1	4264	7599	5950	108553	126366
CDH 2	3437	5951	4732	76673	90793
CDH 4	4365	5641	6619	128029	144654

	CD31	EPCAM	NG2	Mes. Cell
Control	4.38	4.49	3.02	89.36
CDH	2.88	4.85	5.82	78.17

Figure 1. Expression analysis of the four different cell populations used for RNA sequence

A E13 lung tissue stained for EPCAM (magenta, open arrow heads), CD31 (red, arrows) and NG2 (green, closed arrow heads), showing the epithelial cells in the airways and the endothelial cells and perivascular cells in the vasculature (V). **B** Representative FACS plots of sorted endothelial (CD45- CD31+), epithelial (CD45-, CD31-, EPCAM+), perivascular cells (CD45-, CD31-, EPCAM-) and mesenchymal cells (CD45-, CD31-, EPCAM-, NG2-) at E13 Control versus CDH. The table shows the average of the percentages of the four different cell populations in control and CDH. **C** Total numbers of sorted cells of control and CDH at E13 according to the FACS sort. **D** Heatmaps with the average number of Fragments Per Kilobase of transcript per Million mapped reads (FPKM) for the three homogenous cell populations for the specific markers; upper for endothelial cells, middle for perivascular cells and bottom for epithelial cells.

Results

The distinctive cell populations present in the E13 mouse lung

Previously, we and others showed that the CDH mouse model[17] (unpublished data) showed comparable pathological characteristics to the well-established nitrofen rat model and to human CDH patients[18]. In order to not only delineate the molecular pathways, but also the differences at the cellular level in CDH we focused on individual cell populations involved in vascular and airway development. Therefore, the spatial distribution of the individual cell populations was analyzed by immunofluorescent labeling of early mouse lungs (E13) (Fig.

1A). The immunofluorescent labeling revealed that the EPCAM antibody specifically labeled the epithelial cells of the airways (indicated by open arrow heads Fig. 1A). Furthermore, endothelial cells were specifically labeled with the CD31 antibody (indicated with white arrows Fig. 1A) and perivascular cells were specifically labeled with NG2 (indicated with closed arrow heads Fig. 1A).

Since the antibodies appeared very specific, they were subsequently used to isolate the various cell populations from early pseudoglandular stage lungs of control and CDH mouse embryos. The CD45- fraction was subsequently used to purify the endothelial (CD31+), epithelial (EPCAM+) and perivascular (NG2+) cells, while also the, heterogeneous CD31-, EPCAM-, NG2- population was collected (subsequently referred to as the mesenchymal population). FACS plots showed that the size of these cell populations was variable between control and CDH samples (Fig 1B). The cell percentages of the endothelial, epithelial and perivascular cells varied between 2.8% - 5.86%, whereas the mesenchymal cell population was around 86 %. An increase in the number of perivascular cells and in the ratio pericyte/endothelial cell was observed, which further verified our previous study (manuscript in preparation) (Fig 1B and Fig. 1C). The transcriptome of these isolated populations was generated and the purity of the populations was determined by the analysis of genes specific for endothelial, epithelial and perivascular cells, leading to heatmaps with the specific gene expression for each population (Fig. 1D). This revealed that the endothelial specific markers CD34, CDH5, PECAM1 and VEGFR2 had the highest number of reads in the CD31+ endothelial cell population (Fig. 1D). The FPKM values of the perivascular markers Desmin, NG2, PDGFRβ and PDGFRα showed the highest expression in the NG2+ perivascular population, while the gene expression levels of DR1, EPCAM, FOXA1 and NKX2-1 revealed that these markers were expressed the highest by the epithelial (EPCAM $^{+}$) population (Fig. 1D). Taken together, we identified specific populations in situ and subsequently isolated these different cell populations with high purity as indicated by the heatmaps for the different markers. We furthermore detected a significant lower cell number of each sorted populations in the CDH samples, reflecting the clear pulmonary hypoplasia in the CDH lungs.

Expression of CDH-associated genes assigned to specific cell populations

Until now, several studies have focused on identifying genes that may be involved in the pathogenesis of CDH, either identified by exome studies in CDH patient or they have been linked to CDH in mouse studies [12, 19 19, 20]. Therefore, we first focused the analysis of the transcriptomes on those genes that have previously been associated with CDH in human and mouse studies. A heatmap was generated for these so-called CDH -associating genes in order to elucidate the cell populations that differentially expressed these genes (Fig. 2A). For example, previously identified de novo mutations were found in the transcriptional co activators *EYA1*, *EYA2* and transcription factor *ZFPM2* (*FOG2*) of CDH patients [21]. Sequencing studies of CDH patients furthermore revealed mutations in the genes *STRA6*, *GPC3*, *CHD7* and

GATA4 (Reviewed in Holder et al)[11]. The broad variety of genes indicates the heterogeneous nature of CDH and the difficulty of understanding the onset of CDH. *CHD7* was expressed higher in the endothelial and epithelial cell population but had a lower fold change in the perivascular population (Fig. 2A). Mutations in EYA1 and EYA2 have not only been shown in patient studies, but also mice lacking two alleles of the transcriptional co-activators *Eya1* and one allele of *Eya2* show diaphragmatic defects, indicating the importance of appropriate expression levels of these genes[22]. The transcriptome analysis showed that *Eya1* expression was decreased in the epithelial and perivascular population. Additionally, *Eya2* was higher expressed in CDH samples in the epithelial cell population but did not show differences in expression in the other populations. An imbalance in the expression levels of *Eya1* and *Eya2* can thus be related to the development of CDH.

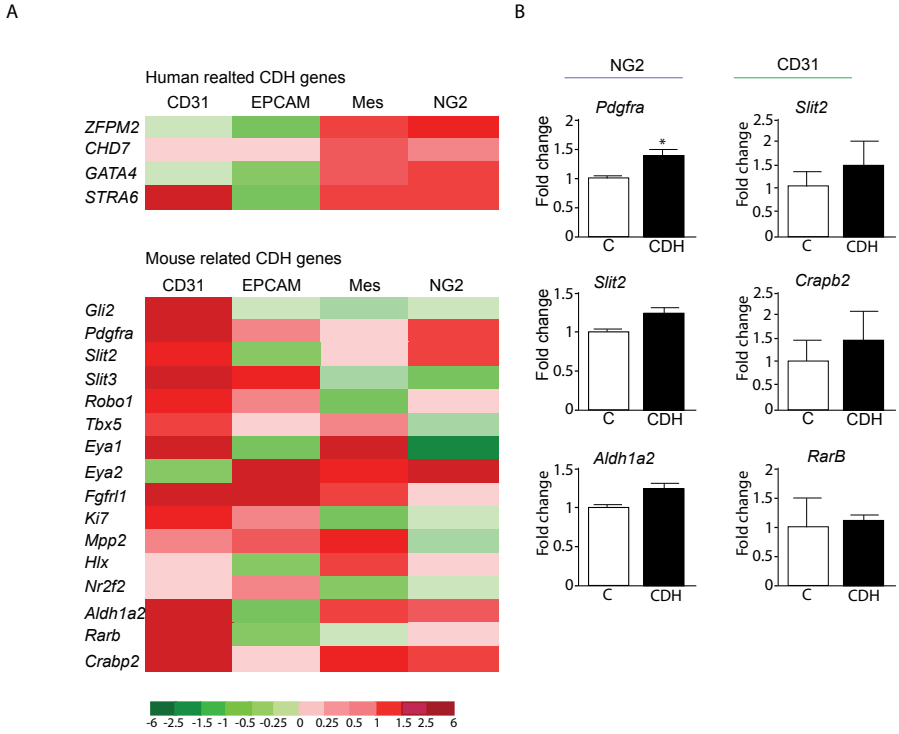


Figure 2 Heatmap of fold changes of CDH related genes for different cell populations

A FPKM values of genes of which are known to be involved in CDH from previous studies in mouse or human samples. Colors indicate the degree of fold change, green indicates downregulation and red indicates up regulation. **B** qPCR validation of RNA sequence results of selected CDH genes in the NG2 and CD31 population.

Genes of the Roundabout (ROBO) -SLIT pathway have also been implicated in CDH[23, 24]. The expression of *Robo* is increased in the endothelial and epithelial cell population and decreased in the mesenchymal and perivascular population in CDH. *Slit3* was expressed differentially higher in the epithelial and mesenchymal population and lower in the endothelial and perivascular cell population.

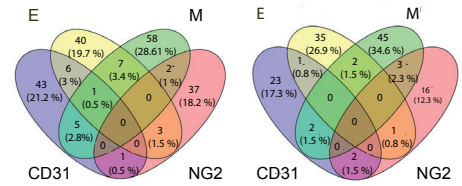
To further confirm the RNA sequence results, the expression of PDGFRα, SLIT2, ALDH1A2, RARB and CRAPB2 was quantified by qPCR in independent samples (Fig. 2B). The same expression patterns between control and CDH samples were observed with the RNA sequence results and the qPCR analysis.

Taken together, assigning genes that are involved in CDH to specific cell population is a step forward in understanding how CDH develops. The RNA sequence data of the four different cell populations isolated at E13 from the CDH mouse model showed that CDH-causing genes are differently expressed. These genes can now be assigned to specific cell populations, which may contribute to understand the role of these genes in CDH. Moreover, the up regulation of *Robo1* in the endothelial population in combination with the down regulation of *Slit3* in the perivascular population could lead to changes in migration and guidance of these cells and thereby leading to impaired angiogenesis and the alterations in the pulmonary vasculature.

Highly differentially expressed genes of molecular pathways assigned to specific cell populations in CDH

To further analyze specific changes in gene expression in the cell populations, an evaluation of transcriptional regulators was performed using Ingenuity pathway analysis (IPA). The complete overview of the transcription factor encoding genes that are either upregulated or downregulated in the four different cell populations is shown in a Venn diagram (Fig. 3A.). Very little overlap was observed between the populations in both situations, indicating that the observed changes were population specific.

Next, we performed a Gene Ontology (GO) analysis using IPA to further investigate which pathways were differentially regulated in early development. The GO terms for the mesenchymal and epithelial cell populations did not overlap with each other and had very little overlap with the other two cell populations. The top 3 GO terms for the mesenchymal population were EIF2 Signaling, mitochondrial dysfunction and regulation of eIF4 and p7056K signaling. The top 3 GO terms for the epithelial cell population were GNRH signaling, Molecular mechanism of Cancer and B Cell Receptor Signaling (SFig. 1).

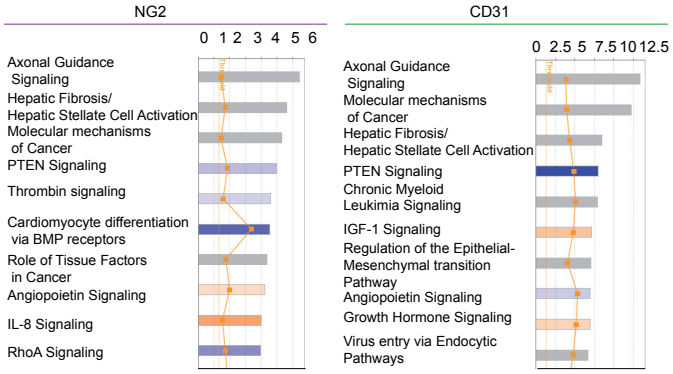


Transcription factors up

CD31	Mes	Epcam	NG2
Prrx1	*Jazf1*	*Hoxd3*	*Irf5*
Myocd	*Zfat*	*Zxdc*	*Hivep3*
Ebf2	*Myog*	*Tbx18*	*Arid3b*
Foxa2	*Foxp3*	*Erg*	*Isl1*
Wt1	*Isl1*	*Rfxap*	*Ebf2*

Transcription factors down

CD31	Mes	Epcam	NG2
Zfp369	*Jfkbie*	*Irf6*	*Prox1*
Zhx3	*Tlx3*	*Atoh8*	*Stat4*
Prox1	*Hoxa11*	*Nmi*	*Actn2*
Zfp398	*Foxr1*	*Jazf1*	*Trim16*
Hdac9	*Gata3*	*Vdr*	*Zfp174*



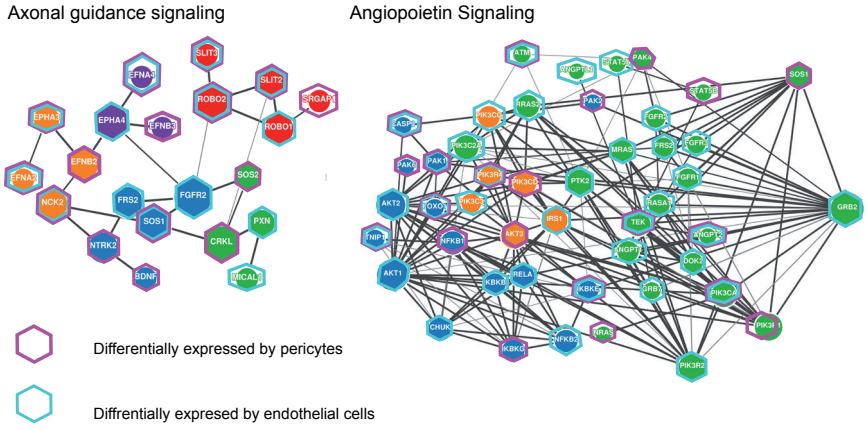


Figure 3 Canonical pathway analysis of RNA sequence of four cell populations

A Venn diaghrams of top 5 fold changes upregulated and downregulated transcription factors in the four different cell populations. Each cell population has its unique top5 indicating the purity of the cell populations. B Top 10 of canonical pathways with the highest number of differentially expressed genes in the NG2 population and the CD31 population showing overlap in the canonical pathways. C Detailed analysis of part of the axonal guidance signaling and the angiopoietin signaling. Part of the proteins belonging to these pathways are differentially expressed by pericytes (indicated in magenta), part by endothelial cells (blue) and part by both populations (blue/magenta).

The top 10 differentially regulated pathways of the NG2 and CD31 population showed high similarity in their GO terms. Two GO terms which were in the top 10 of the endothelial cell population and the perivascular cell population were axonal guidance and angiopoietin signaling pathways (Fig. 3B). These two pathways could explain the vascular abnormalities observed in CDH. Moreover, detailed analysis of segments of the molecules which are part of the axonal guidance pathway revealed that SLIT2/3 and ROBO2 were differentially expressed by both populations but ROBO1 and PXN only by the endothelial population (Fig. 3C). Large circles indicate the more central nodes in the pathway and thicker edges indicate interactions with high confidence. Collectively, this shows that signaling between endothelial and perivascular cells is affected and that a lack in migration and guidance in these populations could lead to the abnormal pulmonary vasculature of CDH pups and in that manner eventually lead to lung hypoplasia. A similar analysis was executed for angiopoietin signaling, which acts through the tyrosine kinase 2 receptor (TIE2) and its ligand angiopoetin-1. The ANG-1/TIE2 signaling is controlled by angiopoietin-2 and in this manner the responsiveness of endothelial cells towards cytokines is regulated[25]. Analysis of the molecules of this pathway showed that part of the proteins was only differentially expressed in the endothelial population while other proteins were differentially expressed by the perivascular cell population. The genes FGFR2, FGFR1 and STAT5 were differentially expressed by the endothelial cell population whereas AKT3, NKFB1 and PAK6 were only differentially expressed by the perivascular population and TEK and ANGPT2 were differentially expressed by both populations (Fig. 3C). This supports the hypothesis that the process of angiogenesis is affected in CDH through the angiopoietin signaling and could furthermore explain the inadequate pulmonary vasculature of CDH pups.

CDH lungs have significant alterations in endothelial cell markers

Significant overlap between the GO terms of the endothelial population and the NG2 population was observed. Therefore the differentiated genes of these populations were compared in more detail starting with the comparison of the Disease & Function terms in IPA (Fig. 4A). This could further reveal which processes underlie the abnormal signaling between endothelial cells and NG2 positive pericytes resulting in the incomplete pulmonary vascular development observed in CDH.

We showed before that increased pericyte coverage leads to abnormal vessel development in CDH. Given the close association between endothelial cells and pericytes,[26] it is likely that the endothelial cell population is also different in CDH. Therefore, a more detailed analysis of the gene expression of the endothelial cell population was performed. This analysis showed that the transcription factors Kruppel like factor 4 (*Klf4*) and Forkhead box F1 (*Foxf1*), as well as the G protein-coupled receptor Sphingoside-1-phosphate receptor 3 (*S1pr3*) were differentially regulated in the endothelial population (Fig. 4B). These proteins have previously been linked to endothelial cell behavior and angiogenesis[15, 27, 28]. KLF4 has

been linked as an upstream regulator of NOTCH signaling. Moreover, increased expression of KLF4 leads to reduced expression of NOTCH genes in tumors[15]. NOTCH signaling is known for being important in the competition between the tip and stalk cells during sprouting of endothelial cells to form new tubes in the process of angiogenesis[29].

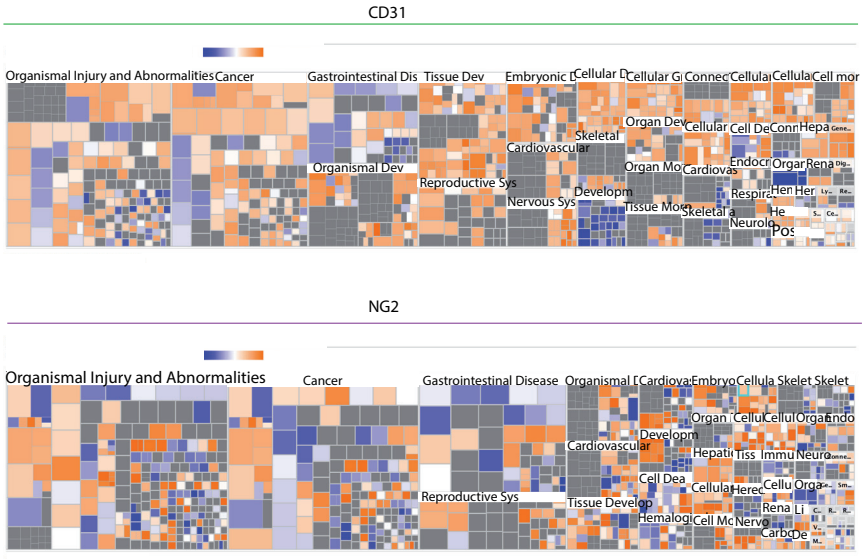
Taken together, the analysis of the transcriptome of the different cell populations revealed differential expression of *Klf4* (Fig. 4B).

Expression of KLF4 is down regulated in CDH and results in malformation of the capillary network in CDH

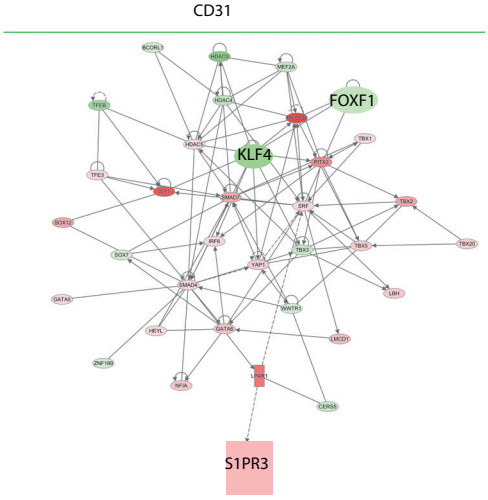
The RNA sequence data showed a decrease of KLF4 expression in the CDH endothelial cell population. Therefore, the spatial and temporal expression pattern of KLF4 was analyzed to evaluate its potential involvement in the occurrence of pulmonary hypertension in CDH. KLF4 expression was decreased in the CDH lung samples isolated at E15 compared to control lungs, and remained lower until E18 (Fig5 A indicated with white arrow heads). To get a better insight of the exact location and effect of reduced KLF4 expression on the vasculature, high resolution images were made of total embryonic lungs stained with specific antibodies. This procedure leaves the lung structure completely intact and the small capillaries can easier be traced back than in lung sections. At E15 a significant lower number of KLF4 positive signal was detected in CDH (Fig. 5B), indicating an early defect in the vascular development in the lungs of CDH pups. The decrease of KLF4 signal was consistent through development, although they were no longer significant (Fig. 5B).

As mentioned in the above section the expression levels of KLF4 have been related to NOTCH signaling [15]. Therefore the expression levels of genes that are part of the NOTCH signaling pathway were analyzed and plotted in a heatmap (Fig 5. C), indeed showing that multiple genes of the NOTCH signaling pathways were differently expressed in the endothelial cell population at E13 in the mouse CDH model. qPCR for HEY2, NOTCH1 and NUMBL1 showed the same expression trends as the RNA sequence analysis. Indicating that disturbed NOTCH signaling caused by downregulation of *Klf4.* The disturbed NOTCH signaling leads to a decreased number of sprouting endothelial cells what results in a reduced number of newly formed tubules in the vascular bed in CDH.

A



B



Gene	Fold change in CD31 pop.
FOXF1	-3.13
KLF4	-6.78
S1PR3	5.21

Figure 4 Disease and function analysis of CD31 and NG2 population leads to specific pathway analysis of the CD31 population

A Disease and function analysis of the CD31 and NG2 population showed that both populations appear to have differentially expressed genes in the same terms. **B** Pathway analysis of the CD31 population of transcription factors and G-coupled proteins showed KLF4, FOFXF1 and S1PR3 are part of the same pathway and all three had a high fold change and are involved in regulation endothelial cells in angiogenesis.

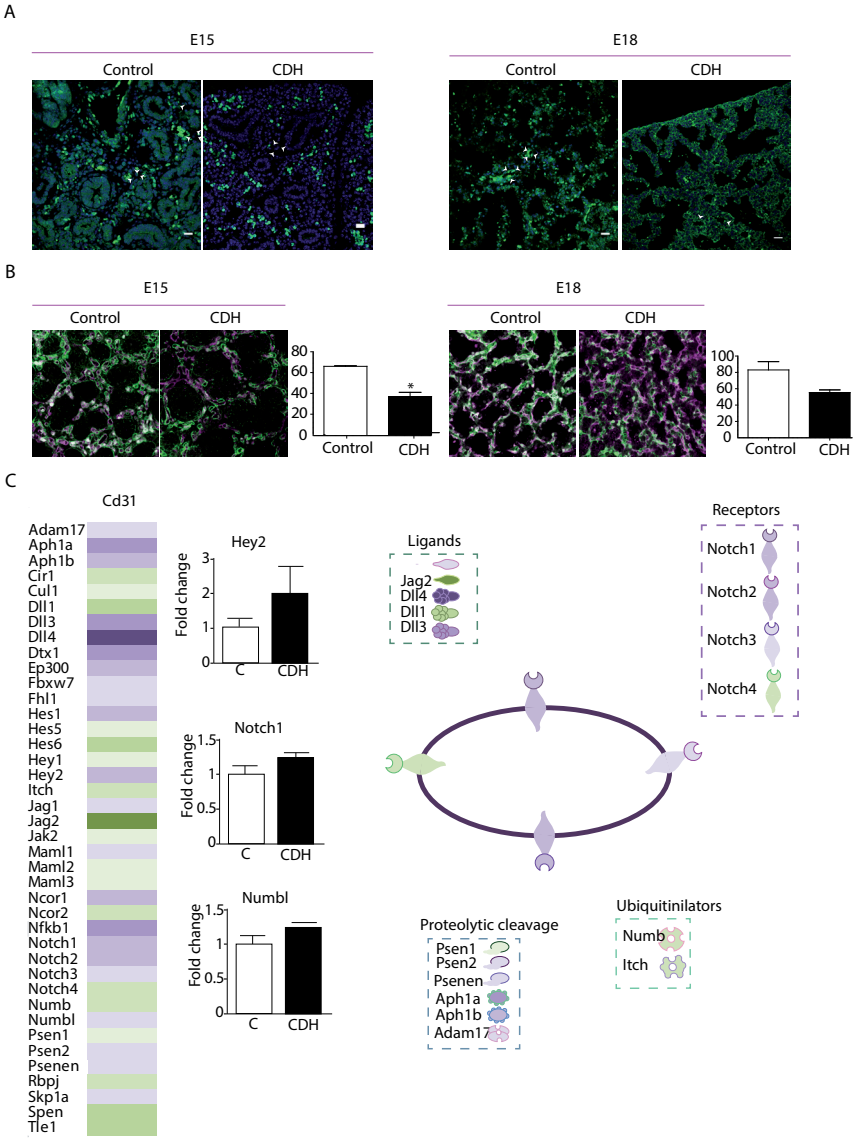


Figure 5 KLF4 expression in the developmental lung vasculature and the expression of its downstream targets of the NOTCH pathway in RNA sequence data

A Expression pattern analysis of KLF4 suggest reduced expression of KLF4 at E15 and E18 in the CDH mouse model. B High magnification analysis of whole mount immunofluorescent labeled embryonic lung samples at E15 show a significant decrease in KLF4 expression and a trend in reduced KLF4 expression at E18, as indicated in the bar diagrams. C The fold change in expression of different members of the NOTCH signaling pathway in the CD31 population. The diversity in the fold change of the affected members of the NOTCH signaling pathway emphasizes the broad underlying mechanism in the developmental defects of the pulmonary vasculature in CDH

Discussion

CDH is a rare disease, which, however, remains difficult to treat. More specifically, the pulmonary hypertension associated with this disease results in long hospitalization and potential life-long problems. The onset of the vascular changes that correspond with the disease are still not well understood. Studies towards genes involved in CDH have mainly been focused on either patient sequencing or mouse knockout studies[30] [31].A number of sequencing studies revealed mutations in CDH patients but the used input is from late stages in lung development. In these studies it often remains unclear what the effect of the mutations is on the number of cells and their behavior[32]. Our study shows for the first time that differences in gene expression are related to individual cell populations. How structural changes associated with PH arise and how this is related to changes in gene expression and how this relates to different cell types appeared in this study for the first time. Previously we showed that the NG2 positive pericytes are the underlying cause of the structural vascular changes that accompany CDH. In this study, we further confirm the strong endothelial/pericyte interaction and how this interaction is affected in CDH. Moreover, the endothelial and pericyte populations revealed high similarity in GO terms. This suggests that the genes of certain signaling pathways are differentially expressed in CDH, resulting in aberrant communication between pericytes and endothelial cell. More specifically, the appearance of the ROBO/SLIT pathway in the GO term analysis indicated that the guidance signaling of the endothelial cells and perivascular cells was affected which could be an explanation for the simplification of the pulmonary vasculature observed in CDH.

Besides the changes in genes that are involved in both cell populations, we also found new genes in the endothelial cell population, namely FOXF1 and KLF4, which have not been shown before to be involved in CDH. Mutations in the FOXF1 gene or in the regulatory elements of FOXF1 have been associated with Alveolar Capillary Dysplasia (ACD). ACD is a severe and lethal congenital lung disease with misalignment of the pulmonary veins [33-35].

It has been demonstrated that there is increased KLF4 expression in SMC in the pulmonary arterioles of pulmonary hypertension patients without CDH. It has been hypothesized that this increase is required for dedifferentiation and clonal expansion of SMCs in the pulmonary hypertension mouse model[36]. This study showed no difference in the expression of KLF4 in the perivascular population but a significant decrease in KLF4 expression in the endothelial population. The observed difference further suggests that pulmonary hypertension of the newborn and pulmonary hypertension secondary to cardiac malformations are intrinsically different from each other.

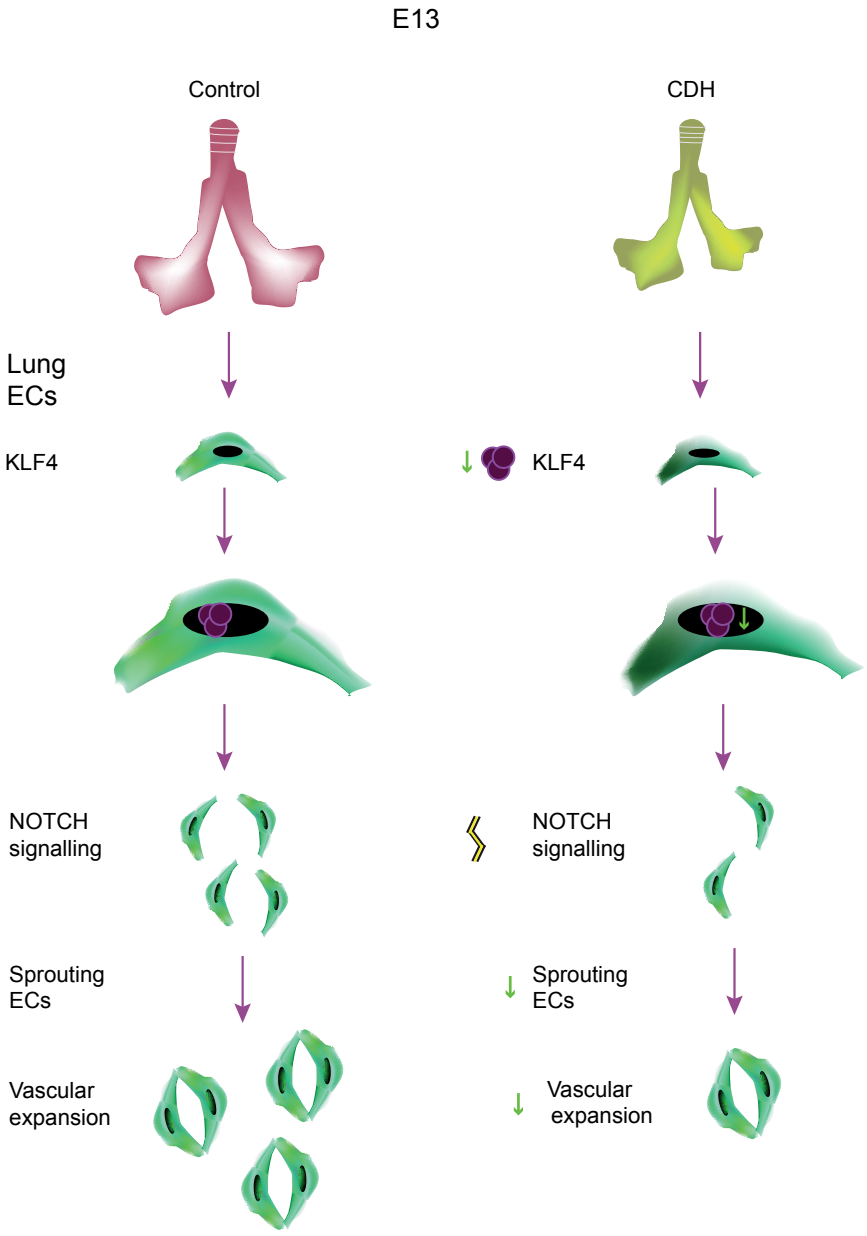


Figure 6 Summary of developmental events that happen during the pulmonary vascular developmen in the mouse model for CDH.

Early inhibition of the retinoic acid pathway (E8.5) results in downregulation of KLF4 at E13. The downregulation of KLF4 leads to disturbance in NOTCH signaling which leads to a reduction in the formation of the lung capillary bed.

The role of KLF4 in angiogenesis has been linked to NOTCH signaling. KLF4 is thought to be an upstream regulator of NOTCH and in that way a regulator of angiogenesis [15]. The importance of KLF4 in the regulation of NOTCH signaling, and thereby the number of sprouting endothelial cells during angiogenesis, together with the observed decrease in KLF4 expression in the CDH mouse model could explain the vascular abnormalities associated with CDH. Since a reduced number of capillaries was observed in the lungs of the CDH mouse model we assume that this could be the result of decreased KLF4 expression by endothelial cells leading to disturbed NOTCH signaling leading to a decrease in active sprouting cells during angiogenesis (Fig. 6).

In summary, this study shows whole trancrtiptome analysis of four different populations of lungs of control and CDH samples. Analysis of these data showed high similarity between the endothelial and pericyte populations, which could explain the altered pulmonary vascular development in CDH. Furthermore, we identify two new candidates involved in angiogenesis and impaired during the development of the pulmonary vasculature in CDH. Taken together, revealing involved genes in these mechanisms could help to early intervene with pulmonary vascular growth in CDH patients.

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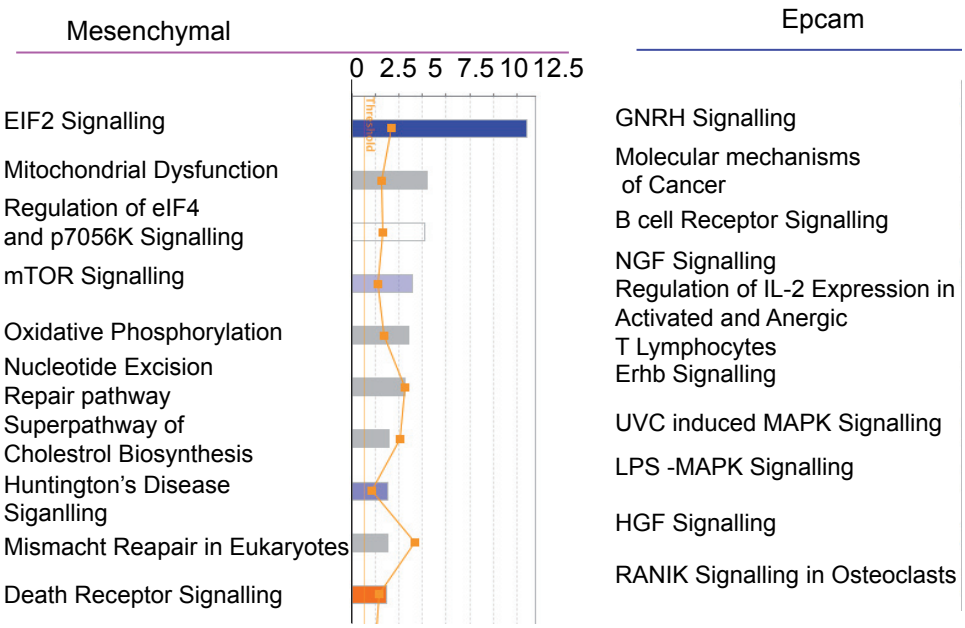
Supplemental figures



CHAPTER 4

Clinically relevant timing of antenatal sildenafil treatment reduces pulmonary vascular remodeling in congenital diaphragmatic hernia

Daphne S Mous, Heleen M Kool, Marjon J Buscop-van Kempen, Anton H Koning, Oleh Dzyubachyk, Rene MH Wijnen, Dick Tibboel, Robbert J Rottier

Abstract

Patients with congenital diaphragmatic hernia (CDH) suffer from severe pulmonary hypertension due to altered development of the pulmonary vasculature, which is often resistant to vasodilator therapy. Current treatment starts postnatally even though significant differences in the pulmonary vasculature are already present early during pregnancy. We examined the effects of prenatal treatment with the phosphodiesterase-5 inhibitor sildenafil on pulmonary vascular development in experimental CDH starting at a clinically relevant time. The well-established, nitrofen induced CDH rodent model was treated daily with 100 mg/kg sildenafil from day 17.5 until day 20.5 of gestation (E17.5-20.5). Importantly, this timing corresponds perfectly to the developmental stage of the lung at 20 weeks of human gestation, when CDH is detectable by 2D-ultrasonography and/or MRI. The lungs were isolated at E21.5 and analyzed using immunostaining, real-time PCR and volume measurements. Prenatal treatment with sildenafil improved lung morphology and attenuated vascular remodeling with reduced muscularization of the smaller vessels. Pulmonary vascular volume was not affected by sildenafil treatment. We show that prenatal treatment with sildenafil within a clinically relevant period improves pulmonary vascular development in an experimental CDH model. This may have important implications for the management of this disease and related pulmonary vascular diseases in human.

Introduction

Congenital diaphragmatic hernia (CDH) is a developmental defect characterized by an incomplete diaphragm and lung hypoplasia [1]. CDH patients have a high risk of mortality and morbidity due to the associated pulmonary hypertension, which is the result of altered development of the pulmonary vasculature and disordered pulmonary vascular remodeling [2-4]. Advancement has resulted in early detection of CDH by ultrasonography at 20 weeks of gestation, but the severity of clinical symptoms postnatally remains poorly predictable at this stage due to significant differences in pulmonary vascular resistance and flow after birth. In addition, the pulmonary hypertension in CDH is often unaffected by standard vasodilator therapy and the lack of randomized controlled trials prevents the implementation of alternative drugs. Trials with Nitric Oxide (NO), one of the most commonly used drugs in newborns, failed to show consistently positive effects in CDH patients [5]. The impaired responsiveness to NO may be due to rapid degradation of the intracellular messenger cyclic guanosine monophosphate (cGMP) by phosphodiesterase-5 (PDE5) [6]. Binding of cGMP to PDE5 stimulates the phosphorylation and activation of PDE5 by cGMP dependent protein kinase G (PKG), which results in the conversion of cGMP into GMP [7]. Sildenafil is a potent PDE5 inhibitor, leading to an accumulation of cGMP and thus the continuous activation of PKG. PKG has several physiological substrates, which are involved in smooth muscle cell (SMC) relaxation by lowering intracellular calcium [8, 9]. Sildenafil also reduces inflammation, improves early postnatal survival and prevents pulmonary vascular remodeling in different experimental animal models of pulmonary hypertension without CDH [10-12] and prolongs survival and improves lung structure in a neonatal hyperoxia rat model [13]. It has been successfully used in the postnatal treatment of persistent pulmonary hypertension of the newborn (PPHN) [14-19] and pulmonary hypertension in patients with congenital heart disease [20]. There are no randomized controlled trials of sildenafil in CDH patients, but there are case reports showing positive effects after postnatal treatment [21, 22]. Previously, we showed thickening of the smooth muscle cell layer in arterioles, neomuscularization of small capillaries and phenotypic changes of the smooth muscle cells in the vascular wall in lungs of CDH patients at 30 weeks of gestation, indicating that significant differences in vascular structure are already present in unborn children that will develop PH after birth [23]. The premature differentiation of vascular smooth muscle cells and the early structural changes in pulmonary vascular development suggest that antenatal treatment of CDH patients could be beneficial. Recently, Luong et al. showed a reduced pathology in experimental CDH after prolonged antenatal treatment with sildenafil [24]. However, they started the daily treatment with sildenafil already at day 10.5 of gestation, when the lung bud is just emerging from the primitive foregut. At this embryonic phase of lung development there are no signs of CDH pathology, yet. Hence, it is unclear whether the prophylactic treatment prevented the development of pathological features, or that the sildenafil indeed regressed the clinical signs. Since human CDH can be diagnosed at 20 weeks of gestation, the canalicular phase of lung development, we analyzed the therapeutic effects

of sildenafil in the nitrofen induced rat CDH model starting at the corresponding gestational age (E17.5). In rat, the CDH pathology is already noticeable from E13.5 on with a defective diaphragm and affected lungs [25-27]. We show that starting the treatment of the CDH rat model with sildenafil at the clinically relevant time point improved lung morphology and attenuated or reversed the vascular remodeling of the smaller vessels. These findings may directly be valuable for future treatment modalities of severe CDH patients.

Methods

Animal Model

Pregnant Sprague-Dawley rats received either 100 mg nitrofen dissolved in 1 ml olive oil or just 1 ml olive oil by gavage on gestational age day E9.5. Nitrofen induces CDH in approximately 70% of the offspring, while all pups have pulmonary hypertension. Administration of nitrofen at this time point results in mainly left sided hernias [28]. Pregnant rats were divided into 4 groups: control, nitrofen (CDH), control+sildenafil and nitrofen+sildenafil (CDH + sildenafil). Sildenafil (100 mg/kg/day, Pfizer, New York, USA) dissolved in water was administered via oral gavage for 4 consecutive days from day E17.5 to day E20.5. At day E21 pups were delivered by caesarean section and euthanized by lethal injection of pentobarbital.
All animal experiments were approved by an independent animal ethical committee and according to national guidelines.

Plasma Sildenafil Concentration

Maternal and fetal rat blood samples were collected directly after caesarian section. Fetal blood samples were pooled, and plasma (50 µl) from 6 maternal and 9 fetal samples was isolated by centrifugation (10.000 RPM, 15 minutes) and sildenafil and its metabolite N-desmethyl-sildenafil (DMS) concentrations were analyzed using ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS).

Lung Morphology

Fetal rat lungs were isolated, fixed overnight in 4% PFA and embedded in paraffin. Serial 5 µm thick sections were made through the middle of the left lobe and stained with haematoxylin and eosin (HE). Sections were imaged at 40x magnification using a BX41 research stereomicroscope system (Olympus; Tokyo, Japan). Four non-overlapping images in different parts of each lung were acquired. Major airways and vessels were excluded from the analysis. The airspace size was automatically quantified using an index (D_2-score) that is based on the alveolar airspace diameter and takes into account the first three central moments of the airspace size distribution. This measurement is designed to account for airspaces of different sizes by assigning them different weights. Compared to the well-known mean linear intercept (L_m), this method is more reliable in the presence of a large variability in airspace sizes [29, 30].

Immunohistochemistry and Immunofluorescence Staining

Immunohistochemistry (IHC) was performed on 5 µm paraffin sections of the lungs according to standard protocols, using the Envision™ detection system (Dako Cytomatic, Glostrup, Denmark) [31]. Primary antibodies used for IHC were smooth muscle actin (α-SMA; MS-113-P1; 1:1200, Thermo Scientific, Fremont, CA, USA), phosphodiesterase-5 (PDE5A; PD5A-101AP; 1:300, Fabgennix, Frisco, TX, USA) and phosphorylated phosphodiesterase-5 (phospho-PDE5; PPD5-140AP; 1:100, Fabgennix). Antigen retrieval with Tris-EDTA buffer (pH 9.0) was used for α-SMA.

Primary antibodies used for immunofluorescence (IF) staining on 5 µm paraffin sections were smooth muscle actin (α-SMA; MS-113-P1; 1:500, Thermo Scientific), smooth muscle actin (clone 1a4) (α-SMA direct labelled FITC; 1:200, Sigma, The Netherlands) and platelet derived growth factor β (PDGFrβ; 1:100). Secondary antibodies against mouse (α-SMA) and rabbit (PDGFrβ) were used. Negative controls were performed by omitting the primary antibody.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

RNA isolation, cDNA synthesis and subsequent qPCR analysis was performed as previously described [31]. The gene-specific primers were custom designed using PerlPrimer 1.1.21 [32] and all retrieved sequences were blasted using Ensembl (RLS 84) [33]. The primer combinations used in our qPCRs are listed in Table 1. Both *Actb* and *Hprt* were used as housekeeping genes and all represented data are based on *Actb*.

Table 1: Primer sequences

Gene	Sequence (forward 5'- 3')	Sequence (reverse 5'- 3')
Pde5	TCAACAACGGATAGCAGAACTC	CCCTGTTCATTAGATCAGCGG
Prkg1	AACTATGCAGGGACAACCCA	CCTTCCCAGTTAAAGCCCTC
Prkg2	ACTAGGCATTATCTACAGAGACC	TCCAAAGTCAACCAACTTAAGG
Sma	TGACCCAGATTATGTTTGAGAC	AGAGTCCAGCACAATACCAG
Pdgfr-β	AACGACCAGTTCTACAATGCC	CATGATCTCATAGATCTCGTCGG
Pecam-1	GCAGTCCCACTTCTGAACTC	GTTCTGGGAGTCGTAATGGC
Actb	AGATGACCCAGATCATGTTTGAG	GTACGACCAGAGGCATACAG
Hprt	AGACTGAAGAGCTACTGTAATGAC	CAACAATCAAGACGTTCTTTCCAG

Volume Measurements Pulmonary Vascularity

Lungs of pups were perfused through the right ventricle with Microfil contrast agent (Microfil, Flow Tech; Carver, MA, USA) and imaged with a micro Computed Tomography (micro-CT) scanner (Quantum FX, PerkinElmer; Waltham, MA, USA; pixel size 10–295 µm). Subsequently, the images were analyzed with the I-Space (Barco, Kortrijk, Belgium), a CAVE™-like Virtual Reality system in which 3D holograms can be viewed with depth perception by wearing a pair of stereo glasses with polarizing lenses. Volumes were calculated by semi-automatic region growing using the V-Scope volume-rendering software (Department of Bioinformatics, Erasmus MC, Rotterdam, The Netherlands) as previously described [34, 35]. Volume of the pulmonary vasculature was measured in relation to the total lung volume. Results obtained with the I-Space were validated using a computer program, Analyze Direct (Kansas City, US).

Statistical Analyses

Data are presented as percentages, means (SD) and univariate analyses were performed using two-way ANOVA tests for normally distributed variables. The analyses were performed using SPSS 21.0 for Windows (Armonk, NY, USA: IBM Corp.). All statistical tests were two-sided and used a significance level of 0.05.

Results

Sildenafil Effectively Crosses the Placental Barrier

In order to investigate potential effects of oral sildenafil on fetuses, we first analyzed the levels of sildenafil and its major metabolite N-desmethyl-sildenafil (DMS) in blood plasma of the mothers and pups approximately 24 hours after the last dose of sildenafil. These measurements showed that the oral application facilitated efficient uptake of sildenafil in the bloodstream and subsequent passage through the placental barrier into the fetal circulation (Table 2, Figure 1A).

Table 2: Plasma level of sildenafil and N-desmehtyl-sildenafil (DMS)

Mothers		Pups	
Sildenafil (ng/ml)	*DMS (ng/ml)*	*Sildenafil (ng/ml)*	*DMS (ng/ml)*
7.00	14.30	15.00	1.80
1.60	4.40	19.80	1.60
1.30	2.70	4.60	1.50
2.70	3.70	4.70	1.90
0.90	2.80	13.20	1.40
1.30	10.80	7.70	0.90
		5.00	1.20
		6.70	3.10
		5.20	1.70

Since the administration of sildenafil to the rats was started late in gestation after the development of the diaphragm, we did not observe a reduction in the incidence of CDH after treatment (Figure 1B). The effect of sildenafil on the general development of the fetuses was analyzed by assessing the body weight. Fetuses of nitrofen treated mothers (CDH) had a significantly lower body weight compared to control at E21.5, but antenatal treatment with sildenafil resulted in a significant increase in body weight in control and CDH fetuses (Figure 1C). The lung weight-to-kidney weight ratio (LW/KW) was used as an indicator for lung hypoplasia, since the kidney weight is less affected by treatment with sildenafil than the body weight. This LW/KW ratio was significantly reduced in CDH fetuses compared to control, indicating severe lung hypoplasia in the CDH fetuses. Antenatal sildenafil treatment reduced the hypoplasia as indicated by the significant improvement of the LW/KW ratio in CDH fetuses.

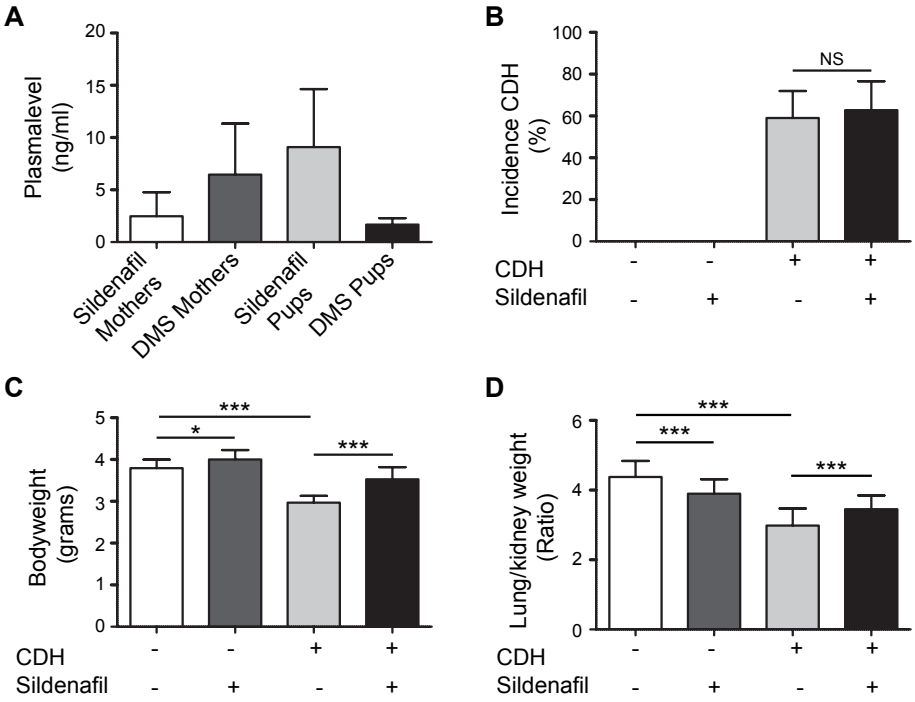


Figure 1: Effects of maternal sildenafil on pups.

(A) Levels of sildenafil and its metabolite desmethylsildenafil (DMS) measured in plasma of mother rats and her fetuses indicate effective placental passage of sildenafil, median (IQR), n=6 (mothers), n=9 (pups). (B) The incidence of CDH is not affected by sildenafil treatment (63% vs 59%, p=0.665); n=5 for all groups. (C) Bodyweight is decreased in pups with CDH (3.0 gr vs 3.8 gr, p<0.001), which is reversed by treatment with sildenafil (3.5 gr, p<0.001). Sildenafil also caused an increase in bodyweight in control pups (4.0 gr, p<0.05); n=13 (control), n=23 (CDH), n=16 (control + sildenafil), n=24 (CDH + sildenafil). (D) LW/KW is decreased in CDH (3.0 vs 4.4, p<0.001), and slightly improves after sildenafil treatment in CDH (3.5, p<0.001). Sildenafil caused a decrease in the ratio in controls (3.9, p<0.001); n=33 (control), n=38 (CDH), n=23 (control + sildenafil), n=28 (CDH + sildenafil). *p<0.05, **p<0.01, ***p<0.001. Error bars represent standard error (SD). LW Lung Weight, KW Kidney Weight.

However, sildenafil induced a mild hypoplasia in control fetuses (Figure 1D).
Sildenafil inhibits PDE5, so we analyzed if sildenafil had an effect on the expression of its target. RNA expression of *Pde5* was increased in fetal CDH lungs, but sildenafil did not reduce this elevated expression. In addition, the downstream targets of Pde5, *Prkg1* and *Prkg2*, were not affected in any of the groups (Figure 2A). Since we did not find differences in expression level, we analyzed the distribution of Pde5 in the lungs of the fetuses. The expression pattern of Pde5 was primarily in the very large (>100 µm) vessels in some of the control samples, but this pattern was expanded in the CDH lungs to a number of small (<50 µm) and larger (50-100 µm) vessels (Figure 2B). Remarkably, treatment with sildenafil resulted in a reduction of the number of Pde5 positive vessels in CDH, and the staining pattern was comparable to control lungs, being primarily around some of the larger vessels. The activated, phosphorylated Pde5 was detected in part of both small and larger vessels of all samples with no clear differences between all groups.

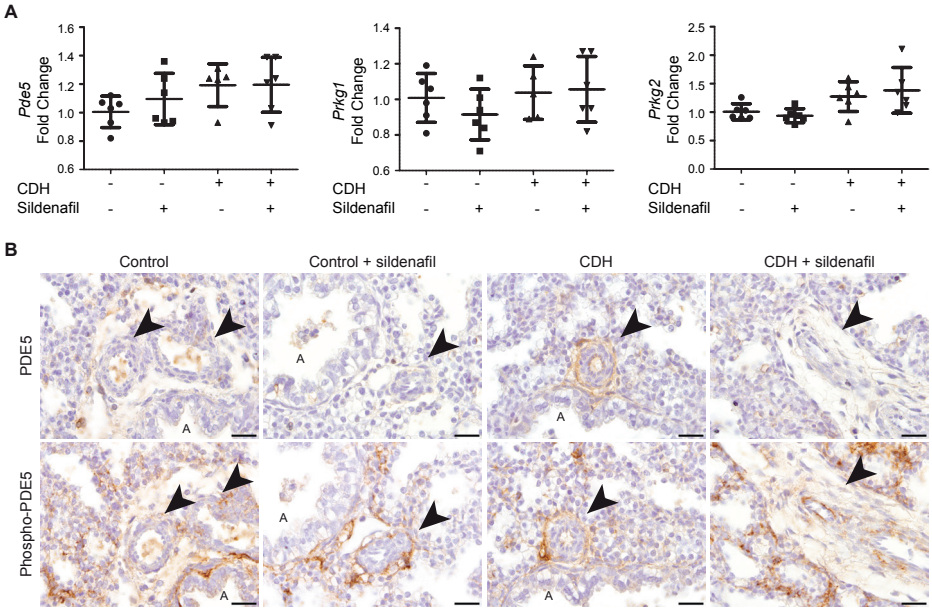


Figure 2: Expression of phosphodiesterase-5 (Pde5) in the lungs of rat fetuses.

(A) Expression of *Pde5*, *Prkg1* and *Prkg2* RNA shows no significant differences. For all groups 6 independent lung samples were used. Error bars represent SE. (B) Representative images of immunohistochemistry staining show expression of Pde5 around the vessels of CDH lungs (top) and phosphorylated Pde5around the vessels in all groups (bottom). Arrows indicate vessels, A indicates airways. Scale bars represent 20 µm. For all groups 3 independent lung samples were used.

Sildenafil Improves Lung Morphology and Attenuates Pulmonary Vascular Remodeling in CDH

We analyzed the histology of the lungs of the different treated pups, which clearly showed differences in cellular density of the lung structure, with thicker septa and smaller alveolar airspaces in CDH (Figure 3A). The alveolar airspace diameter (D_2-score) was used to quantify the alveolar airspaces. Both alveolar density and the number of alveoli were significantly increased in CDH rats compared to control and returned to normal after treatment with sildenafil (Figure 3B,C).

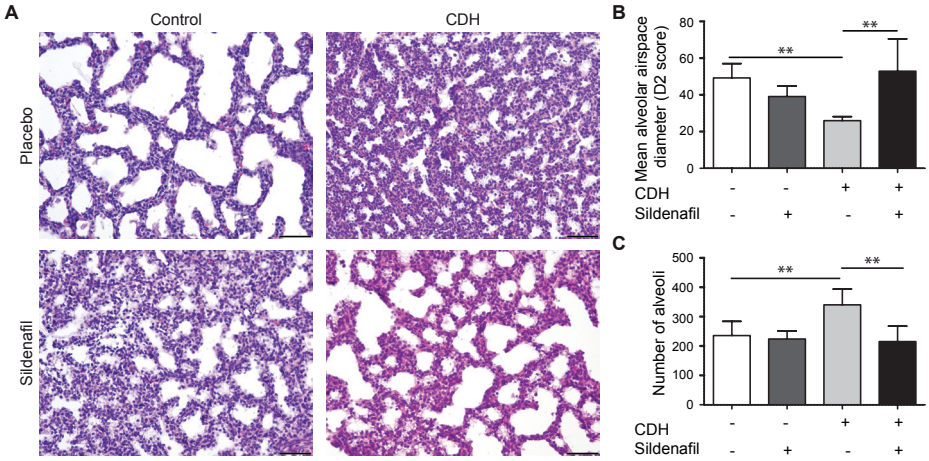


Figure 3: Prenatal sildenafil improves alveolar development in CDH.

(A) Representative images of HE stained sections show a significant decreased mean alveolar airspace diameter in CDH rats compared to control. Scale bars represent 50 µm. (B) Quantification of alveolar development in control and CDH using the D_2-score (49.3 µm (7.8) and 25.9 µm (2.2), respectively, p=0.002). Sildenafil clearly showed an increase in alveolar airspace diameter in CDH (52.9 µm (17.6), p=0.001), taking into account the D_2-score (in µm) that incorporates the first three central moments of airspace distribution. (C) The number of alveoli is significantly increased in CDH (p=0.003), while treatment with sildenafil reverted the alveolar abnormality to normal (p=0.001). For all groups 4 non-overlapping images were used of 5 independent lung samples. *p<0.05, **p<0.01, ***p<0.001. Error bars represent SD.

We and others have previously shown that the nitrofen rat model phenocopies the vascular defects observed in human CDH patients with increased muscularization of the arterioles [23]. To analyze whether nitrofen and/or sildenafil would affect the development of the vascular tree, we measured the total pulmonary vascular volume. Three dimensional volume measurements done in the I-Space showed a significant decrease in total lung volume (LV), in pulmonary vascular volume (PVV) and in the ratio of PVV to LV in CDH fetuses compared to controls (Figure 4A,B). We observed no significant improvement of the vascular volume in CDH fetuses treated with sildenafil, but antenatal sildenafil decreased pulmonary vascular volume and PVV/LV in control fetuses (Table 3). This indicates that starting sildenafil treatment at a clinical relevant time point did not improve the vascular tree.

Table 3: Pulmonary vasculature volume in rat fetuses

	Control (n=7)	*CDH (n=5)*	*Control + sildenafil (n=9)*	*CDH + sildenafil (n=8)*
Lung volume mm³ (SD)	134.0 (22.8)	95.2 (22.3)*	110.4 (22.8)	93.4 (8.3)***
Vasculature volume mm³ (SD)	11.3 (3.3)	4.8 (2.3)**	6.9 (3.1)*	3.6 (0.5)***
Ratio vasculature/lung	0.084 (0.019)	0.048 (0.019)**	0.060 (0.017)*	0.039 (0.005)***

Results are shown as mean (SD). * p<0.05, ** p<0.01, *** p<0.001 compared to control.

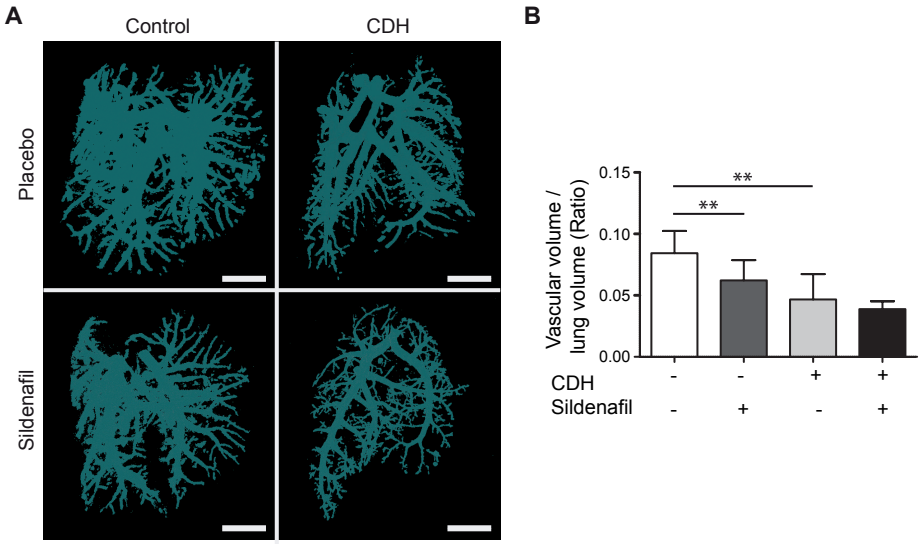
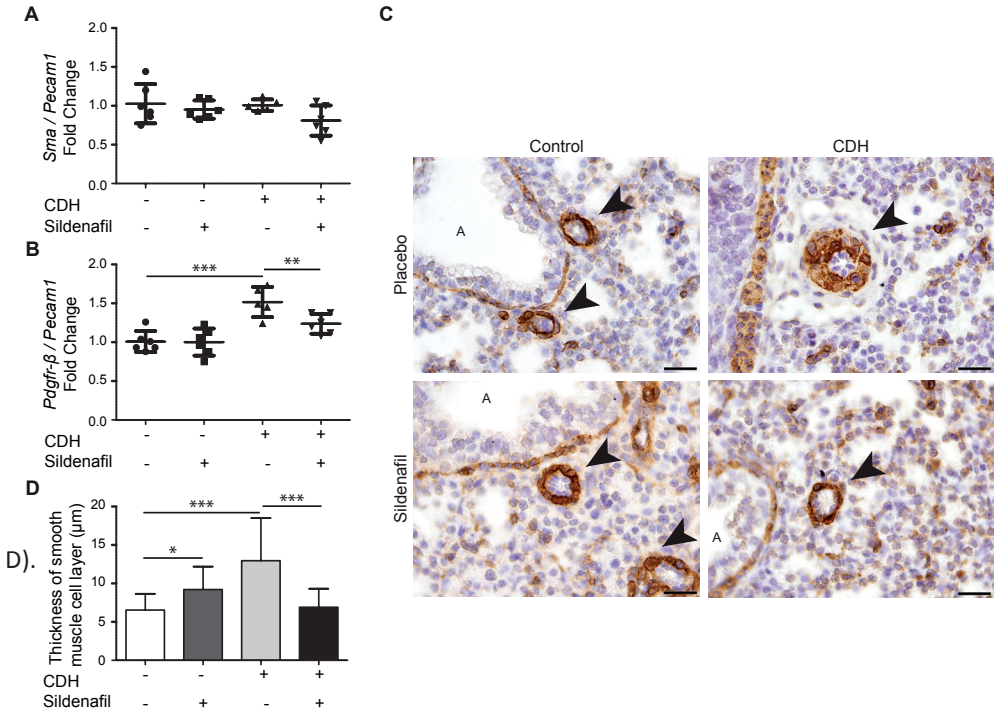


Figure 4: Sildenafil does not affect vasculature volume.

(A) Representative images of computed tomography scans of microfil-injected pulmonary vessels analyzed with the I-Space. Scale bars represent 2 mm. (B) The ratio of the pulmonary vasculature volume to total lung volume is significantly decreased in CDH rats (0.048 vs 0.084, p=0.001) and control rats treated with sildenafil (0.060, p=0.005); n=7 (control), n=5 (CDH), n=9 (control + sildenafil), n=8 (CDH + sildenafil). *p<0.05, **p<0.01, ***p<0.001. Error bars represent SD.

Previously, we showed a thickening of the smooth muscle cell layer in small capillaries in rats with PH [36], and a more extensive peripheral distribution of contractile vascular smooth muscle cells in human CDH [23]. Based on these results, we analyzed gene and protein expression of several vascular-associated markers to study the effects of sildenafil treatment on vascular remodeling. Gene expression analysis of α-Smooth Muscle Actin (*α-Sma*) and Platelet-Derived Growth Factor receptor β (*Pdgfr-β*, pericyte marker) in relation to endothelial cells (*Pecam-1/CD31*) showed a significant increase of *Pdgfr-β* in CDH lungs compared to control, indicative for an increase of differentiating perivascular cells in the CDH lungs. Treatment with sildenafil significantly reduced these markers, suggesting a restoration of normal pulmonary vascular development. However, the expression of *Pdgfr-β* did not revert completely to the control levels (Figure 5A,B). Analysis of the distribution pattern showed

an increased thickening of the α-Sma$^+$ smooth muscle cell layer in small pulmonary vessels (<50 µm) in CDH fetuses. Sildenafil treatment reduced this thickening of the media in CDH lungs, corresponding with the RNA expression data. Remarkably, sildenafil slightly increased the media in control rats (Figure 5C,



D).

Figure 5: Sildenafil decreases pathological muscularization in CDH.

(A) RNA expression of smooth muscle actin (*Sma*) in relation to platelet endothelial cell adhesion molecule (*Pecam1*) shows no significant changes between the groups. (B) RNA expression of platelet derived growth factor β (*Pdgfr-6*) in relation to *Pecam1* shows a significant increase in CDH (p<0.001), which is slightly improved after treatment with sildenafil (p=0.009). (C,D) Representative images of immunohistochemistry staining (C) and quantitation (D) show increased expression of Sma and a significant thickening of the vessel wall of small pulmonary vessels (<50 µm) in CDH (12.96 µm vs 6.55 µm, p<0.001), which is completely reversed by antenatal treatment with sildenafil in CDH (6.91 µm, p<0.001) and thickened in control (9.21 µm, p=0.030). Arrows indicate vessels, A indicates airways. Scale bars represent 20 µm. For all groups 15 to 20 vessels of 3 independent lung samples were measured. *p<0.05, **p<0.01, ***p<0.001. Error bars represent SD.

Immunofluorescence staining of control lungs showed expression of α-Sma almost exclusively in the media of the large vessels and in the subepithelial layer of the airways, and a peripheral, parenchymal staining of Pdgfr-β (Figure 6). Interestingly, α-Sma and Pdgfr-β co-localized around the smallest vessels in CDH lungs, most likely staining differentiating perivascular cells. Thus, in contrast to control lungs, the small capillaries are muscularized in CDH fetuses. Sildenafil treatment of CDH fetuses resulted in a reversion of the staining

pattern of α-Sma and Pdgfr-β to the control situation, indicating that sildenafil may reduce the pulmonary hypertension. This would suggest a beneficial effect of sildenafil on reducing the pulmonary hypertension at the cellular level (Figure 6).

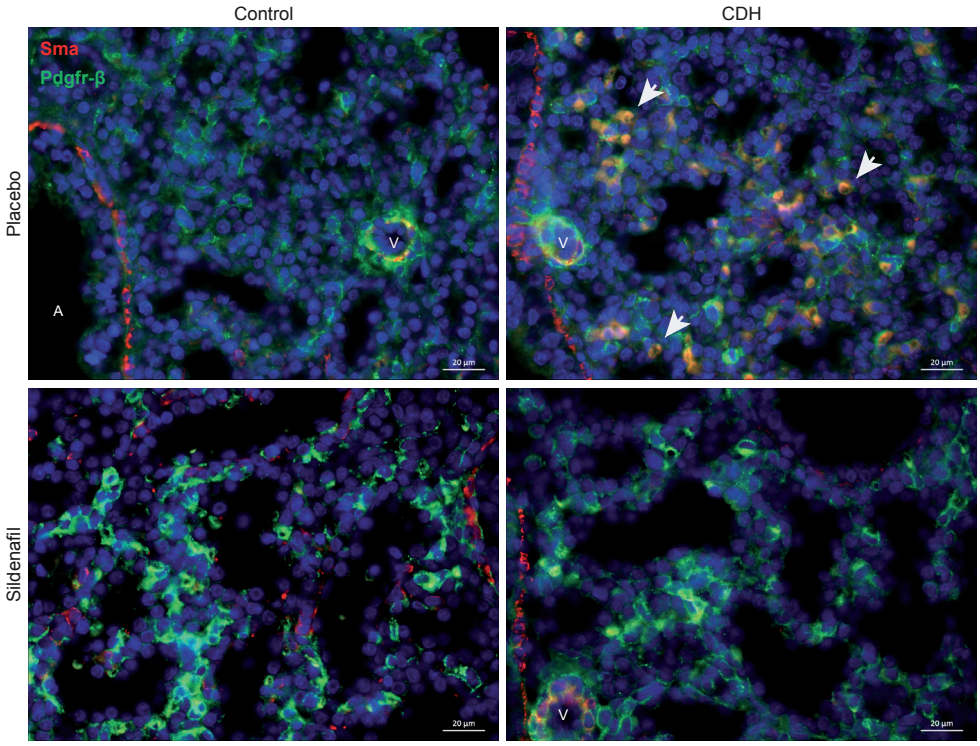


Figure 6: Abnormal smooth muscle cells surround arterioles in CDH.

Representative immunofluorescence staining images of all 4 groups show colocalization of Sma (red) and platelet derived growth factor β (Pdgfr-β; green) in the parenchyma of CDH lungs. Arrowheads indicate examples of capillaries with colocalization, A indicates airways, V indicates vessels. Scale bars represent 20 µm.

Discussion

Our study shows that antenatal treatment of CDH pups with the PDE5 inhibitor sildenafil starting at the clinically relevant time point results in reduced lung hypoplasia and reduced vascular abnormalities. Administration of sildenafil was started at the canalicular stage of lung development in the rat, which corresponds with the time point when human CDH can be detected by routine ultrasound at 20 weeks of gestation. The diaphragm of the rat is already formed and the major pulmonary vessels are already developed at the time the administration of sildenafil was started. Therefore we did not observe a reduction in the incidence of CDH, or an improvement of the pulmonary vascular volume in the major

branches of the vascular tree. However, sildenafil improved the body weight and LW/KW ratio, indicating a better lung growth development compared to untreated CDH pups. Furthermore, the alveolar airspaces increased in diameter, which could be related to the formation of the primary and secondary septa later in prenatal lung development. Moreover, sildenafil reduced the thickening of the smooth muscle cell layer in arterioles normally present in CDH, and prevented the frequently observed aberrant differentiation of pericytes in CDH as indicated by the loss of the co-localization of α-Sma and Pdgfr-β in capillaries [23, 36].

Table 4: Overview of studies with antenatal sildenafil treatment in the nitrofen rat model

	Our study	*Luong et al* [24]	*Kattan et al* [39]	*Lemus-Varela et al* [40]	*Yamamoto et al* [41]
	100 mg/kg Oral 24 h E17.5 – E20.5	**100 mg/kg Subcutaneous 24 h E10.5 – E20.5**	**45 mg/kg Oral 12 h E14 – E22**	**100 mg/kg Oral 24 h E16 – E20**	**100 mg/kg Subcutaneous 24 h E11.5 – E20.5**
Fetal body weight	CDH: significantly decreased CDHsil: significant improvement	CDH: significantly decreased CDHsil: no improvement			
Lung weight	CDH: decreased lung/kidney weight CDHsil: significant improvement Cosil: decreased lung/kidney weight	CDH: decreased lung/body weight CDHsil: no improvement Cosil: no significant differences			CDH: decreased lung/body weight CDHsil: significant improvement
Morphology	CDH: decreased alveolar airspaces CDHsil: significant improvement Cosil: No significant differences	CDH: increased mean linear intercept CDHsil: significant improvement Cosil: no significant differences		CDH: decreased alveolar airspaces CDHsil: no improvement	CDH: decreased alveolar airspaces CDHsil: significant improvement
Vasculature	CDH: decreased vascular volume CDHsil: No improvement Cosil: decreased vascular volume	CDH: less pulmonary vessels CDHsil: significant improvement Cosil: less pulmonary vessels	CDH: less arterioles CDHsil: significant improvement		
Vessel wall	CDH: increased SMC[§] layer CDHsil: significant improvement Cosil: increased SMC[§] layer	CDH: no significant differences CDHsil: no significant differences Cosil: no significant differences		CDH: increased SMC layer CDHsil: significant improvement	

In this study we proved the placental crossing of sildenafil into the fetal circulation with higher levels of plasma sildenafil and lower levels of plasma DMS in the pups compared to the mother. Sildenafil is known to be catalyzed by hepatic CYP3A4 and CYP2C9. Prenatal and early postnatal CYP-mediated N-demethylation is less prominent when compared to adults, which causes less clearance of sildenafil. This reduced clearance in fetuses results in a longer terminal half-life [37]. The dose of sildenafil chosen for this study was 100 mg/kg/d, which was based on a previous study on the pharmacokinetics of sildenafil in rats. Since the metabolism in rats is faster than in human, this dose is a lot higher than the normal dose used in the clinical setting. Although the oral bioavailability of sildenafil in female rats is only 44%, which is similar to humans (38%), it is preferred over other methods keeping in mind the potential clinical application [38].

Luong et al[21] had previously shown that antenatal sildenafil crosses the placenta without affecting the PDE5-expressing organs of the pups in the nitrofen-rat model. Consistent with our study, they show improvement in lung structure in the nitrofen-induced rat model after antenatal sildenafil. However, Luong et al treated rats for 10 days, starting already at day 10.5 of gestation, which is only one day after the start of lung development in rats and can therefore be seen as prophylactic [24]. Furthermore, an increase in the number of arterioles [39], a decrease in vascular remodeling [40] and improvement in pulmonary vascular response and lung growth [41] were shown after antenatal treatment with sildenafil of different duration in the nitrofen rat model. Improvement in parenchymal and lung abnormalities after antenatal sildenafil was shown in a rabbit model of CDH [42] and in a lamb model, downregulation of eNOS was shown to be normalized after antenatal treatment with tadalafil, another PDE-5 inhibitor [43]. However, the treatment strategies in all these studies were already initiated very early during pregnancy, at a time when human CDH would not yet be detectable and before CDH symptoms and pathology develop. A summary of the relevant sildenafil studies in the nitrofen rat model is shown in Table 4.

We found that sildenafil caused a decreased LW/KW ratio, increased muscularization of the arterioles and decreased pulmonary vascular volume in healthy control rats. Differences in lung structure in control rats and rabbits treated with sildenafil were also reported by Luong et al [24] and Russo et al [42]. The pathophysiology of these side-effects is still not clear, but might involve the increase in cGMP after PDE-5 inhibition, since increased cGMP can also lead to toxicity and interfere with normal cellular proliferation [44]. Extreme vasodilation caused by increased cGMP might also have a deleterious effect on the development of the pulmonary vasculature. However, sildenafil has been used as a treatment for preeclampsia in pregnant women with no significant adverse effects in both mother and fetus during follow up of 30 days post-delivery [45]. Furthermore, recently a trial has started for the antenatal use of sildenafil in pregnancies complicated by early-onset extreme fetal growth restriction (STRIDER; NCT02277132 (clinicaltrials.gov)) [46].

In the present study we focused on pulmonary vascular development, since pulmonary hypertension in CDH is the major cause of morbidity and mortality. The major strength of this study is the timing of the sildenafil treatment at day 17.5 of gestation, which corresponds to 20 weeks of gestation in human pregnancy, when CDH is detectable by routine ultrasound in many countries. Sildenafil has never been tested in a clinical trial as an antenatal treatment to target the pulmonary vascular growth. However, the possibility of prenatal diagnosis of CDH offers a unique opportunity to treat fetuses antenatally. So far, sildenafil is only used postnatally as a treatment for severe pulmonary hypertension and in some of the most severe CDH patients who are resistant to current therapies, in an attempt to prevent extracorporeal membrane oxygenation (ECMO). Therefore, it is not possible to directly compare the pathological changes seen in these patients with the effects of the antenatal sildenafil treatment in the CDH rat model.

The potential to treat CDH already antenatally might be a big improvement in the management of this disease in humans. So far the approach for antenatal modulation of the severity of pulmonary hypoplasia is through mechanical interference with pulmonary fluid drainage. To this effect antenatal tracheal plugging has been advocated [47, 48]. An alternative approach can be the provision of antenatal sildenafil in selected high risk prenatally diagnosed CDH fetuses. However, even for postnatal sildenafil no solid safety data are available [49]. Even more for antenatal sildenafil (STRIDER; NCT02277132 (clinicaltrials.gov)) questions remain on safety, dosage as well as repeated prescription and optimal timing of the drug.

In conclusion, our study demonstrates that antenatal treatment with sildenafil started at a clinically relevant time point improves bodyweight, decreases lung hypoplasia and attenuates vascular remodeling in nitrofen-induced CDH in rats. Antenatal use of sildenafil might improve morbidity and mortality in CDH patients by improving lung structure. However, it is important to determine the optimum dosage for this therapy in a potential phase I trial.

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CHAPTER 5

Prenatal treatment with sildenafil and selexipag at a clinically relevant period improves pulmonary vascularity in the congenital diaphragmatic hernia rat model

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Abstract

Patients with congenital diaphragmatic hernia (CDH) often suffer from severe pulmonary hypertension and the choice of current vasodilator therapy is mostly based on trial and error. Since pulmonary vascular abnormalities are already present early during development, we performed a study to modulate these pulmonary vascular changes at an early stage during gestation. Pregnant Sprague-Dawley rats were treated with nitrofen at day 9.5 of gestation (E9.5) to induce CDH in the offspring and subsequently the phosphodiesterase-5 inhibitor sildenafil and/or the novel prostaglandin-I receptor agonist selexipag (NS-304) were administered from E17.5 until E20.5. The clinically relevant start of the treatment corresponds to week 20 of gestation in human, when CDH is usually detected by ultrasound. CDH pups showed increased density of air saccules which was reverted after the use of only sildenafil. The pulmonary vascular wall was thickened and right ventricular hypertrophy was present in the CDH group and improved both after single treatment with sildenafil or selexipag, whereas the combination therapy with both compounds did not have additive value. In conclusion, antenatal treatment with sildenafil improved airway morphogenesis and pulmonary vascular development, while selexipag only acted positively on pulmonary vascular development. The combination of both compounds did not act synergistically, probably because of a decreased efficiency of both compounds caused by CYP interaction and induction. These new insights create important possibilities for future treatment of pulmonary vascular abnormalities in CDH patients already in the antenatal period of life.

Introduction

Congenital diaphragmatic hernia (CDH) is a rare developmental anomaly characterized by an incomplete diaphragm, lung hypoplasia and pulmonary hypertension (PH), which is often unresponsive to current vasodilator therapy [1]. Although the postnatal therapeutic approach is highly protocolized [2] the pharmacotherapy of PH in CDH is mainly trial and error and is based on the modulation of three major vasoactive pathways: the nitric oxide (NO), endothelin (ET) and prostacyclin (PGI_2) pathways. Inhaled NO (iNO) is the most frequently used drug followed by (i.v.) sildenafil, a phosphodiesterase-5 (PDE5) inhibitor acting on the same pathway by inhibiting the conversion of cyclic guanosine monophosphate (cGMP). Currently drugs acting on the PGI_2 pathway are used only in a compassionate way, showing contradicting results [3-5]. Although the ET pathway has shown to be affected in patients with CDH [6, 7], targeting this pathway is even more challenging because of the clinical availability of oral formulation only. A recent Cochrane review showed no improvement in patients with CDH after iNO treatment [8]. However, properly designed trials are lacking while no systematic research has been performed into the different pathways involved. An overview of all studies performed in humans using postnatal vasodilator therapy in CDH is presented in Table 1

Table 1: Studies on vasodilatory drugs in CDH

Compound	Pathway	Patients	Effect	Reference
Inhaled NO	Nitric Oxide	34	No effect	Kinsella et al., 1997 [35]
		53	No effect in mortality and ECMO	NINOS, 1997 [36]
		31	No effect in mortality and ECMO	Clark et al., 2000 [37]
		84	No effect in mortality and ECMO	Finer et al., 2003 [38]
Sildenafil	Nitric Oxide	9	Improved oxygenation index	Bialkowski et al., 2013 [39]
		7	Improved cardiac output Reduced PVR	Noori et al., 2007 [40]
Milrinone	Prostacyclin	6	Improved RV function Improved oxygenation index No effect on PVR	Patel et al., 2012 [41]
Prostacyclin	Prostacyclin	9	Improved oxygenation index	Bos et al., 1993 [42]
Bosentan	Endothelin		No studies performed	

ECMO = extracorporeal membrane oxygenation, PVR = pulmonary vascular resistance, RV = right ventricle.

Furthermore, changes in the pulmonary vasculature, leading to PH, have previously been shown to be present already early during gestation [9], while treatment is only offered postnatally. Over the last years we and others showed improvement in lung development after antenatal treatment with sildenafil in different animal models of CDH [10-12]. However, the pulmonary pathology in these animals was not totally reversed. Since CDH-associated

abnormalities may not be limited to only one pathway, antenatal targeting of more pathways could possibly provide new approaches for therapeutic strategies. Antenatal use of all endothelin receptor antagonists have shown to be teratogenic [13, 14]. However, prenatal monotherapy with a slow-release synthetic prostacyclin agonist in a rat model of CDH showed improvement of the diminished development of alveolar and capillary networks [15]. Until recently most available prostacyclin analogues could only be administered by continuous intravenous infusion or inhalation, and had limited stability and a very short half-life [16] Selexipag is a novel highly selective long-acting oral PGI_2 receptor agonist that has recently been approved for the treatment of PH in adults. The active compound of selexipag, NS-304, is hydrolyzed by the liver to its active metabolite ACT-333679, which has an even higher affinity for the PGI_2 receptor [17, 18].

Here, we analyzed for the first time the effects of antenatal treatment targeting both the NO pathway and the PGI_2 pathway in the nitrofen-CDH rat model, starting at a clinically relevant time point.

Methods

Animal Model

Pregnant Sprague-Dawley rats received either 100 mg nitrofen dissolved in 1 ml olive oil or just olive oil by gavage on gestational age day E9.5. Administration of nitrofen at exactly this time point induces mainly left sided CDH in approximately 70% of the offspring, while all pups have PH [19]. This study included only pups with an observable diaphragmatic defect. Pregnant rats were divided into 8 groups: control, nitrofen (CDH), control+sildenafil, nitrofen+sildenafil (CDH+sildenafil), control+NS-304, nitrofen+NS-304 (CDH+NS-304), control+sildenafil/NS-304 and nitrofen+sildenafil/NS-304 (CDH+sildenafil/NS-304). Sildenafil (100 mg/kg/day, Pfizer, New York, NY, USA) and NS-304 (1 mg/kg/day, MedChem Express, Monmouth Junction, NJ, USA) were dissolved in 0.8% ethanol in water and administered via oral gavage for 4 consecutive days from E17.5 to E20.5. At E21 pups were delivered by caesarean section and euthanized by lethal injection of pentobarbital (Figure 1). The dosage of sildenafil was based on our previous study [10], whereas for NS-304 a dose study was performed.

All animal experiments were approved by an independent animal ethical committee and according to national guidelines.

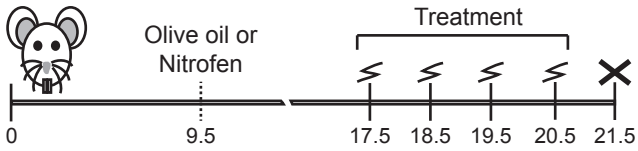

Figure 1 Schematic overview of the study design

Overview of study design showing interventions at different time points. X-axis shows days during gestation. Flashes indicate time point of intervention, cross indicates time point of termination. The different treatments are placebo, sildenafil, NS-304 and sildenafil + NS-304. n= 2 litters for control and nitrofen with placebo (n= 26 and 24 pups, respectively) or sildenafil (n= 15 and 24 pups, respectively), n= 3 litters for control and nitrofen with NS-304 (n= 35 and 37 pups, respectively) or sildenafil+NS-304 (n= 36 and 37 pups, respectively).

Lung Morphology

Fetal rat lungs were isolated, fixed overnight in 4% PFA and embedded in paraffin. Serial 5 µm thick sections were made through the middle of the left lobe and stained with haematoxylin and eosin (HE). Sections were imaged at 40x magnification using a BX41 research stereomicroscope system (Olympus; Tokyo, Japan). Four non-overlapping images in three different sections of each lung were acquired. Major airways and vessels were excluded from analysis. The airspace size was automatically quantified using the two following measures: the D_2-score, as previously described [10], and the mean linear intercept L_m. Approximate value of the latter was calculated as proposed by Muñoz-Barrutia et al [20], using both horizontal and vertical test lines. However, previous comparisons between both methods have shown a higher accuracy of the D_2-score [21].

Immunohistochemistry and Immunofluorescence Staining

Immunohistochemistry (IHC) was performed on 5 µm paraffin sections of lungs according to standard protocols, using the Envision™ detection system (Dako Cytomatic, Glostrup, Denmark) [22]. Primary antibody used for IHC was smooth muscle actin (α-SMA; MS-113-P1; 1:1200, Thermo Scientific, Fremont, CA, USA). Antigen retrieval with Tris-EDTA buffer (10 mmol Tris, 1 mmol EDTA; pH 9.0) was used.

Primary antibodies used for immunofluorescence staining were smooth muscle actin (α-SMA; MS-113-P1; 1:500, Thermo Scientific) and Ki-67 (1:100, Abcam, Cambridge, UK). Secondary antibodies against mouse (α-SMA) and rabbit (Ki-67) were used. Negative controls were performed by omitting the primary antibody. Antigen retrieval with Citric Acid buffer (11.2 mmol; pH 6.0) was used.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

RNA isolation of whole lungs, cDNA synthesis and subsequent qPCR analysis were performed as previously described [22]. Primer combinations for the qPCR reactions are listed in Table 2. *Actb* was used as housekeeping gene.

Table 2: Primer sequences

Gene	Sequence (forward 5′- 3′)	Sequence (reverse 5′- 3′)
Ptgir	CACGAGAGGATGAAGTTTACCA	AATCCTCTGATCGTGAGAGGC
Ptgis	CATCAAACAGTTTGTGGTCCT	CAAAGCCATATCTGCTAAGGT
eNos	CATACTTGAGGATGTGGCTG	CCACGTTAATTTCCACTGCT
Pde3	CCAGCAACCGAATATTGACCA	AATCTGAAAGTTCCAGTTGCTC
Pde5	TCAACAACGGATAGCAGAACTC	CCCTGTTCATTAGATCAGCGG
Prkg2	ACTAGGCATTATCTACAGAGACC	TCCAAAGTCAACCAACTTAAGG
Sma	TGACCCAGATTATGTTTGAGAC	AGAGTCCAGCACAATACCAG
Pdgfr-β	AACGACCAGTTCTACAATGCC	CATGATCTCATAGATCTCGTCGG
Actb	AGATGACCCAGATCATGTTTGAG	GTACGACCAGAGGCATACAG

Cardiovascular measurements

Lungs and heart of pups were perfused through the right ventricle with Microfil contrast agent (Microfil, Flow Tech; Carver, MA, USA) and imaged with a micro Computed Tomography (micro-CT) scanner (Quantum FX, PerkinElmer; Waltham, MA, USA; pixel size 10–295 µm). Subsequently, images of the hearts were analyzed with Dataviewer (Skyscan, Bruker, BioSpin, Ettlingen, Germany).

Statistical analysis

Data are presented as means (SD). For the results of the dose study, one-way analysis of variance (ANOVA) was applied to compare bodyweight and lung-to-kidney weight ratio (LW/KW) between dose levels (placebo, 0.1, 1 and 10 mg/kg), followed by Tukey's method for post-hoc multiple comparisons. For the data of the intervention study, two-way ANOVA with factors disease (control versus CDH) and treatment (placebo, sildenafil, NS-304 and combination of sildenafil and NS-304) was used to compare the results of the experiments between groups. The interaction effect of disease and treatment was included in the model in case this effect was statistically significant. The normality assumption of the ANOVA models was assessed by creating histograms of the model residuals. The analyses were performed using SPSS 21.0 for Windows (Armonk, NY, USA: IBM Corp.). All statistical tests were two-sided and used a significance level of 0.05.

Results

Dose study

We first established an effective antenatal dose of NS-304 by analyzing the effects on the pups and monitoring possible side-effects of this compound. Therefore we started a dose study in control rat pups using 3 different dosages based on previous studies in adult rats [23, 24].

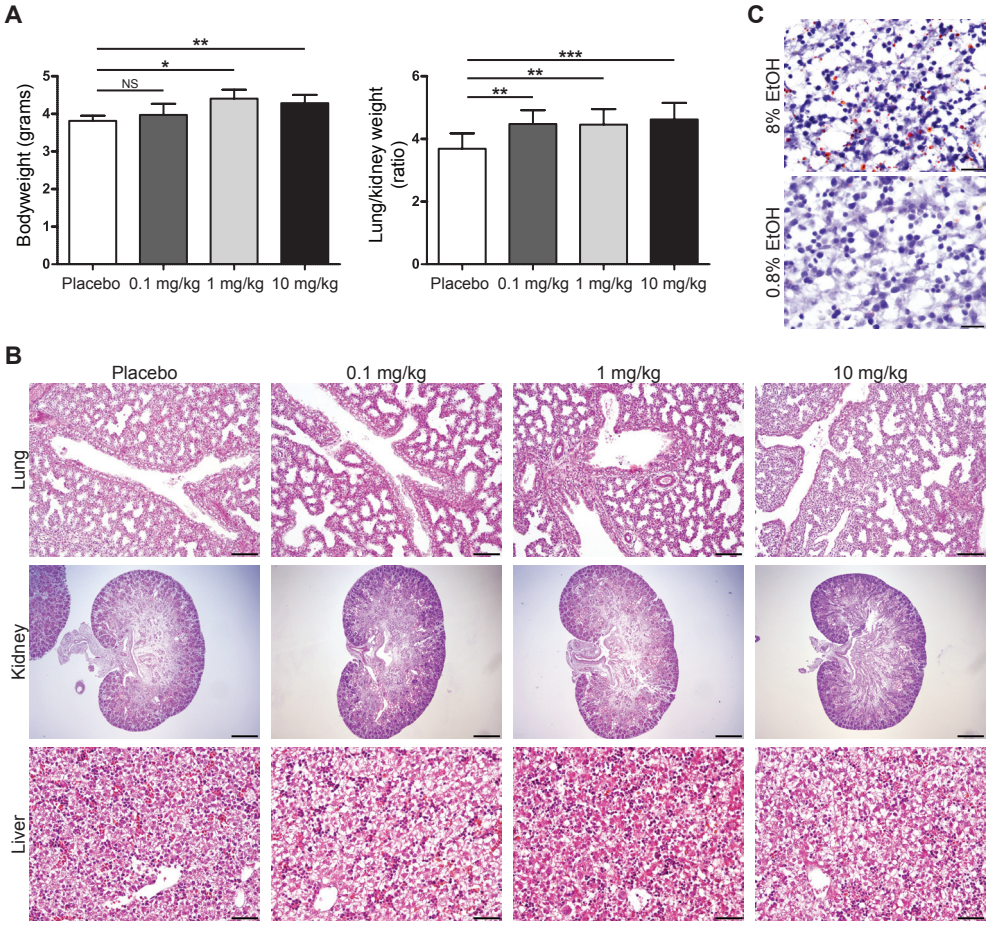

Figure 2 Dose study of NS-304

A: Bodyweight is significantly increased after treatment with NS-304 at 1 mg/kg (p=0.036) and 10 mg/kg (p=0.009) (n= 12, 13, 10 and 14, respectively). Lung-to-kidney weight ratio is significantly increased in all treated groups (p=0.001, p=0.005 and p<0.001, respectively) (N = 13, 14, 11 and 15, respectively). B: Representative images of H&E staining on lung and kidney show no abnormalities in all groups. Representative images of the liver show vacuoles in all samples. Scale bars represent 200 µm (lung), 500 µm (kidney) and 50 µm (liver). C: Representative images of Oil-Red-O (ORO)-staining showing steatosis of the liver after use of 8% EtOH, but not after use of 0.8% EtOH. Scale bars represent 20 µm. * p<0.05, ** p<0.01, *** p<0.001. Bars represent means (SD).

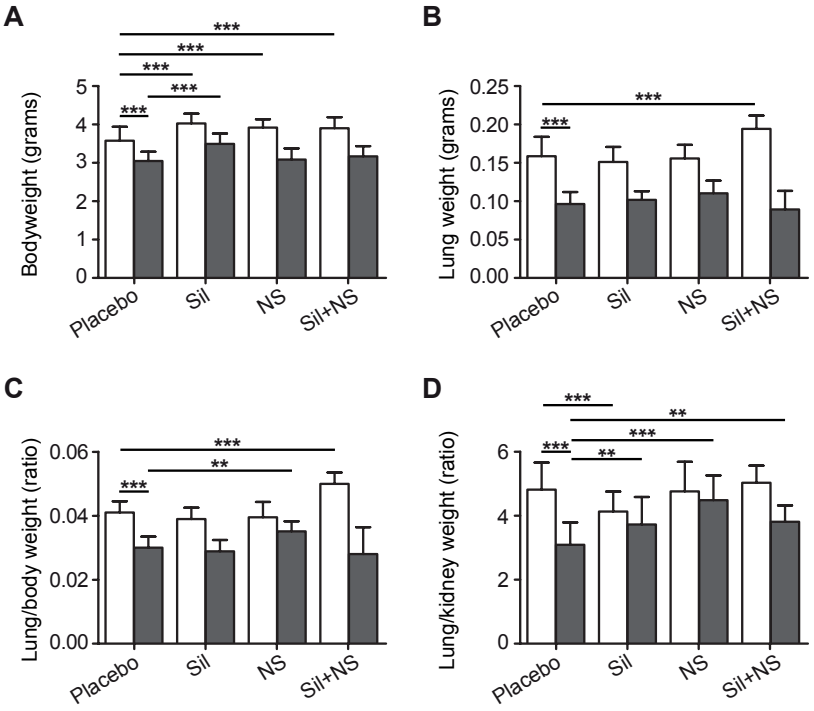

Figure 3 Body and lung weight

A: Bodyweight is decreased in pups with CDH ($p<0.001$). Sildenafil increases bodyweight significantly in both control and CDH pups (both $p<0.001$), where NS-304 and the combination of both compounds only increases bodyweight in control pups (both $p<0.001$). N= 39, 37, 35 and 36 in the control groups and 19, 31, 14 and 21 in the nitrofen groups, respectively. B: Lung weight is significantly decreased in CDH pups ($p<0.001$) with no difference after treatment. N= 53, 50, 26 and 27 in the control groups and 24, 32, 10 and 16 in the nitrofen groups, respectively. C: The lung-to-body weight ratio is significantly decreased in CDH ($p<0.001$) with improvement only after NS-304 ($p=0.003$). N= 33, 35, 26 and 27 in the control groups and 17, 28, 10 and 16 in the nitrofen groups, respectively. D: Lung-to-kidney weight ratio is significantly decreased in CDH ($p<0.001$) and improved in all treatment groups ($p=0.002$, $p<0.001$ and $p=0.004$, respectively). N= 44, 50, 24 and 27 in the control groups and 23, 32, 10 and 15 in the nitrofen groups, respectively. Weights of pups from our previous experiment 10 were included to enlarge the data. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Bars represent means (SD). White bars represent control pups, grey bars represent CDH pups. Sil means sildenafil, NS means NS-304.

Pups of mothers treated with NS-304 did not show any malformations of face, palate, limbs or other organs. Bodyweight was increased in pups treated with 1 and 10 mg/kg/day and LW/KW was increased in all treated pups (Figure 2A). No differences in histology were observed in both lungs and kidneys between all dose levels (Figure 2B). The livers of all pups showed steatosis, which was resolved after adjusting the percentage of ethanol in which the compound was dissolved from 8% to 0.8% (Figure 2C). Based on this dose study the optimal dosage of NS-304 was found to be 1 mg/kg/day. The dosage of sildenafil was based on our previous study [10].

Lung morphology

In accordance with our previous study [10], CDH pups had a decreased bodyweight, which increased after treatment with sildenafil. Lung weight was also reduced in CDH pups, but this did not improve after treatment of sildenafil, NS-304 or the combination of sildenafil and NS-304 (Figure 3A, B). Both lung-to-bodyweight ratio (LW/BW) and LW/KW were significantly reduced in CDH pups. LW/BW increased only after treatment with NS-304, where LW/KW increased in all three groups receiving treatment (Figure 3C, D).

In correspondence with our previous results [10], the density of the air saccules was increased in lungs of CDH pups (Figure 4A). Statistical analyses showed a significant positive correlation between disease and treatment for the D_2-score, the L_m and the number of air saccules ($p=0.005$, $p=0.024$ and $p<0.001$, respectively). The lower D_2-score and L_m in CDH were significantly higher in the pups treated with sildenafil, while NS-304 alone or the combination of sildenafil and NS-304 were not significantly different (Figure 4B, C). The number of air saccules was increased in CDH and diminished after treatment with sildenafil or the combination of sildenafil and NS-304 (Figure 4D). Combined, these results show a therapeutic effect on the formation of air saccules after antenatal targeting mainly by sildenafil.

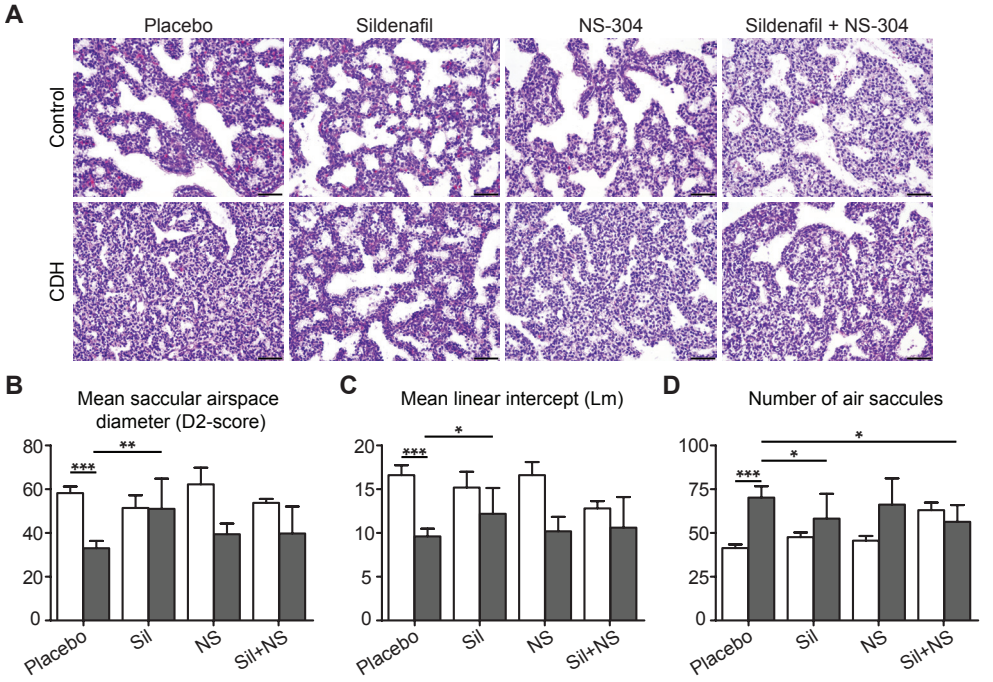

Figure 4 Disrupted lung morphogenesis is partly resolved after sildenafil treatment

A: Representative images of H&E stained lungs. Scale bars represent 50 µm. B: Mean saccular airspace diameter (D_2-score) is significantly decreased in CDH ($p<0.001$), with a significant increase after treatment with sildenafil only ($p=0.001$). C: The mean linear intercept (L_m) is significantly decreased in CDH ($p<0.001$), with a significant increase after treatment with sildenafil only ($p=0.048$). D: The average number of air saccules in 1 image per lung was increased in CDH ($p<0.001$) and decreased after sildenafil ($p=0.036$) and combination therapy ($p=0.017$). For each group, 4 non-overlapping images on 3 different sections for 5 different animals were used. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Bars represent means (SD). White bars represent control pups, grey bars represent CDH pups. Sil means sildenafil, NS means NS-304.

Since NS-304 acts on the IP-receptor (Ptgir), we checked the expression of this receptor and its synthase at mRNA level. We found an increase in *Ptgir* and a decrease in *Ptgis* in CDH with only a trend to improvement after treatment in case of *Ptgir* (Figure 5A, B). The expression of endothelial NO synthase (*eNos*), an important enzyme in the production of vasoactive NO, was increased in CDH and did not change after treatment (Figure 5C). Phosphodiesterase-3 (*Pde3*), which hydrolyses and thus inactivates the secondary messengers cyclic adenosine monophosphate (cAMP) and cGMP, was not expressed differently in control and CDH, but was decreased after treatment with NS-304 in both control and CDH (Figure 5D). Phosphodiesterase-5 (*Pde5*), the enzyme which hydrolyses cGMP is inhibited by sildenafil, and its downstream target protein kinase G2 (*Prkg2*) were both increased in CDH. Prkg2 was decreased in CDH in all treatment groups (Figure 5E, F). All these changes in the expression of these factors in both therapeutic pathways confirm the importance of these pathways in CDH.

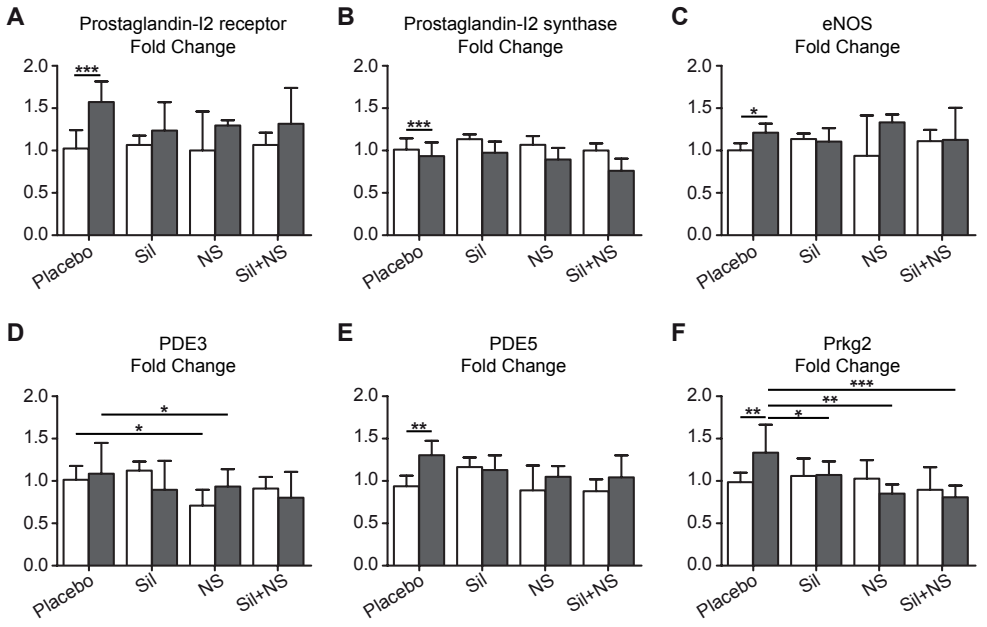

Figure 5 RNA expression levels of rate-limiting factors in the NO and PGI_2 pathways

A: Quantitative PCR shows a significant increase in *Ptgir* in CDH (p<0.001) with no differences after treatment. B: *Ptgis* is decreased in CDH (p<0.001) with no improvement after treatment. C: *eNos* expression is increased in CDH (p=0.041) and shows no improvement after treatment. D: No differences were found in *Pde3* between control and CDH pups, but treatment with NS-304 decreased *Pde3* expression in both control and CDH (both p=0.030). E: *Pde5* is increased in CDH (p=0.007) with no improvement after treatment. F: *Prkg2* is increased in CDH (p=0.013) and decreased after treatment with sildenafil, NS-304 and the combination of both (p=0.045, p=0.002 and p<0.001, respectively). N= 6 for all groups. * p<0.05, ** p<0.01, *** p<0.001. Bars represent means (SD). White bars represent control pups, grey bars represent CDH pups. Sil means sildenafil, NS means NS-304. No interaction model was used for *Ptgir*, *Ptgis*, *eNos*, *Pde3* and *Pde5*.

Pulmonary vasculature

Next, we analyzed the pulmonary vascular development by whole mount imaging after infusion of a contrast agent (Figure 6A). This revealed a decrease in the vascular branching and total vasculature volume in CDH. None of the applied treatment modalities showed significant improvement (Figure 6B), as we previously reported after prenatal sildenafil monotherapy [10]. However, histological analysis of the lungs revealed an increased thickening of the smooth muscle layer of the small pulmonary vessels (25-50 µm) in CDH pups, comparable to our previous results [10]. This augmented thickness of the vascular wall was significantly reduced after treatment with sildenafil as well as with NS-304 alone or the combination of both. Remarkably, all treated control groups showed an increase in thickness of the medial smooth muscle layer (Figure 6C,D). Immunofluorescence staining showed an increase in Ki67/Sma double-positive cells in the small pulmonary vessels in CDH pups, which was reversed to normal in all three treatment groups, indicating reduced proliferation of these cells after treatment (Figure 6E, F).

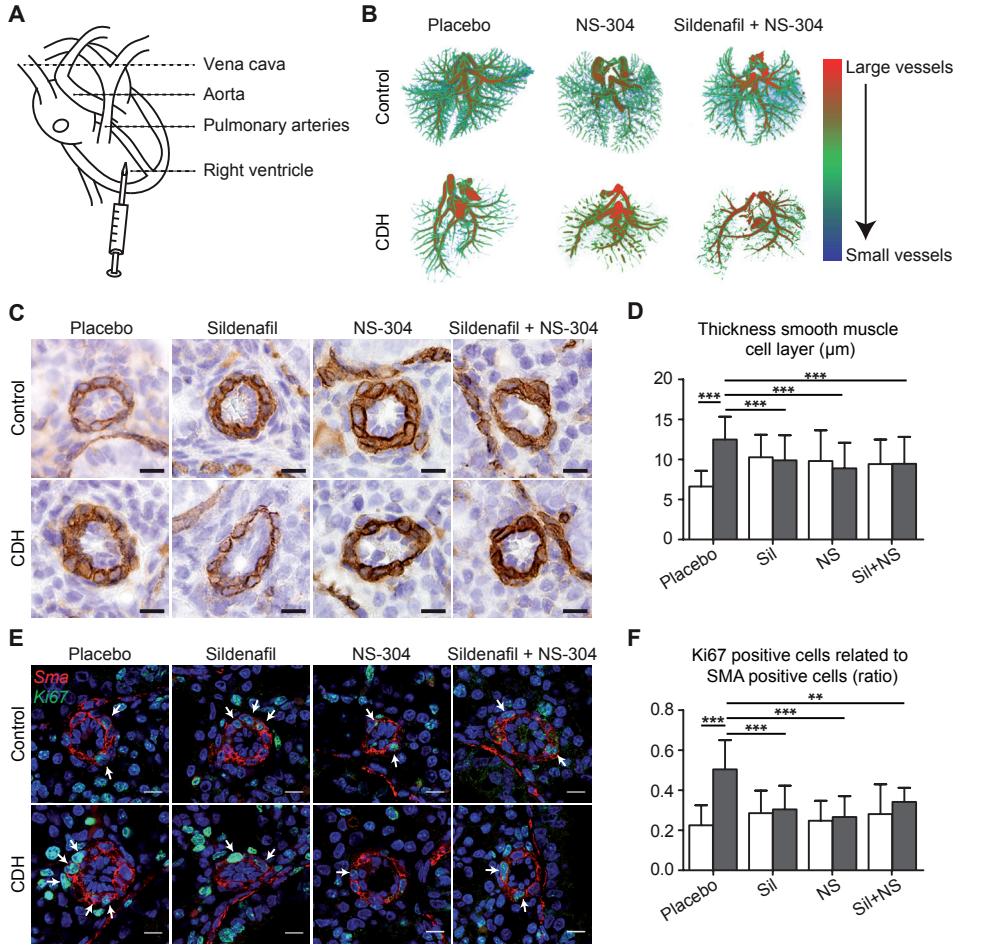

Figure 6 Both sildenafil and NS-304 reduce muscularization defects in CDH

A: Schematic image of injection through the right ventricle. B: Representative images of Microfil-injected pulmonary vessels show decreased branching and volume in CDH with no improvement after treatment with NS-304 or the combination of sildenafil and NS-304. C+D: Representative images of immunohistochemistry staining show increased expression of Sma and an increased thickening of the vascular wall of small pulmonary vessels (25-50 µm) in CDH ($p<0.001$) and control lungs treated with all compounds (all $p<0.001$). In CDH this thickening is decreased after all treatments (all $p<0.001$). n= 34, 30, 33 and 34 in the control groups and 32, 33, 38 and 33 in the nitrofen groups, respectively. E+F: Representative images of immunofluorescence staining show an increase in Ki-67/Sma double-positive cells in small pulmonary vessels in CDH ($p<0.001$) with improvement to normal in all treated groups ($p<0.001$, $p<0.001$ and $p=0.001$, respectively). n=12 for all groups. Scale bars represent 10 µm. ** $p<0.01$, *** $p<0.001$. Bars represent means (SD). White bars represent control pups, grey bars represent CDH pups. Sil means sildenafil, NS means NS-304.

Since we found reduced muscularization of the pulmonary vasculature in CDH lungs after antenatal vasodilator therapy, we checked the effect of the treatment on the heart. Since right ventricular hypertrophy is an indication for pulmonary hypertension postnatally [25], we measured the myocardium of the right ventricle in relation to the total diameter of the heart. This showed a significant increase in CDH with reversion to normal after treatment in all 3 groups (Figure 7A, B) showing a potential effect of treatment on the already higher pulmonary vascular resistance before birth.

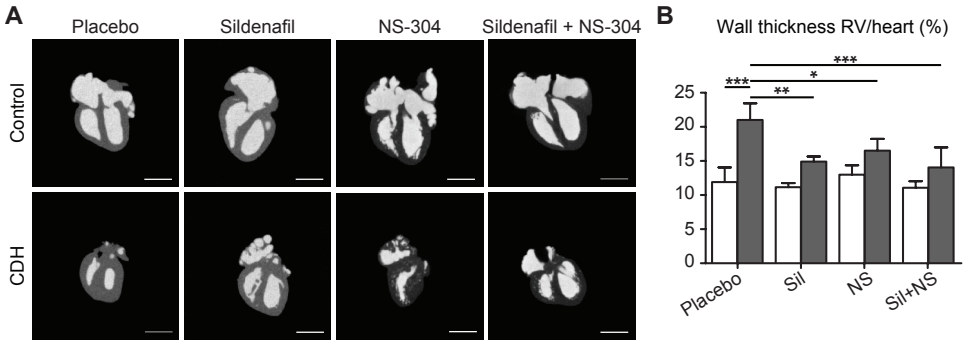

Figure 7 Prenatal treatment improves cardiovascular defects

A + B: Representative images of with Microfil filled hearts show increased thickness of the right ventricle wall in relation to the total diameter of the heart ($p<0.001$). This thickening improved after treatment with sildenafil, NS-304 and the combination of both ($p=0.001$, $p=0.023$, $p<0.001$, respectively). Scale bars represent 2mm. n= 5, 3, 5 and 4 in the control groups and 2, 3, 2 and 4 in the nitrofen groups, respectively. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Bars represent means (SD). White bars represent control pups, grey bars represent CDH pups. Sil means sildenafil, NS means NS-304.

Discussion

In this paper we confirm that antenatal treatment with sildenafil, starting at a clinically relevant time point, results in a partial reversal of the abnormal development of the lung morphology and pulmonary vasculature in the nitrofen-induced CDH rat model. However, we show for the first time that the novel PGI_2 receptor agonist selexipag improves the pulmonary vasculature. Combination therapy with both compounds did not have a synergistic effect. This is the first study combining therapies targeting both the NO and PGI_2 pathway for antenatal use in CDH.

In accordance with our previous work [10], sildenafil increased bodyweight and LW/KW ratio and improved the lung morphogenesis in CDH pups. LW/KW ratio was even reversed to normal after treatment with selexipag, but sildenafil did not have a synergistic effect when combined with selexipag. This may be explained by a combined inducing effect of sildenafil and selexipag on the activation of CYP3A4. of activity. This is supported by a phase 3 study on selexipag, which predicted a 30% lower exposure to the active metabolite ACT-333679 when used in combination with a PDE-5 inhibitor [26]. Furthermore, it is known that the clearance of drugs by CYP3A4 is increased during pregnancy [27]. A recent study showed no measurable levels of radioactivity in fetal tissues after the use of selexipag in a pregnant rat [28]. However, they only measured radioactivity after just one single dose. The saccular airspaces did not increase after the use of selexipag and, when added together with sildenafil, it only seemed to reverse the positive effects of sildenafil, suggesting the enhanced clearance of both compounds. In contrast to the lung morphology, selexipag caused a decrease in muscularization and proliferation of the smooth muscle layer and a reduced myocardial thickness of the right ventricular wall. These effects were also observed after the use of sildenafil as well as the combined sildenafil/selexipag therapy. The increased proliferation of Sma positive cells in CDH might relate to the pulmonary hypertension in these pups and was previously shown by others as well [15]. Hypertrophy of the right ventricular wall has been known to be an early sign of PH postnatally followed by right ventricular dilatation and eventually heart failure [25]. Normally, early during gestation the right and left cardiac ventricles are approximately identical in size, whereas later in pregnancy the right ventricle becomes slightly more dilated [29]. However, in case of right ventricular outlet obstruction the myocardial mass can already increase antenatally [30]. A previous study in adult rats with monocrotaline-induced PH showed reduced hypertrophy of the pulmonary arterial wall and less thickening of the right ventricle after the use of selexipag [23]. These combined results indicate a potential effect of this drug on the severity of the PH. Reduced pulmonary vascular pathology was also observed in a prophylactic study by Umeda et al, who applied a prostacyclin agonist antenatally in CDH [15]. They showed an increased LW/BW ratio and reduced thickening of the medial wall of pulmonary arteries, as we show as well. In contrast to our results, they found an improvement of the alveolar and capillary networks with an

increased mean linear intercept at E21. These differences can possibly be explained by the thromboxane inhibitory activity of their compound or the early start of treatment at day E9.5, when development is still at an earlier stage and deviations in lung development have not yet started. The major advantage of our approach is the start of our treatment at E17.5, a phase of lung development comparable to 20 weeks of gestation in human when CDH can be detected, which makes it potentially more clinically relevant. Furthermore, we used an orally available approved medicine for our treatment, which could be extrapolated easier to clinical use. As previously described by our group [10], pulmonary vascular volume is decreased in CDH. Apart from sildenafil, selexipag and combination therapy with both compounds did not increase vascular volume and branching. This may well be expected since the majority of the pulmonary vessels is already developed at the start of treatment and treatment will therefore mostly affect the vascular remodeling.

Confirming the results previously shown by us and others [10-12], sildenafil caused unanticipated differences in lung structure in healthy controls, with a thickening of the smooth muscle layer in the pulmonary vessels. However, selexipag induced similar effects in healthy subjects, which strengthens the idea that inducing vasodilation in already healthy vessels might be deleterious for the development of the pulmonary vasculature [10].

The possibility of early detection of CDH by ultrasound makes this disease suitable for antenatal therapies. Some studies have already been performed in the nitrofen rat model on the antenatal use of sildenafil alone [10, 11] or in combination with steroids [31, 32] or the endothelin antagonist bosentan [33]. However, the combination with a prostacyclin agonist, which can safely be used antenatally, has never been studied in this disease. Although antenatal treatment with selexipag or the combination of selexipag and sildenafil did not seem advantageous over monotherapy, at least in the nitrofen rat model, the addition of a second drug might still be of interest because of the variable response to vasodilator therapy of CDH patients in the clinical setting [1] (Table 1). The variability between patients and the altered expression of different vasoactive factors in CDH strengthens the need for ongoing evaluation of the developmental sequences of the pathways involved and subsequent, a more precision medicine approach.

We analyzed for the first time the feasibility and effects of antenatal use of the novel PGI_2-receptor agonist selexipag in the nitrofen-induced CDH rat model. The positive effects on the pulmonary vasculature show that the compound successfully entered the fetal circulation. Embryotoxicity studies in rats and rabbits have shown no malformations, irregularities or neurological differences after the use of selexipag during pregnancy [26, 34]. Indeed, no malformations or abnormalities in histology were seen in different organs in our study.
In conclusion, this study demonstrates improvement of lung morphogenesis after antenatal treatment with sildenafil monotherapy and a reduction in vascular remodeling after

antenatal treatment with both sildenafil and selexipag monotherapy, where no synergistic effect was present after combination of both compounds. This knowledge creates important possibilities in the therapy of pulmonary hypertension in CDH patients. Ideally future research has to reveal antenatal differences in expression of vasoactive factors in specific individual CDH patients before clinical trials on precision medicine with these compounds can be performed.

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CHAPTER 6

General discussion

Congenital diaphragmatic hernia a vascular disease of the newborn

Congenital diaphragmatic hernia is a congenital abnormality, which occurs approximately in 1:2500 live births. CDH is associated with a defect in the diaphragm, a variable bilateral pulmonary hypoplasia and pulmonary hypertension (PH), which still leads to mortality and potential life-long morbidity [1]. The severity of the defect in the diaphragm and the PH fluctuates per patient and the response of the patient to the treatment modalities also differs from patient to patient. This leads to difficulties in the choice of the optimal treatment of these critically ill newborns. Development of a standardized treatment protocol in the past decade has increased the survival chances of CDH patients significantly[2] +referentie? The development of new treatment modalities awaits a better understanding of the disease, which requires new insights in how and when during development the structural changes associated with PH arise and which molecular mechanisms are involved in these changes. In order to reveal the molecular pathways involved in the development of the structural changes in PH it is important to first understand how the pulmonary vasculature normally develops. Although many studies have been performed both clinical and experimental using a variety of animal models such as sheep, rabbits and rodents [3-5] very few have focused on early pulmonary vascular development

Development of the pulmonary vasculature in CDH

The pathological changes in the pulmonary vasculature of PH associated with CDH are characterized by thickening of the smooth muscle cell layer in the tunica media and muscularization of the small capillaries, a process called neo-muscularization [6]. Moreover, these structural vascular changes as well as the expression of markers associated with more contractile smooth muscle cells are already present early in lung development [7]. Indicating that these pathological changes in CDH are caused by aberrant developmental processes, making the PH associated with CDH an early developmental anomaly.

Description and function of pericytes

Pericytes are prime candidates to underlie the structural changes in PH. Pericytes are located around the vessels and are in direct contact with the endothelial cells located in the capillaries [8]. During angiogenesis, the process where the vasculature expands through sprouting of endothelial cells to form new tubules, pericytes are recruited in a PDGF-depended manner [9-11].

Fluctuations and alterations in the number of pericytes that surround the vasculature (pericyte coverage) have been linked to multiple pathological conditions such as diabetic renopathy, cancer biology, pulmonary fibrosis and pulmonary hypertension in the adult [8,

[12-14]. To study the potential role of pericytes in the vascular pathological conditions, it is necessary to understand the biology of these cells. We used a cell based approach and in Chapter 2 we first characterized the pericyte population during lung development in CDH and age matched controls. Analysis of multiple markers at different gestational ages revealed that NG2 and PDGFRβ were the most specific in identifying pericytes in the lung in mice and human respectively. The diversity in the specificity of the pericyte markers indicates the variance in the nature of these cells. In the developing lung pericytes most likely differentiate from a mesenchymal pool of cells. Suitable markers to identify this progenitor pool are not yet revealed. Next steps will be to characterize the mesenchymal pool and identify the signaling pathways involved in the differentiation of the mesenchymal cell pool into pericytes during lung development. Characterization of the progenitor pool will give an even earlier target to influence the vascular growth and may also be helpful in early diagnosis of CDH, assuming that this pool is also affected in CDH.

The role of pericytes in PH associated with CDH

In chapter 2, we hypothesized that pericytes are potential candidates to be the precursor cells for the smooth muscle cells due to their location and function. Confocal microscopic analysis in combination with Fluorescence-activated cell sorting (FACS) analysis showed an increase in pericyte coverage from E15 onwards in CDH. Firstly, this confirms the hypothesis that pericytes are the source of the hypermuscularization and neomuscularization in PH. Secondly, it shows for the first time that not only the number of smooth muscle cells is higher in CDH, but also the number of the possible precursor cell, the pericyte, is higher (Chapter 2). Increased pericyte coverage is shown to be a consequence of downregulation of BMPR2 and an upregulation of WNT/ β-Catenin signaling [15]. An increased number of pericytes is associated with a decrease in functional pericytes. This leads during the active growth of the pulmonary vasculature to improper stabilization of newly formed tubes and an incomplete capillary bed

The differentiation of pericytes into multiple cell types such as chondrocytes, adipocytes and smooth muscle cells has been shown *in vitro* [16-18], indicating the multipotent nature of pericytes. However, it has been shown recently that pericytes do not differentiate into other cell types *in vivo* [19]. The observation in chapter 2 that pericytes express more Smooth muscle actin (ACTA2) in CDH at E18 indicates that they start to differentiate into smooth muscle cells in a malignant manner, leading to the excessive muscularization of the pulmonary vessels. The increase of pericyte coverage and their aberrant differentiation is also observed in the mouse model for arterial pulmonary hypertension in the adult [12], however the role of perciytes in the developmental disease CDH and its associated PH has not been studies before.

Unraveling the cellular changes that lead to the underdeveloped capillary bed in CDH could help to develop new treatments. Previous studies and therapies have focused on the

postnatal treatment of the hypertension associated with CDH. Given our data that clearly show an early developmental process, a new approach could be to interfere with the altered behavior and maturation of pericytes in the early onset of lung development. The observation of increased pericyte coverage and disturbed angiogenesis could potentially be translated to other diseases.

For instance, alterations in pericyte coverage also plays a distinctive role during vascular growth and thereby angiogenesis in tumors [20, 21]. Both an increase as well as a decrease in pericyte coverage in tumor vasculature is observed[22, 23]. Moreover, an increase, but not a decrease, in pericyte coverage has been linked to resistance to therapy and poor clinical outcome for patients (reviewed in Ribeiro et al [24]). However, a decreased number of pericytes have been linked to increased vessel permeability and thereby favors tumor cells to metastasize. The, observed increased pericyte coverage in CDH may increase the resistance to PH therapy and thereby be comparable to the effect in tumor biology. Indeed there is one case report in the literature on the positive effect of Glivec [25] a well-known anticancer drug that rescued a patient with CDH [26].

Further support of disturbed angiogenesis in CDH as described in chapter 2, came from the observation of decreased Collagen IV (COLIV) expression at E15 and E18. COLIV is a major component of the basal membrane between endothelial cells and pericytes. The expression of COLIV indicates integrity of the basal membrane [27 28].

The diaphragmatic hernia in CDH is the result of an underdeveloped diaphragm [29]. Reduced COLIV expression could also lead to a higher vulnerability of the tissue. The severity and thereby the size of the diaphragmatic defect could be a result of the reduced COLIV expression.

Thus, the observed increased number of pericytes early during lung development in CDH gives a new insight into the development of the pathological changes in the vasculature in CDH.

Molecular analysis of the structural changes in the pulmonary vasculature in CDH

To further investigate which genes are involved in the disturbed development of the pulmonary vasculature in CDH, the transcriptome of the endothelial cells and pericytes was analyzed as described in Chapter 3. Besides the endothelial and pericyte population, the epithelial cells and mesecnhymal cells were also used for RNA sequence analysis. Analysis of the four different populations at E13 revealed that there was high similarity in the GO terms between the endothelial and pericyte population. Moreover, detailed analysis of the ROBO/

SLIT pathway and the angiopoietin pathway revealed differentially expression of some members of these pathways by endothelial cells, of other members by the pericytes, and of a number of members in both populations This indicates that signaling between endothelial cells and pericytes early in lung development is affected in CDH, and that multiple pathways are involved which could explain the heterogeneity of the clinical presentation and the degree of the pulmonary hypoplasia of CDH.

The role of KLF4 in the development of the pulmonary vasculature in CDH

Since the development of the pulmonary vasculature is mostly based on two cell types, the endothelial cells and pericytes, we further focused on genes of the endothelial cell population. A novel gene, which has not been linked to CDH before is *Kruppel like factor 4 (Klf4*) which we identified by RNA sequence analysis.

KLF4 is shown to be an upstream regulator of NOTCH signaling [30], which is active during angiogenesis, regulating the competition between the tip and the stalk cell to be the guidance cell to form new tubes [31]. Decreased KLF4 expression in CDH could thus lead to disturbed NOTCH signaling and thereby causing reduced sprouting of endothelial cells. A lower number of sprouting endothelial cells lead to an arrest in growth of the capillary network of the pulmonary vasculature, which we describe in Chapter 2.

Thus, decreased KLF4 expression may be a key in the mechanism that underlies the underdevelopment of the pulmonary vasculature in CDH. Revealing these mechanisms could help to intervene early with pulmonary vascular growth in CDH patients, thereby preventing the development of severe pulmonary hypertension. KLF4 has been described as a regulator of dedifferentiation of perivascular cells in pulmonary arterial hypertension in adults [32]. However, we did not find differences in expression of KLF4 in the pericyte population or in the mesenchymal population. The pathology of pulmonary arterial hypertension in adults is different from the pathology of PH in the newborn. Moreover, in adult pulmonary arterial hypertension an increase in proliferating endothelial cells is observed while no signs of increased apoptosis were observed. Endothelial cells in pulmonary arterial hypertension are indeed hyperproliferating and in that manner similar to cancer cells. Moreover, proliferating endothelial cells associated with pulmonary arterial hypertension have been used as a histological hallmark for pathology [33]. Although we did not observe differences in proliferating endothelial cells in PH associated with CDH, alterations in specific endothelial gene expression were observed. This indicates that endothelial cells in CDH are intrinsically different, similar to endothelial cells in pulmonary arterial hypertension.
Furthermore, pulmonary hypertension associated with CDH is caused by a developmental defect and pulmonary hypertension in adults is due to other causes[34] [35]. An alternative explanation may be that the aberrant pulmonary vasculature in CDH is primarily caused

by ‘malignant’ differentiation of multipotent cells and less by dedifferentiation of smooth muscle cells.

Targeted therapy influencing the behavior of endothelial cells during angiogenesis could lead to more appropriate vascular growth. Therapy of CDH patients will have to go through the mother. Finding cell surface markers that are specific for the endothelial cells in the lung of the CDH patient will help to develop targeted therapy. Moreover, drugs developed for specific cell surface markers only expressed on ‘pathological’ endothelial cells could be downstream or upstream of KLF4 and thereby influence the KLF4 activity.

Retinoic acid and pulmonary vascular development

Retinoic acid signaling is of high importance during embryogenesis. It has been shown to be essential for limb development, neuronal differentiation and organogenesis [36]. Likewise, the importance of proper retinoic acid signaling is shown for normal lung development. The inhibition of retinoic acid signaling by the chemical compound BMS493 resulted in reduced budding and branching of the early lung buds [37]. The role of the retinoic acid pathway in the development of CDH has been shown in multiple studies in mice, rat and human [38, 39]. Furthermore the expression of retinoic acid receptors has been thoroughly studied during the development of the diaphragm, in the pleuroperitoneal folds and the primordial diaphragm [5]. Indeed, retinoic acid signaling is active in the structures from which the diaphragm develops and inhibition of the retinoic acid signaling results in diaphragmatic defects. The timing of the administration of the chemical compounds is crucial. Moreover, the timing determines the sidedness of defect in the diaphragm [5]. The chemical compounds that are used to induce CDH interfere with the retinoic acid pathway. When CDH inducing compounds are administered, the activity of Aldehyde dehydrogenase 1 family, member A2 (RALDH2) is inhibited. The RALDH2 enzyme is required for the conversion of retinaldehyde into retinoic acid. Thus, retinol is present in the serum and is transported by the Retinol binding protein 4 (RBP4), it then binds the extracellular receptor Stimulated by retinoic acid gene 6 (STRA6). Then retinol gets internalized and through enzymatic conversion steps is converted into retinoic acid. Retinoic acid is secreted and taken up by retinoic acid responsive cells where it is transported to the nucleus and binds to the retinoic acid receptors thereby activating retinoic acid responsive genes [40].The inhibition of the enzyme results in less retinoic acid and reduced activation of the retinoic acid receptors [40].

Studies related to retinoic acid signaling and CDH have been performed both in rodent models and with patient material. Moreover, newborns with CDH show significant lower levels of the retinol binding protein compared to controls [39]. Further evidence of the involvement of retinoic acid signaling in CDH comes from retinoic acid receptor knockout studies[41]. Retinoic acid receptor knockout mice show only 25% of CDH in their offspring. The

low numbers of CDH in the retinoic acid receptor knockouts supports the idea that reduced activity of the retinoic acid pathway is not the only molecular mechanism, which is causing CDH. The effect of retinoic acid inhibition on the growth of the pulmonary vasculature has not been studied before. In Chapter 2 we found for the first time the effect of the inhibition of retinoic acid on the development of the pulmonary vasculature in the mouse CDH model. The cellular effects were determined which included an increase in pericyte coverage in the pulmonary vasculature in early lung development. However this observation, did not link any molecular mechanisms to the effect of retinoic acid pathway inhibition. Studying the transcriptome of different cell population revealed which cells express retinoic acid receptors and new candidates that are important for the development of the pulmonary vasculature, but it could not directly be linked to the retinoic acid pathway. This may be because the whole transcriptome analysis was performed 4 days after the nitrofen administration. Nonetheless, this not only shows the difficulty of understanding the retinoic acid pathway, but also indicates the complexity of the natural history and variability of CDH. Genes whose expression is regulated by retinoic acid receptors may have other activators and are therefore called retinoic acid responsive genes. Thus, their expression is related to retinoic acid activity, but not exclusively.

Previously, it has been shown that a decreased active retinoic acid led to an increase in active Transforming Growth Factor β (TGFβ)[37]. The idea that TGFβ signaling becomes more active in our studies is an interesting finding in relation to our finding that pericyte differentiation is accelerated in CDH. Moreover, in our model the inhibition of retinoic acid could lead to an increase in TGFβ signaling. TGFβ signaling is a major regulator of a wide range of signaling pathways [42]. The finding of reduced COLIV expression in chapter 2 could be an effect of disturbed TGFβ signaling. Furthermore, increased activity of the TGFβ signaling pathway could be the molecular pathway which leads to disturbed differentiation of pericytes into smooth muscle cells and the hyper –and neomuscularization. However, the decrease in retinoic acid signaling and thereby the increase in TGFβ signaling is probably a direct effect, creating a signaling cascade from which the effects are observed later in development. It is likely that this imbalance in signaling pathways in the onset of lung development hampers the pulmonary vascular development.

Diaphragm development in CDH

The other pathological characteristic of CDH is the incomplete diaphragm. Many studies have focused on either the lung or the diaphragm and not the combination of the two structures. The diaphragm and lung develop from different embryonic structures. The major diaphragmatic components, the muscle layer and the connective tissue surrounding it, develop from three major structures, the septum transversum, the pleuroperitonial fold and the somites [29, 43]. These structures are all mesodermally derived and thereby have the same origin as pericytes. Pericytes in the developing diaphragm have not been studied in detail,

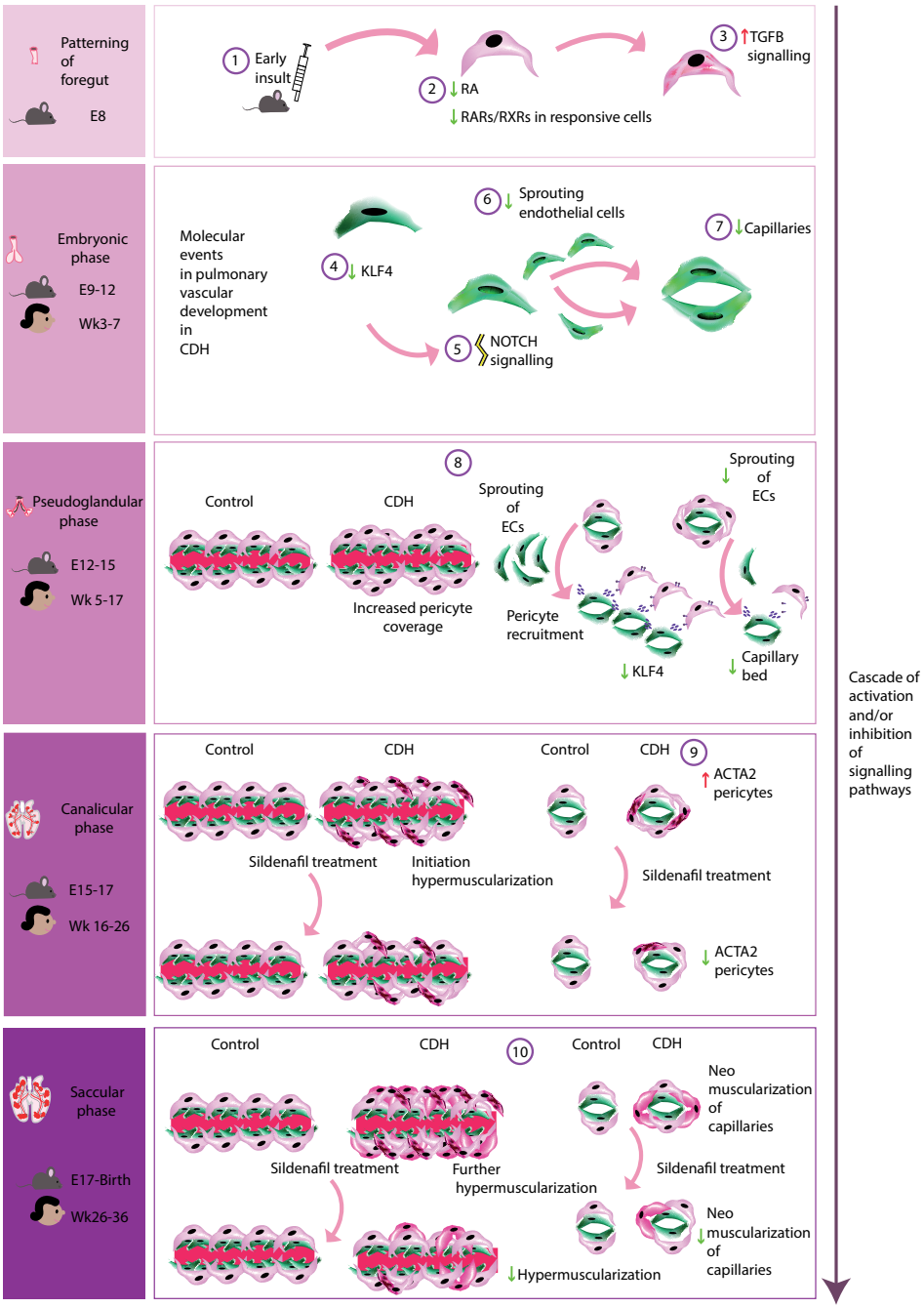

Figure 1

During the development of the pulmonary vasculature in congenital diaphragmatic hernia multiple events lead to pathological changes in the pulmonary vasculature. Moreover, a cascade of activation and/or inhibition of signaling pathways lead to a decrease in newly formed capillaries and thereby an underdeveloped capillary bed, neo-muscularization of capillaries and hyper- muscularization.of the mid-sized vessels.

however they have been characterized in the postnatal diaphragm [44]. Thus, pericytes are present in the diaphragm and share their origin with the developmental structures of the diaphragm. This makes them attractive candidates to understand not only the pulmonary vascular abnormalities but also the diaphragmatic defects. Thus further studies should be done to add insights in the role of pericytes in the development of the diaphragmatic defects in CDH. Lineage tracing studies could help to reveal whether pericytes in the diaphragm and pericytes in the lung have the same cellular origin, and are perhaps even derived from the same progenitor pool. In the CDH rodent model, the retinoic acid pathway is inhibited at E8 and one the targets may be the pericyte progenitor pool. During development this progenitor pool will invade the lung and diaphragm and differentiate into pericytes. However, the microenvironment and the local signaling cascades in the lung and diaphragm are different, resulting in organ-specific responses in the pericytes, which may explain the multiple pathological characteristics of CDH. Thus in the lung the signaling cascade will result in pericyte hypermuscularization whereas the effect in diaphragm may be opposite and leads to insufficient muscularization or uneven muscularization leading to rupture of the diaphragm [45]

New possibilities for treatment of pulmonary hypertension in CDH rat model

Progress in medicine has resulted in early detection of CDH by ultrasonography usually during population screening around 20 weeks of gestation. However, the severity of clinical postnatal symptoms remains difficult to predict. This is due to the different gradations of pulmonary vascular resistance of CDH patients postnatally and their unpredictable responsiveness to current treatment modalities such as nitric oxide (NO). The impaired responsiveness to NO may be due to rapid degradation of the intracellular messenger cyclic guanosine monophosphate (cGMP) by phosphodiesterase-5 (PDE5) [46]. Binding of cGMP to PDE5 stimulates the phosphorylation and activation of PDE5 by cGMP-dependent protein kinase G (PKG), which results in the conversion of cGMP into GMP [47]. Sildenafil is a potent PDE5 inhibitor, leading to an accumulation of cGMP and thus the continuous activation of PKG. PKG has several physiological substrates, which are involved in smooth muscle cell (SMC) relaxation by lowering intracellular calcium [48, 49]. Sildenafil also reduces inflammation, improves early postnatal survival, prevents pulmonary vascular remodeling in different experimental animal models of pulmonary hypertension without CDH [48, 50, 51], and prolongs survival and improves lung structure in a neonatal hyperoxia rat model [52].

To study the effect of sildenafil in the CDH rodent model pregnant rats were treated with nitrofen at E9.5 and received sildenafil later in gestation. The administration of sildenafil during the canalicular stage of lung development resulted in an improved body weight and lung to kidney ratio. Furthermore, the alveolar airspaces increased in diameter, which could be related to the formation of the primary and secondary septa later in prenatal lung

development. Moreover, sildenafil reduced the thickening of the SMC layer in arterioles normally present in CDH and prevented the frequently observed aberrant differentiation of pericytes in CDH as indicated by the loss of the colocalization of ACTA2 and PDGFRβ in the capillaries. However, the control group also showed decreases of the alveolar airspaces. The effect in the control group indicates the importance of the balance in the NO pathway. Another compound what has been tested for prenatal therapy of CDH in the nitrofen rat model is ONO -1301RS. The compound was proven to reduce lung hypoplasia and did not affect heart and kidney function. However, the limitation of compound studies is that the organs are analyzed post mortem. These analyses will not show any side effects, which may occur during birth and postnatal life. It therefore would be beneficial before using these compounds in the clinic to study the effect on post-natal function of organs in pups.

In conclusion

Here, we present new insights into the development of the pulmonary vasculature in the retinoic acid deficient mouse model and although the general concept of lung development in human and mouse is comparable[53], little is known about the differences and the involved signaling pathways. Therefore, a next step would be to study how these cells behave during human lung development. A possibility to study this is to use in vitro culture systems, like 3D organoids or a 'lung on a chip' principle. The lung on a chip provides a method to culture multiple cell layers and thereby create an artificial environment to mimic airflow. These cell culture approaches give the possibility to culture patient material, which may help to identify the affected processes in lung development associated with CDH. Using multiple cell types will give insight in cell -cell interactions during human lung development. The lung on a chip technique and organoid cultures with patient material are useful for drug discovery and can serve as a diagnostic tool since this culture system provides a method to test multiple drugs directly. In addition, an immediate read out can be made of patient response to the drug.

A targeted approach for therapy development for CDH should be based on the knowledge of developmental processes and mechanisms. This thesis describes new detailed insights into the development of the pulmonary vasculature in CDH, revealing increased pericyte coverage, which is the first sign of developing pulmonary hypertension. We furthermore show that during early lung development (E13), the endothelial cell population in CDH shows the biggest difference in gene expression compared to other sequenced populations. Down regulation of KLF4 in CDH may explain the reduced extension of the capillary network in CDH.
This work is a first step in revealing the early molecular and cellular mechanisms, which underlie pulmonary hypertension in CDH. New studies can be designed with this knowledge and provide opportunities to treat pulmonary hypertension associated with CDH from future perspectives to actual therapy.

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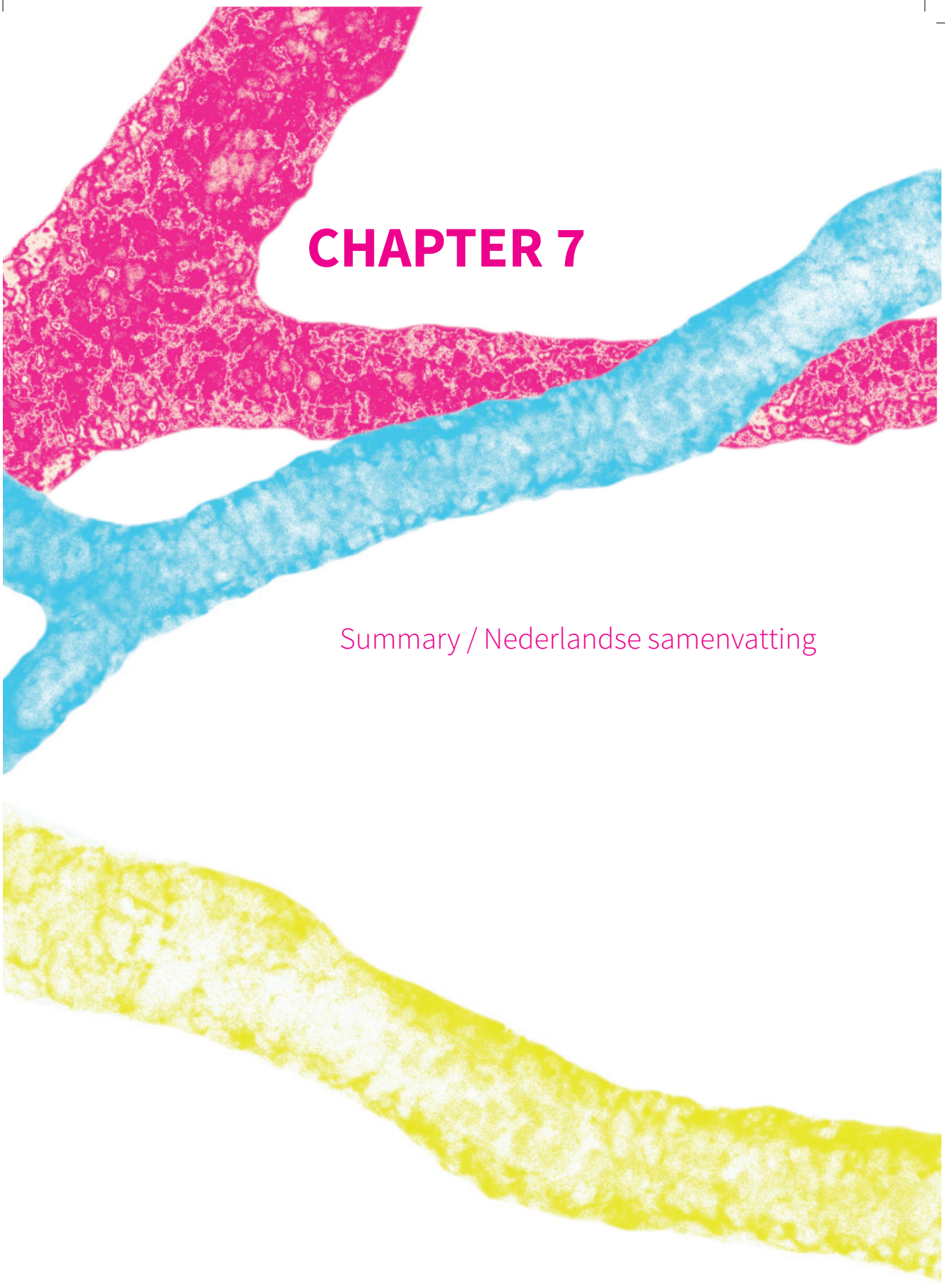

CHAPTER 7

Summary / Nederlandse samenvatting

Summary

Congenital diaphragmatic hernia (CDH) is a life-threatening congenital disease which occurs approximately 1:2500 life births. CDH is characterized by a defect in the diaphragm, pulmonary hypoplasia and pulmonary hypertension (PH).

The PH associated with CDH is the cause of long term hospitalization and life-long medical treatment. The difficulty of treating CDH lays in the fact that CDH patients respond differently to therapy for PH.

PH is characterized by a thickening of the smooth muscle cells layer within the tunica media of the arterioles, this is a process called hyper-muscularization. The small capillaries in the distal end of the lung are also muscularized, a process what is called neo-muscularization. Previous it has been showed that these changes occur already early during development.

Pericytes are prime candidates to underlie the muscularization of the vasculature in the PH associated with CDH. During growth of the pulmonary vasculature pericytes are recruited in a PDGFβ dependent manner by endothelial cells to stabilize newly formed tubules. As a consequence of the interaction between pericytes and endothelial cells, the pericytes start to release Collagen IV (COLIV), a key component of the basement membrane (BM).

To study the role of pericytes in the development of PH a mouse CDH model was established. First, NG2 was identified as the proper marker for pericytes during lung development. An increase in pericyte coverage was observed with Fluorescence- activated cells sorting and confocal microscopy analysis of immunofluorescent labeled whole mount lung samples from E15 onwards in the CDH mouse model. Reduced expression of COLIV was observed, indicating that the basal membrane between pericytes and endothelial cells is affected in CDH. Furthermore, pericytes in CDH intent to express more smooth muscle actin (ACTA2) and loose proliferation and migration capacity.

Samples of CDH patients confirmed that an increase in PDGFRβ expression, and thus an increase in pericytes, is linked to aberrant expression of COLIV at young gestational ages. Thus, the increased pericyte coverage is an important factor in the pathogenesis of CDH and a lack of COLIV in the BM leads to reduced stabilization of the vessel bed and therefore insufficient development of the pulmonary vasculature.

For further understanding of the pathogenesis of PH associated with CDH whole transcriptome analysis on FACS sorted cells was performed at E13 in the CDH mouse model. The transcriptome of four different populations, the epithelial, the mesenchymal, the endothelial and pericytes were analyzed. The analysis of the RNA seq revealed high overlap in the differentially expressed genes between the endothelial cell population and

the pericyte, indicating that the signaling between endothelial cells and pericytes is affected causing the aberrant vascular development in CDH. Detailed analysis of the RNA sequence data revealed that the expression of the gene Kruppel like factor 4 (*Klf4*) is reduced in the endothelial cell population in CDH. The reduced expression of KLF4 was confirmed with whole mount fluorescent analysis. KLF4 acts upstream of NOTCH and therefore members of the NOTCH signaling pathway were further analyzed. This confirmed disturbed NOTCH signaling indicating that the aberrant vascular development in CDH is a result of decreased KLF4 expression resulting in disturbed NOTCH signaling

To explore the new possibilities to treat PH associated with CDH sildenafil was administered to pregnant female rats after they received nitrofen at E9.5, in the canalicular phase of lung development. The administration of sildenafil resulted in improved body weight and improved lung to kidney ratio. Furthermore, the alveolar airspaces increased in diameter, which could be related to the formation of the primary and secondary septa later in prenatal lung development. Moreover, sildenafil reduced the thickening of the SMC layer in arterioles normally present in CDH and prevented the frequently observed aberrant differentiation of pericytes in CDH as indicated by the loss of the colocalization of ACTA2 and PDGFRβ in the capillaries. However, the control group also showed decreases of the alveolar airspaces, Indicating the presence of unwanted side effects in the control group.

A targeted approach for therapy development for CDH should use the knowledge of developmental processes and mechanisms. This thesis describes new detailed insights into the development of the pulmonary vasculature in CDH, revealing increased pericyte coverage, which is the first sign of developing pulmonary hypertension. We furthermore show here that during early lung development (E13), the endothelial cell population in CDH shows the biggest differences in gene expression compared to the other sequenced populations. Down regulation of KLF4 in CDH may explain the reduced extension of the capillary network in CDH.

This work is a first step in revealing the early molecular and cellular mechanisms, which underlie pulmonary hypertension in CDH.

Samenvatting

Congenital hernia diafragmatica (CHD) is een levensbedreigende ziekte die ongeveer 1:2500 geboortes plaats vindt. CHD wordt gekarakteriseerd door een defect in het diafragma, long hypoplasia and pulmonaire hypertensie (PH). De PH die geassocieerd wordt met CDH is de oorzaak van lange termijn ziekenhuis opname en levenslange behandeling van de PH. De behandeling van de PH die gepaard gaat met CHD is moeilijk, dit komt doorrdat patienten verschillend reagageren op dezelfde behandeling.

PH wordt gekarakteriseerd door een verdikking van de gladde spiercellen in de tunica media van de arteriolen, dit process wordt hyper muscularizatie genoemd. De kleine capilalairen zijn ook gemusculariseerd, dit proces wordt neo muscularisatie genoemd. Eerder is al laten zien dat deze veranderingen al vroeg in de otnwikkeling zijn te vinden.

Pericyten zijn geschikte kandidaten om aan de grondslag van de muscularisatie te liggen geassocieerd met PH en CDH. Pericyten zijn nodig tijdens de groei van de vaatboom van de long. Pericyen worden aangetrokken door endotheel cellen in een PDGFβ afhankelijke manier om nieuw gevormde buisjes te stabilizeren. Door de interactie tussen de endotheel celle en de pericyten gaan de pericyten Collageen IV produceren, dit is een van de hoofd componenten van het basale membraan.

Om de rol van pericyten in de ontwikkeling van PH te bestuderen werd er gebrukt gemaakt van een muis model voor CDH. Als eesrte werd NG2 geindentificeerd als de meest gechikte marker voor pericyten tijdens de long ontwikkeling. Verder werd een toename in het aantal pericyte in verhouding met het aantal endotheel cellen. Deze observatie werd gemaakt in het muis model voor CDH vanaf E15 doormiddel van Fluorescent-activated cell sorting (FACS) en analyse van complete longen die fluorescent gelabeled waren. Er werd vermidenderde expressie van Collageen IV geobserveerd. Dit suggereert dat het basale membrane tussen de pericyten en de endotheleel cellen aangedaan is. Verder werden in het CDH muis model meer pericyten gevonden die Aplpha smooth muscle actin tot expressive brachten en deze pericyen verloren hun capiciteit om te prolifereren en migreren. Preparaten van human CHD patienten bevestigden de eerdere bevindingen uit het muis model. In de humane preparaten werd meer expressie van PDGFRβ gevonden (dus meer pericyten) en minder expressie van Collageen IV vroeg tijdens de ontwikkeling. Samengevat, de vermeerdering van pericyten is een belangrijke factor in de pathogenesis van CHD. De vermindering van Collageen IV in het basale membraan leidt tot verminderde stabilizatie en daardoor minder uitgebreide ontwikkeling van de groeiende vaatboom in CHD.

Om de pathogenese van PH beter te begrijpen werd het complete transcriptoom van FACS geisoleerde cellen op E13 geanalyseerd in het muis CHD model. Het transcriptoom van de epiteel, de mesenchymale cellen, de endotheel cellen en de pericyten werd geanalyseerd.

De analyse van de RNA sequence onthulde een grote overlap tussen de anders gereguleerde genen in CHD tussen de endotheel populatie en de pericyten. Dit suggereert dat de signalering en communicatie tussen endotheel cellen en pericyten aangedaan is en dat dit de oorzaak is van de verminderde vaatgroei in CHD. Gedetialleerde analyse van de RNA sequence data onthulde verder dat het gen Kruppel like factor 4 (*Klf4*) verminderd tot expressive komt in de endotheel populatie in CHD. Dit werd verder bevestigd met de analyse van complete immunofluorescent gelabelde longen. KLF4 reguleert de signaal route van NOTCH. Hierdoor werden leden van de NOTCH signaal route verder geanalyseerd. Deze analyse bevestigde dat leden van de NOTCH signaal route veranderd tot expressie komen in CHD. Dit te samen laat zien dat de onder onwikkeling van de long vaatboom in CHD wordt veroorzaakt door verminderde *Klf4* expressie en de ontregelde NOTCH expressie.

Om verdere mogelijkheden voor de behandeling van PH te onderzoeken werd sildenafil gegeven aan zwangere ratten die op E9.5 nitrofen hadden gehad. De toediening van sildenafil resulteerde in de pups met een verbetering van het lichaamsgewicht en verbetereing van de long tot nier ratio. Verder werd er een vergroting van de alveolaire ruimte geobserveerd, wat relateert aan de ontwikkeling van de eerst en tweede septa later in de long ontwikkeling. Behandeling met sildenafil resulteerde verder in een vermindering in de verdikking van de gladde sper cel laag om de arteriolen en verder voorkwam behandeling met sildenafil verdere differentiatie van pericyten. Desalniettemin, de groep die geen nitrofen had ontvangen maar wel sildenafil, de controle groep, lie teen vernauwing van de alveolaire ruimte zien. Dit suggereert dat behandeling met sildenafil ongwilde bijeffecten kan induceren.

Therapeutische studies naar de ontwikkeling van de behandeling van CHD moet gebruik maken van de kennis die beschikbaar is over onwtikkelings processen en moleculaire mechanismens. Dit proefschrift beschrijft een gedetialleerd inzicht over de ontiwkkeling van de longvaatboom in CHD. Het onthuld een verhoging in de pericyte/endotheel ratio, wat het eerste teken is van de ontwikkeling van pulmonale hypertensie. Verder laten we voor het eerst zien dat gedurende de vroege ontwikkeling van de long (E13), de endotheel populatie in CHD de meeste grote verschillen heeft in gen expressie in CHD. Verminderde expressive van KLF4 in CHD kan de verminderde groei van het capillaire network verklaren wat gerelateerd is met CHD. Dit proefschrift is een eerste stap in het onthullen van de vroege moleculaire en cellulaire mechanismen die ten grond slag liggen aan de pulmonale hypertensive geassocieerd met CHD.

APPENDICES

Curriculum Vitae
PHD Porfolio
List of publications
Dankwoord

Curriculum Vitae

EXPERIENCE	**Postdoctoral Reseacher at Cambridge University** The Gurdon Institute. Research on lung development and lung regeneration. Group of Dr. Emma Rawlins Sep' 17- **PHD CANDIDATE AT ERASMUS MEDICAL CENTER** Department of Biomedical Sciences, Cell biology and pediatric surgery May'12 – Sep'17 Promotor: Prof. Dr. D. Tibboel, Co-promotor: Dr. R.J. Rottier Thesis: 'The cellular origin of CDH and potential translational approaches' **MASTER INTERNSHIP AT HUBRECHT INSTITUTE** Group of Prof. Stefan Schulte-Merker, under daily supervision of Dr. Terhi Karpanen Thesis: 'Identifying novel regulators of lymphangiogenisis in zebra fish' **MASTER INTERNSHIP AT ACADEMICAL MEDICAL CENTRE AMSTERDAM** Group of Dr Coert Zuurbier, under daily supervision of Willeke Brandt Thesis: 'The innate Immune system and ischemia'
EDUCATION	**MASTER OF SCIENCES – Biomedical sciences** University of Amsterdam, 2009 - 2012 **BACHELOR OF SCIENCES – Biomedical sciences** University of Amsterdam, 2005 - 2009 **STUDENT EXCHANGE PROGRAMME – Molecular Biotechnology** Lund University (Sweden), 2007 - 2008 **HIGHER LABORATORY EDUCATION** Hogeschool Rotterdam, 2003 - 2005 **HAVO, profile Nature and Health with History and German** Sint Stanislascollege Delft, 1998 - 2003

PHD Porfolio

PhD PORTFOLIO: Heleen Kool
ERASMUS MC DEPARTMENT: Cell biology and pediatric surgery
RESEARCH SCHOOL: MGC graduate school
PERIOD: May'12 – Sep'17
PROMOTOR: Prof. Dr. D. Tibboel
CO-PROMOTOR: Dr. R.J. Rottier

1. PhD TRAINING			
GENERAL COURSES	**PLACE**	**YEAR**	**ECTS**
Course on animal handling and competence (Article 9)	Rotterdam	2012	3
Adobe Photoshop and Illustrator course	Rotterdam	2013	0,5
Biostatistics	Rotterdam	2015	2
Adobe InDesign	Rotterdam	2017	0,5
SPECIFIC COURSES	**PLACE**	**YEAR**	**ECTS**
Biochemistry and Biophysics	Rotterdam	2012	3
Cell and Developmental Biology	Rotterdam	2012	3
Genetics	Rotterdam	2012	3
Literature course	Rotterdam	2013	2
Microscopic Image Analysis: From Theory to Practice	Rotterdam	2015	2
Basic course in R	Rotterdam	2016	2
SEMINARS, CONFERENCES AND WORKSHOPS	**PLACE**	**YEAR**	**ECTS**
MGC PhD workshop	Luxemburg	2013	1
Congenital diaphragmatic hernia meeting	Rotterdam	2013	2
NRS meeting: Animal models in science	Utrecht	2013	1
Pediatric surgical meeting	Cape Town	2014	2
Congenital diaphragmatic hernia meeting	Toronto	2015	2
PRESENTATIONS	**PLACE**	**YEAR**	
Monday morning work discussions cell biology department	Rotterdam	2012-2017	10
Presentation during NRS meeting	Utrecht	2013	1
Presentation during Congenital diaphragmatic hernia meeting	Rotterdam	2013	2
Grant application Sophia foundation for medical research	Rotterdam	2013	1
Bi-weekly PhD meetings	Rotterdam	2014-2016	4
Presentation during Pediatric surgical meeting	Cape Town	2014	2
Presentation during Congenital diaphragmatic hernia meeting	Toronto	2015	2
Grant application Sophia foundation for medical research	Rotterdam	2015	1
2. TEACHING			
INTERNSHIP SUPERVISOR	**PLACE**	**YEAR**	
Supervision of a Master student	Rotterdam	2013	2
Supervision of a student from practical laboratory school	Rotterdam	2015-2016	3
TOTAL ECTS			**57**

List of publications

H.M. Kool, D.S. Mous, D. Tibboel, A. de Klein, R. Rottier. Pulmonary vascular development goes awry in congenital lung abnormalities. *Birth Defects Research Part C Embryo Today Reviews 2014*

D.S. Mous, **H.M. Kool**, M.J. Buscop van Kempen, A.H. Koning, O. Dzyubachyk, R.M.H. Wijnen, D.Tibboel, R.Rottier. Clinical relevant timing of antenatal sildenafil treatment reverses pulmonary vascular remodeling in congenital diaphragmatic hernia. *AJP Lung Cellular and Molecular Physiology 2016*

J. Kapere Ochieng, K. Schilders, **H.Kool**, A. Boerema-De Munck, M. Buscop-van Kempen, C. Gontan, R. Smits,F. Grosveld, R.M.H. Wijnen, D. Tibboel, R. Rottier. Sox2 Regulates the Emergence of Lung Basal Cells by Directly Activating the Transcription of Trp63

J. Kapere Ochieng, K. Schilders, **H.Kool**, M. Buscop-van Kempen, A. Boerema-De Munck, F. Grosveld, R. Wijnen, D. Tibboel, R.Rottier. Differentiated type II pneumocytes can be reprogrammed by ectopic Sox2 expression. *PLoS One 2014*

E. van Mastrigt, I. Reiss, J. de Jongste, C. Cheng, C. van Dijk, J. Samsom, **H.Kool**, R.Rottier, R. W. Hendriks, M. Pijnenburg, I. de Kleer. The role of monocyte derived growth factors in BPD development. *European Respiratory Journal 2016*

Dankwoord

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