Blood Vessel and Airway Development in Normal and Congenital Diaphragmatic Hernia Lungs

CIP-gegevens Koninklijke Bibliotheek. Den Haag

ISBN 90-6734-056-1

Cover: Male surgeons treating a boy with anorectal malformation and female surgeons treating a girl with vaginal atresia.
Source: an ancient surgical book "Cerrahiye-i Ilhaniye", written by Serafeddin Sabuncuoglu in 1460 (Fatih Millet Library, Istanbul).

Publication of this thesis was supported by:
The Turkish Ministry of Health for a scholarship in Pediatric Surgical Intensive Care, Ankara, Turkey.
The David Vervat Foundation, Rotterdam, The Netherlands.

Printed by: Optima Grafische Communicatie, Rotterdam
Lay-out: Margo Terlouw-Willebrand, Nieuwerkerk aan den IJssel

Blood Vessel and Airway Development in Normal and Congenital Diaphragmatic Hernia Lungs

Bloedvat en luchtwegontwikkeling in normale en congenitale hernia diafragmatica longen

Proefschrift

ter verkrijging van de graad van doctor

aan de Erasmus Universiteit Rotterdam

op gezag van de Rector Magnificus

Prof.dr.ir. J.H. van Bemmel

en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op

donderdag 12 december 2002 om 11.00 uur

door

Münevver Hösgör

geboren te Izmir, Turkije

Promotiecommissie:

Promotoren:	Prof.dr. D. Tibboel Prof.dr. W.J. Mooi
Overige leden:	Prof.dr. C. Blanco Prof.dr. F.W.J. Hazebroek Prof.dr. W. Helbing
Copromotor:	Dr. R.R. de Krijger

Table of contents

Chapter 1

Introduction

Congenital Diaphragmatic Hernia: from a surgical defect to developmental biology of the lung

Based on the article:
Congenital Diaphragmatic Hernia: from a surgical defect to developmental biology of the lung
D. Tibboel, M. Hosgor, J.C. Molenaar
Gaslini 2000;32:306-312

1.1 Introduction

Congenital diaphragmatic hernia (CDH) is classically considered as a primary defect of diaphragmatic embryogenesis that often results in antenatal herniation of fetal abdominal contents into the thoracic cavity. Diaphragmatic hernia was first described in 1575 by Ambroise Pare.[49] The introduction of animal models of CDH, aimed to understand the pathophysiology, opened the possibility to perform studies of various treatment modalities including surfactant replacement,[7,22] nitric oxide therapy,[13,21] high-frequency ventilation,[26] and liquid ventilation with perfluorocarbon,[42] in utero surgical repair,[17,36] and more recently the so called PLUG procedure (plug the lung until it grows).[16,30,31] The fetal lamb model of CDH was created by de Lorimier in 1967,[12] while the nitrofen rat model of CDH was first described by Iritani,[22] in 1984, and brought to attention to pediatric surgeons by Kluth and Tibboel, in 1990.[32] In this review we focus on recent advances in the clinical treatment of congenital diaphragmatic hernia and experimental research relevant to this subject.

1.2 Epidemiology

The reported incidence of CDH ranges from 0.08 to 0.45 per 1000 births.[39] Four population-based studies showed a prevalence of 0.17 to 0.36 per 1000 births.[5,54,64,65] In Torfs' epidemiological study, 95.8% of CDH's were posterolateral hernias (Bochdalek), 84% of these were left-sided, 13% were right-sided and 2% bilateral.[68] The incidence of familial cases of CDH is estimated to be approximately less than or equal to 2% of all forms of CDH.[34] Autosomal recessive, multifactorial, sex-linked or autosomal dominant inheritance modes are proposed, but up till now these modes of inheritance could not explain all the cases of familial CDH.[65] Associated malformations are documented in 27% to 47% of cases of CDH[34] depending on case selection. The distribution of associated anomalies in sporadic cases of CDH is reported as follows: CNS anomalies, 56% to 75%, cardiovascular anomalies, 15% to 25%, genitourinary anomalies, 5% to 10%.[10,61] The incidence of chromosomal abnormalities is reported as 11.6% - 27.2%.[50]

Since the cardiovascular malformations (CVM) are a group of lethal extrapulmonary abnormalities determining survival rate in live born infants with CDH, a pathogenetic link between CDH and CVM has been suggested.[37] Another group of extrapulmonary abnormalities, skeletal malformations, which are occasionally seen in infants with CDH, were also studied in the nitrofen animal model of CDH to identify a common pathway resulting in both types of malformations.[40]

1.3 Pathogenesis

The cause of CDH is still unknown. There are two theories explaining the pathogenesis of CDH.[43] In one theory failure of formation or fusion of the pleuroperitoneal membranes results in a posterolateral diaphragmatic defect. If the pleuroperitoneal canals remain open during the 9th and 10th gestational week, when the midgut returns to the abdominal cavity, the abdominal viscera herniates into the ipsilateral thoracic cavity. According to this theory, in utero compression of the developing lung(s) by herniated viscera impairs pulmonary growth and leads to pulmonary hypoplasia. In fetal lamb model, originally studied by de Lorimier and Harrison,[12,18] compression of the developing lung by an intrathoracic balloon indeed produced lung hypoplasia. In accordance with this, Iritani using the nitrofen treated mice model, described the development of the so-called posthepatic mesenchymal plate (PHMP) in association with growth of the lung bud into the pleuroperitoneal cavity.[24] By Greer and co-workers a detailed analysis is undertaken to understand the role of diaphragmatic development in this respect.[14] Recently, Keijzer postulated the dual-hit hypothesis explaining the pathogenesis of pulmonary hypoplasia in CDH cases by two developmental insults. The first insult occurs early in development, before diaphragm development and affects both lungs during branching morphogenesis in a similar fashion. After defective development of the diaphragm, the second insult affects the ipsilateral lung only at a later stage of development due to interference with fetal breathing movements.[29] Abnormal growth and differentiation of the lungs in infants with CDH have been documented as retarded development of the pulmonary acinus, fewer alveoli in the ipsilateral and the contralateral lung, markedly thickened alveolar walls, increased interstitial tissue, markedly diminished alveolar air space and gas-exchange surface area.[3,25]A recent report of Lipsett et al indicated increasing relative hypoplasia as gestation proceeds and they found that the left upper lobe was affected most in CDH-affected preterm lamb lungs.[35] As an integral part of the pathology in CDH, lungs of CDH patients show a number of pulmonary vasculature abnormalities consisting of a reduced total pulmonary vascular bed, decreased number of vessels per volume unit lung, medial hyperplasia of pulmonary arteries together with peripheral extension of the muscle layer into small arterioles, and arterial adventitial thickening.[4,23] Taira suggested that the structural changes in the adventitial of the pulmonary arteries may be an important factor in the development of persistent pulmonary hypertension (PPHN) in CDH patients.[60] Ting also demonstrated that the lungs in CDH are less vascularized at the alveolar surface due to a reduction in the total alveolar surface area.[63] Evaluation of the expression of α-actin, SMemb (Smooth muscle, embryonic), SM1, SM2 (Smooth muscle actin 1 and 2) and VEGF suggests that the differences in smooth muscle differentiation do not account for the vascular abnormalities in CDH. The increased VEGF expression in small-diameter

and supernumerary pulmonary arteries in CDH cases may reflect an attempt of the developing fetus and the neonate to compensate for the stunted lung vessel growth and/or to stimulate the arterial angiogenesis of the pulmonary pressure-regulating arteries caused by a mechanism which remains to be identified.[45,55] Apart from the morphologic abnormalities in the pulmonary vasculature, an altered expression of various cellular mediators, such as nitric oxide, endothelins, prostaglandins, leukotrienes, catecholamines and the renin-angiotensin system, have been suggested to contribute to the pathogenesis of PPHN in CDH.[44,54,55]

1.4 Prenatal diagnosis

Maternal serum alpha-fetoprotein (MS-AFP) levels and prenatal ultrasonography have been used to diagnose CDH patients.[39] Low levels of MS-AFP have been associated with CDH as well as other conditions such as, trisomy 18 and 21, and as such can not be considered as indicative. A variety of imaging techniques are used for antenatal CDH diagnosis, but because of to its cost and non-invasive nature, ultrasound examination is accepted as the gold standard for antenatal diagnosis of CDH.[15] The value of repeated prenatal MRI to diagnose the amount of pulmonary hypoplasia has to be awaited. In a recent report, Bahlmann suggested that the sonographic prenatal assessment of the lung diameter and the lung diameter/thorax circumference ratio permit a relatively reliable intrauterine diagnosis of severe pulmonary hypoplasia associated with CDH.[1] Doppler ultrasound imaging has also been used to detect lung hypoplasia before birth, an increased pulsatility index has been found as a reliable parameter in lung hypoplasia when cardiac defects are absent.[8,33]

1.5 In utero management of CDH

After the establishment of CDH in the fetal lamb model, studies for unravelling the pathophysiology and in utero correction of CDH were initiated. Recently it has been clear that the best prognostic indicators are presence or absence of liver herniation into the chest (liver "up" or "down") and ultrasonographic measurement of the lung-to-head ratio (LHR).[56] It is now known that fetal tracheal obstruction can correct pulmonary hypoplasia.[2,30,31,48] With ongoing experimental and clinical experience, the tracheal occlusion technique evolved from a polymeric foam to external clip and EXIT procedure (ex utero intrapartum treatment).[56] Recently, a less invasive video-fetoscopic technique (FETENDO) which allows the dissection of the fetal trachea and placement of an

occluding clip without opening the uterus, was developed. Harrison reported a postnatal survival rate of 75% in eight CDH patients with this treatment[16] Fetal TL (tracheal ligation) in CDH results in tremendous lung growth, reversal of pulmonary hypoplasia but decreased total alveolar phospholipid content, decreased type II cellular function and surfactant synthesis.[2,31] The effects of temporary endoscopic fetal tracheal occlusion in humans is under evaluation in so called "liver up" patients. In the nitrofen treated model, Kitano performed tracheal ligation in a nitrofen treated model and concluded that tracheal ligation also accelerates lung growth and increases lung parenchyma.[30] The hemodynamic changes of the vascular tree after tracheal ligation in CDH lungs were reported as reversion of the high impedance to pulmonary blood flow.[59] The mechanism of the growth acceleration after TL is possibly explained by the increased intratracheal pressure provided by the accumulation of lung fluid and secondary airway distension.[31] Induction of four differentially expressed genes, hepatoma-derived growth factor (HDGF), ribosomal protein S24, stathmin and parathyroid hormone (PTH) are also shown to be related with increased intratracheal pressure in organotypic tissue cultures of nitrofen induced CDH in mice.[9] Antenatal glucocorticoid (CS) treatment in premature babies with hyalin membrane disease (HMD) has proved to accelerate pulmonary maturity. Because of the immaturity of the lungs in CDH, Suen et al used antenatal glucocorticoid in the nitrofen rat model and showed that antenatal CS increased lung maturity, surfactant content, reduced the saccular septal thickness and lung glycogen concentration.[27,58] These results were confirmed in the fetal lamb model.[20,46,53] Amongst the various factors involved angiotensin converting enzyme (ACE) activity, which is produced by the vascular endothelium, is implicated in the pathogenesis of pulmonary hypertension. Antenatal CS, by suppressing pulmonary ACE activity is suggested to reduce the risk of PPHN in CDH patients.[6,47,63] By combining the different approaches the effects of antenatal intra-amniotic surfactant or dexamethasone administration on lung development were compared with the changes induced by tracheal ligation alone. While lung weight to body weight ratios were increased by TL, no difference was found with the other methods.[61] Intra-amniotic surfactant or CS administration improved lung compliance and distensibility with the increment of collagen concentration and lung maturity. In humans abnormalities in vitamin A metabolism were suggested to inhibit the in situ differentiation of some cells of the post-hepatic mesenchymal plate into myeloblasts, which are necessary to form the muscular part of the diaphragm.[38]

In the nitrofen model the antenatal administration of vitamin A resulted in significantly increased survival and lung growth with a decreased incidence of CDH.[62] These observations suggest that other factors are also important for antenatal lung development.

1.6 Postnatal therapy and outcome

Until 1980's, it was believed that reduction of the herniated viscera and closure of the diaphragmatic defect should be performed as an emergency procedure after birth to improve hypoxia and hypercarbia. Delayed surgery, with preoperative stabilisation, has now become the widely accepted approach to the management of CDH; however, this approach has had no significant impact on mortality.[19,66] High-frequency oscillatory ventilation (HFOV) provides an additional tool to support neonatal respiratory failure, Reyes reported recently a survival rate of 81% with the management of CDH by delayed surgical repair, early postnatal HFOV, and selective referral for ECMO.[51] Kamata, in contrast, indicated that prolonged preoperative stabilisation using HFOV does not improve the outcome in neonates with CDH.[26] Another mode of ventilatory support, partial liquid ventilation (PLV), uses gas ventilation with a mechanical ventilator at conventional settings with the lungs filled with a volume of perfluorocarbon equal to functional residual capacity, has been demonstrated to result in excellent gas exchange and improvement of lung compliance. Nobuhara reported the postnatal distension with perfluorocarbon is capable of accelerating alveolar proliferation in an experimental study.[42] Extracorporeal membrane oxygenation (ECMO) is widely accepted as an ultimate method for treatment of infants who have CDH and respiratory failure refractory to conventional and high-frequency modes of ventilatory support and are non responders to inhaled NO.[38] In a number of patients with CDH deterioration is due to a reactive pulmonary vascular bed with abnormally muscularized vessels and right to left shunting. As a consequence, it is expected that these changes might regress with ECMO support. Recently Shehata reported that adventitial thickening is partially reversed by ECMO treatment, however medial hyperplasia remained unchanged.[54] A clinical trial of surfactant, given prophylactically before the first breath in infants with a prenatal diagnosis of CDH, is currently in progress.[7] IJsselstijn et al indicated a primary surfactant deficiency is unlikely in infants with CDH.[29] Routine administration of exogenous surfactant in human patients was not supported by the experimental results of Scheffers.[52] Inhaled nitric oxide (INO) is a selective pulmonary vasodilator, which proved to be an effective therapy for the hypoxic term neonate to reduce the occurrence of death and the need for ECMO.[10,13,21] Infants with CDH have an unpredictable response to early INO therapy,[13] probably due to the involvement of the endothelin system in the pathogenesis of pulmonary hypertension in CDH.[64] Although many innovative studies, reviewed in this paper, have provided valuable data for our knowledge of the pathogenesis and natural history of congenital diaphragmatic hernia, the management of the individual patient is often "trial and error". Today more and more clinicians are aware that different ventilatory regimen have very deleterious effects on the hypoplastic lung. The concept of gentle

handling of the lung has indeed resulted in improved survival rates.[28,70] We still have not found the magic bullet for the high risk patient with CDH standardised treatment protocols eventually following prenatal diagnosis, will make randomised clinical trials with large series possible to improve a better insight in this problem and for all improve survival rate and hopefully morbidity.

1.7 Summary

Against the background of the changing concepts related to pulmonary development the pathogenetic aspects of congenital diaphragmatic hernia (CDH) are reviewed. Besides the idea that CDH is often part of a syndrome and major associated anomalies are present, the concepts of prenatal evaluation of the amount of pulmonary hypoplasia and the implications for surgical or hormonal intervention are discussed.

The further treatment of the patient with CDH is guided by increasing knowledge of the regulation of pulmonary vascular tone. Nitric oxide has shown to result in unpredictable vasodilation in groups of patients with CDH, while more invasive strategies as extracorporeal membrane oxygenation has turned out not to be the magic bullet. Based on pathology reports of serious parenchymal damage of the lung following agressive treatment modalities has resulted in enhanced survival rates in recent reports using the concept of "gentle air". For the high-risk patient with CDH standardised treatment protocols eventually following prenatal diagnosis, will make randomised clinical trials with large series possible to improve a better insight in this problem and for all improve survival rate and hopefully morbidity.

1.8 Aim of the studies

The phenotypic expression of congenital diaphragmatic hernia results in major difficulties to standardize the treatment and the order of treatment modalities in the individual patient. As a result even today the treatment of patients with congenital diaphragmatic hernia is "trial and error" based on general assumptions with regards to the amount of pulmonary hypoplasia, the unpredictability of the pulmonary vascular response as well as the vulnerability of the lung parenchyma on different forms of ventilatory support.
As a consequence numerous authors have tried to modulate pulmonary vascular growth by either surgical or chemical means in an attempt to have the fetus born with more mature lungs. The variety of animal models as well as different approaches such as the

antenatal use of corticosteroids, tracheal obstruction of variable length and definitive "repair" of the diaphragmatic defect have been practiced.

Besides the pulmonary hypoplasia the response of the pulmonary vasculature on stress in the postnatal period as well as the unpredictability of the result of attempts to modulate pulmonary vascular tone has resulted in a variety of treatment modalities for pulmonary hypertension.
In contrast to the suggested decreased nitric oxide synthase level in rodent and sheep models of CDH, the use of inhaled nitric oxide has not resulted in a significant improvement in survival rate.
Although the morphology of the pulmonary vasculature has been documented for many decades consisting of medial hypoplasia, adventitional thickening and peripheral extension of the muscular coat, still the question is unanswered whether the altered morphology of the pulmonary vascular bed is the result of a developmental arrest or a disease specific event. Up till now no data are available in humans to reach a definitive answer.

- As a consequence the specific aims of the studies described in this thesis are:
- To evaluate the contribution of vasculogenesis in the process of development of the pulmonary vasculature (chapter 2).
- To describe the ontogeny of the Von Hippel Lindau pathway (VHL) in normal human lungs (chapter 3) and to compare these findings with observations in age-matched human CDH cases (chapter 4).
- To evaluate the expression pattern of the epithelial differentiation marker thyroid transcription factor 1 (TTF-1) in human CDH cases and age-matched controls (chapter 4).
- To evaluate the evidence for the use of a variety of proposed ways to modulate prenatal lung growth both in animals as well as in humans (chapter 5).
- To identify the current state of art with regards to evidence based knowledge in lung development and pre- and postnatal treatment of congenital diaphragmatic hernia patients (chapter 6).

1.9 References

1. Bahlmann F, Merz E, Hallermann C et al. Congenital diaphragmatic hernia: Measurement of fetal lungs to predict pulmonary hypoplasia. Ultrasound Obstet Gynecol 1999;14:162-168.
2. Bambini DA, Irish MS, Kapur P et al. Tracheal ligation in CDH: bigger may not be better. Pediatr Surg Int 1998;13:537-538.
3. Beals DA, Schloo BL, Vacanti JP et al. Pulmonary growth and remodeling in infants with high-risk congenital diaphragmatic hernia. J Pediatr Surg 1992;27:997-1002.
4. Belik J, Keeley FW, Baldwin F et al. Pulmonary hypertension and vascular remodeling in fetal sheep. Am J Physiol; 1998;266:H2303-H2309.
5. Benjamin DR, Juul S, Siebert JR. Congenital posterolateral diaphragmatic hernia: associated malformations. J Pediatr Surg 1988;23:899-903.
6. Bos AP, Sluiter W, Tenbrick R et al. Angiotensin-converting enzyme activity is increased in lungs of rats with pulmonary hypoplasia and congenital diaphragmatic hernia. Exp Lung Res 1995;21:41-50.
7. Bos AP, Tibboel D, Hazebroek FWJ et al. Surfactant replacement therapy in high-risk congenital diaphragmatic hernia. Lancet 1991;338:1279.
8. Chaoui R, Kalche K, Tennstedt C et al. Pulmonary arterial Doppler velocimetry in fetuses with lung hypoplasia. Eur J Obstet Gynecol 1999;84:179-185.
9. Cilley RE, Zgleszewski SE,Chinoy RM. Fetal lung development: airway pressure enhances the expression of developmental genes. J Pediatr Surg 2000;35:113-119.
10. Clark RH, Kueser TJ, Marshall W, et al. Low-dose nitric oxide therapy for persistent pulmonary hypertension of the newborn. N Engl J Med 2000;342:469-474.
11. Cunniff C, Jones KL, Jones M. Patterns of malformation in children with congenital diaphragmatic defects. J Pediatr 1990;116:258-261.
12. De Lorimier AA, Tierney DF, Parker H. Hypoplastic lungs in fetal lambs with surgically produced congenital diaphragmatic hernia. Surgery 1967;62:12-17.
13. Finer NN, Barrington KJ. Nitric oxide therapy for the newborn infant. Semin Perinat 2000;24:59-65.
14. Greer JJ, Allan DW, Babiuk RP, Lemke RP. Recent advances in understanding the pathogenesis of nitrofen-induced congenital diaphragmatic hernia. Ped Pulmonol 2000;29:394-399.
15. Halamek LP, El-Sayed YY. Congenital diaphragmatic hernia: the perinatalogist's perspective. Pediatr Rev 1999;20:e67-70.
16. Harrison RM, Mychaliska GB, Albanese CT et al. Correction of congenital Diaphragmatic hernia in utero IX: fetuses with poor prognosis (liver herniation and low lung-to-head ratio can be saved by fetoscopic temporary tracheal occlusion. J Pediatr Surg 1998;33:1017-1023.
17. Harrison MR, Adzick NS, Longaker MT et al. Succesful repair in utero of a fetal diaphragmatic hernia after removal of herniated viscera from the left thorax. N Engl J Med 1990;322:1582-1584.

18. Harrison MR, Jester JA, Ross Na. Correction of congenital diaphragmatic hernia I. The model: Intrathoracic balloon produces fatal pulmonary hypoplasia. Surgery 1980;88:174-182.
19. Hazebroek FW, Tibboel D, Bos AP et al. Congenital diaphragmatic hernia: impact of preoperative stabilization. A prospective pilot study in 13 patients. J Pediatr Surg 1988;23:1139-1146.
20. Hedrick HL, Kaban JM, Pacheco BA et al. Prenatal glococorticoids improve pulmonary morphometrics in fetal sheep with congenital diaphragmatic hernia. J Pediatr Surg 1997;32:217-222.
21. Henneberg SW, Jepsen S, Andersen PK et al. Inhalation of nitric oxide as a treatment of pulmonary hypertension in congenital diaphragmatic hernia. J Pediatr Surg 1995;30:853-855.
22. IJsselstein H, Zimmermann LJI, Bunt JEH et al. Prospective evaluation of surfactant composition in bronchoalveolar lavage fluid of infants with congenital diaphragmatic hernia and of age-matched controls. Crit Care Med 1998;26:573-580.
23. IJsselstein H, Tibboel D. The lungs in congenital diaphragmatic hernia: do we understand? Pediatr Pulmonol 1998;26:204-218.
24. Iritani I. Experimental study on embryogenesis of congenital diaphragmatic hernia Anat Embryol 1984;169:133-139.
25. Jesudason EC, Connell MG, Fernig DG et al. Early lung malformations in congenital diaphragmatic hernia. J Pediatr Surg 2000;35:124-128.
26. Kamata S, Usiu N, Ishikawa S et al. Prolonged preoperative stabilization using high-frequency oscillatory ventilation does not improve the outcome in /neonates with congenital diaphragmatic hernia. Pediatr Surg Int 1998;13:542-546.
27. Kapur P, Holm BA, Irish MS et al. Lung physiological and metabolic changes in lambs with congenital diaphragmatic hernia after administration of prenatal maternal corticosteroids. J Pediatr Surg 1999;34:354-356.
28. Kays W, Langham MR, Ledbetter DJ, et al. Detrimental effects of standard medcial therapy in congenital diaphragmatic hernia. Ann Surg 1999;230:340-351.
29. Keijzer R, Liu J, Deimling J et al. Dual-hit hypothesis explains pulmonary hypoplasia in the nitrofen model of congenital diaphragmatic hernia. Am J pathol 2000;156:1299-1306.
30. Kitano Y, Yang EY, Allmen D et al. Tracheal occlusion in the fetal rat: a new experimental model for the study of accelerated lung growth. J Pediatr Surg 1998;33:1741-1744.
31. Kitano Y, Flake AW, Quinn MT et al. Lung growth induced by tracheal occlusion in the sheep is augmented by airway pressurization. J Pediatr Surg 2000;35:216-222.
32. Kluth D, Kangah R, Reich P et al. Nitrofen-induced diphragmatic hernias in rats: an animal model. J Pediatr Surg 1990;25:850-854.
33. Laudy JA, Gaillard JL, Anker JN et al. Doppler ultrasound imaging: a new technique to detect lung hypoplasia before birth? Ultrasound Obstet Gynecol 1996;7:189-192.

34. Langham MR, Kays DW, Ledbetter DJ et al. Congenital diaphragmatic hernia. Epidemiology and outcome. Clin Perinatol 1996;23:671-688.
35. Lipsett J, Cool JC, Runciman SI et al. Morphometric analysis of pretem fetal pulmonary development in the sheep model of congenital diaphragmatic hernia. Pediatr Dev Pathol 2000;3:17-28.
36. Losty PD. Recent advances. Pediatric surgery. BMJ 1999;318:1668-1672.
37. Losty PD, Connell MG, Freese R et al. Cardiovascular malformations in experimental congenital diaphragmatic hernia. J Pediatr Surg 1999;34:1203-1207.
38. Major D, Cadenas M, Fournier L et al. Retinol status of newborn infants with congenital diaphragmatic hernia. Pediatr Surg Int 1998;13:547-549.
39. Meurs KV, Short BL. Congenital diaphragmatic hernia: the neonatologist's perspective. Pediatr Rev 1999;20:e79-87.
40. Migliazza L, Xia H, Diez-Pardo J et al. Skeletal malformations associated with congenital diaphragmatic hernia: experimental and human studies. J Pediatr Surg 1999;34:1624-1629.
41. Nagaya M, Kato J, Niimi N et al. Analysis of patients with congenital diaphragmatic henia requiring pre-operative extracorporeal membrane oxygenation (ECMO). Pediatr Surg Int 1998;14:25-29.
42. Nobuhara KK, Ferretti ML, Siddiqui AM et al. Long-term effect of perfluorocarbon distension on the lung. J Pediatr Surg 1998;33:1024-1029.
43. Nobuhara KK, Wilson JM. Pathophysiology of congenital diaphragmatic hernia. Semin Pediatr Surg 1996;5:234-242.
44. Okazaki T, Sharma HS, McCune SK et al. Pulmonary vascular balance in congenital diaphragmatic hernia: enhanced endothelin-1 gene expression as a possible cause of pulmonary vasoconstriction. J Pediatr Surg 1998;33:81-84.
45. Okazaki T, Sharma HS, Aikawa M et al. Pulmonary expression of vascular endothelial growth factor and myosin isoforms in rats with congenital diaphragmatic hernia. J Pediatr Surg 1997;32:391-394.
46. Okoye BO, Losty PD, Lloyd DA et al. Effect of prenatal glucocorticoids on pulmonary vascular muscularization in nitrofen-induced congenital diaphragmatic hernia. J Pediatr Surg 1998;33:76-80.
47. Okoye BO, Losty PD, Fisher MJ et al. Antenatal glucocorticoid therapy suppresses angiotensin-converting enzyme activity in rats with nitrofen-induced congenital diaphragmatic hernia. J Pediatr Surg 1998;33:286-291.
48. Papadakis K, De Paepe ME, Tackett LD et al. Temporary tracheal occlusion causes catch-up lung maturation in a fetal model of diaphragmatic hernia. J Pediatr Surg 1998;33:1030-1037.
49. Pare A. Les oeuvres. Buon, Paris. 1575.
50. Philip N, Gambarelli D, Guys JM et al. Epidemiological study of congenital diaphragmatic defects with special reference to aetiology. Eur J Pediatr 1991;150:726-729.

51. Reyes C, Chang LK, Waffarn F et al. Delayed repair of congenital diaphragmatic hernia with early high-frequency oscillary ventilation during preoperative stabilization. J Pediatr Surg 1998;33:1010-1016.
52. Scheffers EC, Ijsselstijn H, Tenbrick R et al. Evaluation of lung function changes before and after surfactant application during artificial ventilation in newborn rats with congenital diaphragmatic hernia. J Pediatr Surg 1994;29:820-824.
53. Schnitzer JJ, Hedrick HL, Pacheco BA et al. Prenatal glucocorticoid therapy reverses pulmonary immaturity in congenital diaphragmatic hernia in fetal sheep. Ann Surg 1996;224:430-439.
54. Shehata SM, Sharma HS, van der Staak FH et al. Remodeling of pulmonary arteries in human congenital diaphragmatic hernia with or without extracorporeal membrane oxygenation. J Pediatr Surg 2000;35:208-215.
55. Shehata SM, Mooi WJ, Okazaki T et al. Enhanced expression of vascular endothelial growth factor in lungs of newborn infants with congenital diaphragmatic hernia and pulmonary hypertension. Thorax 1999;54:427-431.
56. Skarsgard ED, Harrison MR. Congenital diaphragmatic hernia: the surgeon's perspective. Pediatr Rev 1999;20:e71-78.
57. Steinhorn RH, Kriesmer PJ, Green TP et al. Congenital diaphragmatic hernia in Minnesota. Impact of antenatal diagnosis on survival. Arch Pediatr Adolesc Med 1994;148:626-631.
58. Suen HC, Bloch KD, Donahoe PK. Antenatal glucocorticoid corrects pulmonary immaturity in experimentally induced congenital diaphragmatic hernia in rats. Pediatr Res 1994;35:523-529.
59. Sylvester KG, Rasanen J, Kitano Y et al. Tracheal occlusion reverses the high impedance to flow in the fetal pulmonary circulation and it normalizes its physiological response to oxygen at full term. J Pediatr Surg 1998;33:1071-1075.
60. Taira Y, Miyazaki E, Ohshiro K et al. Administration of antenatal glucocorticoids prevents pulmonary artery structural changes in nitrofen-induced congenital diaphragmatic hernia in rats. J Pediatr Surg 1998;33:1052-1056.
61. Tannuri U, Rodrigeus CJ, Maksoud-Filho JG et al. The effects of prenatal intraamniotic surfactant or dexamethasone administration on lung development are comparable to changes induced by tracheal ligation in an animal model of congenital diaphragmatic hernia: studies of lung glycogen content, elastic fiber density, and collagen content. J Pediatr Surg 1998;33:1776-1783.
62. Thebaud B, Tibboel D, rambaud C et al. Vitamin A decreases the incidence and severity of nitrofen-induced congenital diaphragmatic hernia in rats. Am J Physiol 1999;277:L423-429.
63. Thebaud B, Mercier JC, Dinh-Xuan At. Congenital diaphragmatic hernia. A cause of persistent pulmonary hypertension of the newborn which lacks an effective therapy. Biol Neonate 1998;74:323-336.

64. Thebaud B, Lagausie de, P, Forgues D, Aigrain Y, Mercier J-C, Dinh-Xuan AT. Eta-receptor blockage and Etb-receptor stimulation in experimental congenital diaphragmatic hernia. Am J Physiol Lung Cell Mol Physiol 2000;278:923-932.
65. Tibboel D, Gaag AV. Etiologic and genetic factors in congenital diaphragmatic hernia. Clin Perinatol 1996;23:689-699.
66. Tibboel D, Bos AP, Pattenier JW et al. Pre-operative stabilization with delayed repair in congenital diaphragmatic hernia. Z Kinderchir 1989;44:139-143.
67. Ting A, Glick PL, Wilcox DT et al. Alveolar vascularization of the lung in a lamb model of congenital diaphragmatic hernia. Am J Respir Crit Care Med 1998;157:31-34.
68. Torfs CD, Curry CJR, Bateson TF et al. A population-based study of congenital diaphragmatic hernia. Teratology 1992;46:355.
69. Wenstrom KD, Weiner CP, Hanson JW. A five-year statewide experience with congenital diaphragmatic hernia. Am J Obstet Gynecol 1991;165:838-842.
70. Wung JT, Sahni R, Moffitt ST, et al. Congenital diaphragmatic hernia: survival treated with very delayed surgery, spontaneous respiration and no chest tube. J Pediatr Surg 1995;30(3):406-409.

Chapter 2

Development of the pulmonary vasculature in the mouse embryo limited role of vasculogenesis?

Based on the article:
Development of the pulmonary vasculature in the mouse embryo limited role of vasculogenesis?
Munevver Hosgor, Fons J. Verbeek, P.J. Boon, J. Korving, Carel Meijers, Dick Tibboel, Wolter J. Mooi
Submitted for publication

2.1 Abstract

The development of the pulmonary vascular bed is thought to result from two distinct processes: angiogenesis and vasculogenesis. Angiogenesis consists of vascular sprouting from existing vessels; vasculogenesis has been defined as de novo formation of blood vessels from free angioblasts, which are precursors of endothelium that emerge in the primitive mesenchyme.
We studied the lungs of mice from days E10- E19, and provide data which suggest a more extensive contribution of angiogenesis in the developing murine lung than has hitherto been assumed. On the basis of 3D reconstructions of serial sections, continuity of vessels reaching from the lung hilum to the lung periphery were detected at E10 and E19. Furthermore, the blood cells found the lumina of all vessels were of the type found in the chambers of the heart and in the large vessels at that gestational age, indicating absence of local haematopoiesis in pulmonary vascular lakes, and a presence of connections to central vessels with circulating blood cells.

2.2 Introduction

The pulmonary vascular system is one of two tubular systems that arise within the lung: the epithelial system is known to develop exclusively by sprouting and branching from larger, centrally located epithelium. In contrast, the second tubular system, the vasculature, is thought to result from two distinct processes: vasculogenesis and angiogenesis (Poole & Coffin, 1989; Gale & Yancopoulos, 1999). Angiogenesis consists of "vascular sprouting from existing vessels" (Coffin et al., 1991), in a fashion similar to the development of the airways. In contrast, vasculogenesis is defined as "de novo formation of blood vessels from free angioblasts" (Coffin et al., 1991), which are primitive precursors of endothelium that emerge in the primitive mesenchyme. Both vasculogenesis and angiogenesis have been reported to contribute to the developing pulmonary vasculature, in the mouse (deMello et al., 1997; Akeson et al., 2000; Schachtner et al., 2000) as well as in man (deMello & Reid, 2000).

According to published descriptions of the morphology of the vasculogenetic process, its first recognizable stage consists of "a ring of flattened cells, which will differentiate into endothelial cells and, within the center, cells that will differentiate to hematopoietic cells" (Akeson et al., 2000). Connections to larger vessels are absent at this stage. Ultimately, however, these "vascular lakes" connect to larger vessels, formed by way of angiogenesis, thus resulting in the establishment of a continuous vascular network. In the mouse, the

establishment of these connections between proximal and distal vessels has been reported to start at day E13 or day E14 of embryonal development (deMello et al., 1997; Akeson et al., 2000).
Our morphological observations of embryonic human and murine lungs have led us to question whether vasculogenesis, as outlined above, occurs to the extent that is generally thought. Therefore, we conducted a detailed and systematic histological study, including 3D reconstructions, of serially sectioned murine lungs at 10-19 days of gestation (days E10-19), i.e., from the day that the first recognizable pulmonary vascular structures have been described (Akeson et al., 2000), to the end of the intrauterine period.

2.3 Materials and methods

Pregnant FVB mice were killed by cervical dislocation and foetuses were gently removed from the uterus in a bath of phosphate-buffered saline (PBS), using a dissection microscope. Foetuses between 10 to 13 days of gestational age (E 10 -13) were fixed in toto by overnight immersion in isotonic phosphate-buffered formalin at room temperature; foetuses between 14 to 19 days gestational age (E 14 -19) were carefully dissected under a dissection microscope, avoiding as much as possible any tissue trauma. The heart and lungs were removed en bloc and fixed by immersion. All foetuses were staged according to the age determination system of Goedbloed and Smits-van Prooije (1986). After fixation, specimens were dehydrated in graded alcohols and embedded in paraffin in axial and transverse orientations. Serial 5 mm thin sections were stained with Haematoxylin and Eosin (H&E), and a three-dimensional (3D) reconstruction of the microanatomy of the vascular and airway systems was made, using a computer-assisted reconstruction program, as reported previously (Verbeek et al., 1995; 1999). Briefly, from every section that contained a part of the lung lobe of interest, an image was taken with a CCD camera (JAI M10RS, JAI-Inc, Denmark) attached to a research microscope, equipped with a computer-controlled stage and camera mount, so that at the acquisition phase, the subsequent images could be registered by moving the stage and/or camera mount (Verbeek, 1999, Verbeek, et al., 1999). The user control of the registration process was accomplished by overlaying the subsequent images. If necessary, a post registration was applied on the image sequence. The image sequence of the serial sections was processed to improve contrast and sharpness and used in the second part of the reconstruction process, i.e. the determination of the parts of interest (e.g. lung lobe area, blood vessels, and airways). These annotations were done manually, by drawing the contours on the images displayed on a computer screen, using a digitizer tablet or a LCD digitizer (Verbeek, 1999). The contours were directly rendered as an overlay in the image.

Intermediate results could be visualized as a contour stack, and for the final visualization, volume rendering was applied. To this end the contour stack was converted to a labeled volume model (Verbeek et al., 1995, Verbeek & Huijsmans, 1998). To that end, all data are stored in a graphical database. The image acquisition and the 3D-annotation program is windows based software which was developed at the Hubrecht Laboratory in Utrecht the Netherlands. (www.imaging.niob.knaw.nl). The 3D annotation software (TDR-3Dbase) used runs on a PC without the need for additional hardware.

The reconstruction and visualizations of the lung lobes of the 10 ED embryo were based on 106 sections, in which airways and blood vessels were determined, as well as the oesophagus and aorta; 831 contours were identified. The reconstruction and visualizations of the left lung lobe of the 13 ED embryo were based on 177 sections, in which 4 different structures were determined. In total, 3349 contours were used in the reconstruction.

2.4 Results

Serial sections of embryonal lungs were investigated histologically, with special attention to the shape of intercellular spaces within the primitive mesenchyme, continuity and contour of cells bordering such spaces, and the content of the spaces, in order to distinguish between "vascular lakes" representing an early phase of vasculogenesis, and tissue shrinkage artefacts.

The mesenchyme varied in cellularity according to gestational age and micro-anatomical site: areas in close proximity to developing airways tended to have a slightly more compact architecture (Fig. 1A, on page 91), but there were slight variations, possibly also relating to slight variations in degree of cellular shrinkage during tissue processing. The intercellular spaces varied accordingly; primitive mesenchymal cells often showed irregular extensions traversing irregular, slit-like intercellular spaces. Indeed, there was no positive morphological evidence that these exceedingly numerous, irregular small spaces were in any way related to the emergence of the vascular bed. In addition to these very numerous irregular spaces, there were small numbers of more smoothly contoured empty spaces, which were surrounded by one or several mesenchymal cells forming a distinct - albeit often discontinuous - lining. These spaces quickly increased in number during the E10 - E19 time window of this study, but were already present, and readily identifiable, at E10. The shapes and lining of these latter spaces was similar to those of larger, obviously vascular spaces near the lung hilum, which often contained foetal erythrocytes (Fig. 1B, on page 91), which were also numerous in the lumen of the aorta and the chambers of the

developing heart. Efforts to identify endothelial cell lineage by means of immunohistochemistry, using a panel of markers including Factor VIIIrAg, Cd34 and CD31 were not successful in these small presumably vascular structures (results not shown).
As indicated above, these small but smooth-contoured spaces often contained erythrocytes, which were recognized by the abundant pale bright pink cytoplasm and a central round dark nucleus. Despite strenuous and extensive efforts, we failed to identify any earlier elements of erythropoiesis or of any other haematopoietic lineage within or adjacent to these small smooth-contoured spaces. This was in contrast to the abundant haematopoiesis seen in the liver of the same embryos, where haematopoietic cells of all developmental stages were numerous (Fig. 1C, on page 91). Thus, there was no evidence that the erythrocytes found within the small smooth-contoured spaces of the developing lung had emerged there in situ, since we did not detect any precursor cells at that site.

In the lung, small numbers of erythrocytes were occasionally encountered outside smooth-contoured spaces, which occasionally exhibited discontinuities of their lining, as indicated above. This observation was consistent with extravasation of circulating erythrocytes within the primitive pulmonary mesenchyme, occurring either in vivo or after death as a result of minor trauma caused by tissue handling prior to fixation.

3D reconstruction of vascular tree
Lungs of one 10 ED and one 13 ED embryo were serially sectioned in their entirety and a reconstruction was performed. Although the technical limitations of the procedure precluded an unequivocal assessment of the continuity or discontinuity of very small and very peripheral vessels, the comprehensive identification and 3D positioning of the vascular profiles showed that at both gestational ages investigated, some vessels could be traced from the lung hilar area to the distant periphery of the developing lung (Fig. 1D, on page 91).

2.5 Discussion

As outlined in the introduction, the first recognizable stage of vasculogenesis has been said to consist of "a ring of flattened cells, which will differentiate into endothelial cells and, within the center, cells that will differentiate to hematopoietic cells" (Akeson et al., 2000) (our italics). Such a definition carries the problem that it is based on differentiation characteristics that the cells are expected to display at a later stage, rather than on parameters that can be evaluated unequivocally at the time of observation.

In the very early literature based on observations of chick embryos, bidirectional (endothelial and haematopoietic) differentiation of precursor cells has been postulated to occur (Sabin, 1920; Murray, 1932). Partial support for this idea was obtained later: for instance, so-called embryoid bodies, derived from embryonic stem cells, contain cells which are able to differentiate in endothelial and haematopoietic lineages, under certain conditions in vitro (Choi et al., 1998). Circulating cells of haematopoietic origin have been shown to be able, under certain circumstances, to transdifferentiate into endothelium and thus contribute to angiogenetic sprouting in the adult human (Asahara et al., 1997). This has been shown to occur in granulation tissue and even in normal tissue (Crosby et al., 2000). However, in a quail-chick intracoelomic graft model, haematopoietic cells within the developing lung were shown to be (quail) bone marrow-derived rather than derived from the (chick) primitive lung itself (Pardanaud et al., 1989). Nonetheless,

The morhological essence of the concept of vasculogenesis states that there is an early phase where connections between these early vessel precursors, so-called blood lakes, and the larger vessels have not yet been established. Accordingly, the positive morphological basis of its identification needs to consist of two parts:

1. the space identified is truly a vessel precursor rather than an intercellular space without any relationship to a developing blood vessel, and
2. the space is not connected to larger vessels (if it is, it could have arisen by way of angiogenesis).

Since our own observations point to the existence of a vascular network reaching the lung periphery already at E10, i.e., at a far earlier developmental stage than was reported by previous investigators, we have critically reexamined the published data, from which the presence of pulmonary vasculogenesis has been postulated, with these two questions in mind.

A detailed and elegant study of pulmonary vascular development in lungs of Swiss-Weber mouse fetuses between 9 and 20 days of gestation, by deMello and colleagues (1997), was based on a variety of techniques, including light microscopy, transmission and scanning electron microscopy of lungs, specimen angiography and vascular cast analysis. From their observations, these workers concluded that both angiogenesis and vasculogenesis contribute to the development of the pulmonary vasculature, and that communications between the two (angiogenetic and vaculogenetic) systems occur from day E 13 onwards and are brought about by endothelial cell lysis. In their study, a few intercellular spaces devoid of hematopoietic cells were identified at 9 days gestational age. Ultrastructurally, the spaces appeared to result from discharge of intracytoplasmic vesicles, although we feel that this is not demonstrated conclusively in their manuscript. More importantly,

there was no positive and unequivocal proof that the early spaces illustrated relate to the formation of blood vessels. At 10 days, the spaces were more numerous and now were said to resemble lakes; the description and illustrations of these lakes correspond to the vascular profiles we observed at this gestational age, and which we used for our 3D reconstruction, where they were generally found to be part of continuous vessels extending from the lung hilum to the periphery. These vascular lakes were said to contain haematopoietic cells; our interpretation of these latter cells will be discussed below. Connections to the central vessels were first identified in methyl methacrylate casts at day 13-14. Since these casts could only be made from day 12 onwards, their absence was only reported from casts taken from E12 foetuses. We feel that although casts are useful to demonstrate vascular connections, they are of little or no value in excluding such connections, since, as has been our own experience in previous studies employing the methyl methacrylate cast technique, apparently blind-ending branches can be produced by incomplete penetration of the resin into the microvasculature. The procedure, where the resin compound is injected into the right ventricle and injection is stopped when the compound oozed from the umbilical cord, allows one to conclude that the main blood vessels have been filled with resin, but it does not provide proof that this also applies to all small and possibly blind-ending vascular branches. In view of the delicacy of the immature lung tissue, very careful resin injection is required, which increases the chance of incomplete filling of the vessels. There can be no doubt that 3D reconstructions of histological sections are intrinsically superior to casts in the detection of early vascular continuity.

In addition to the systematic study of murine lungs, deMello and colleagues (2000) reported similar histologic findings in human embryonal and fetal lungs, where vascular lakes of haematopoietic cells were recorded from stage 4+ weeks of gestational age. These blood lakes were similar to those described in the mouse. They were identified using serial sections of the Carnegie collection of human embryos; for obvious reasons, no immunohistochemical evaluations using endothelial markers could be done on this unique archival material.

We have been unsuccessful in our attempts to highlight very early murine endothelial cells by immunohistochemistry, using a number of markers including Farctor VIIIrAg, CD34 and CD31, so that histological morphology had to be the mainstay of our investigation. This is in accordance with Colen et al (1999), who, using Factor VIIIrAg immunohistochemistry, found blood vessels begin in the murine lung only from day 12.5 onwards. Cd34 expression has been documented in developing murine lung (Wood et al., 1997) Since CD34 decorates endothelium as well as some haematopoietic cells but also

various additional, nonrelated cell types such as some fibroblasts and the intestinal cells of Cajal, it cannot be taken as a cell lineage specific marker. Transgenic techniques provide additional possibilities to detect early commitment to endothelial lineage: Schachtner et al. (2000) used mice heterozygous for targeted insertion of the LacZ reporter gene into the flk (VEGF-R2) locus, which is activated very early in developing endothelial cells. Using this sensitive endothelial marker technique, these authors found that continuity between the proximal pulmonary artery and vessels forming in the distal mesenchyme was present even at day E10.5, which was the earliest stage evaluated in that study. Lack of continuity between endothelial cells of the aortic sac and the endothelial cells of the distal lung mesenchyme was not observed at any stage. This continuity is in accordance with the outcome of the 3D reconstructions of our study, and is at variance with the previous reports, discussed above, where connections were said to be absent before days E13 / E14. Schachtner and colleagues (2000) concluded that vasculogenesis was the mechanism of proximal vessel formation, whereas in our opinion, the data of their study, showing continuity of endothelium from central large vascular structures to the lung periphery, is entirely compatible with an angiogenetic mode of development of the peripheral vessels. Indeed, discontinuity instead of continuity of endothelium between central and peripheral lung areas would provide the morphological argument supportive of vasculogenesis.

An additional clue to the mode of vascular development, neglected in the literature of vasculogenesis in the lung, concerns the morphology of blood cells within distal "blood lakes" or vessels. The presence of blood cells at a give site could theoretically mean that either the cells have originated at that site, or that they have arrived from elsewhere. If they have originated at the site, one would need to be able to identify all stages of their development, from primitive precursor cells to the most mature form present at that gestational age. If such precursors are not found, the blood cells must have come from elsewhere. Indeed, it is the presence of blood cells in the absence of precursors that constitutes the most immediate proof that the vessel is in contact with larger vessels containing circulating blood. Probably, this consideration has previously escaped attention because foetal circulating blood cells which are mature for that specific gestational age, but also precursors thereof as seen in liver and spleen, have all been designated "haematopoietic precursors", so that the difference between the two populations, which is visible on routine histological sections, disappears from the descriptions and discussions which are based on a single term that does not discriminate between the two.
In sum, the possibility of a continuum between small peripheral blood vessels and large central ones in early foetal lung has not been rigorously excluded in previous publications. The presence of blood cells in the absence of precursor cells provides

suggestive evidence that such a continuum is present whenever vessels ("blood lakes") are identified in the foetal lung periphery. We therefore conclude that angiogenesis is more extensive and rapid than has been previously assumed.

These findings indicate that previous reports based on the concept of pulmonary vasculogenesis should be re-evaluated; for instance, the two cell lines with endothelial characteristics, derived from pulmonary mesenchyma of E14.5 and E19, reported by Akeson et al. (2000) may be derived from angiogenetic endothelium which has reached the lung, rather than from vasculogenetic precursor cells.
The vascular continuity from lung hilum to lung periphery as well as apparent absence of in situ hematopoiesis by themselves do not exclude the existence of vasculogenesis: induction of endothelial phenotype in primitive mesenchyme may well occur and early connections of such cells quickly leading to continuous vascular structures, which already reach from the lung hilum to the periphery at day E10 may occur, and haematopoiesis may be absent from these structures. Substantiation of such a hypothesis however requires further substantiation.

Finally, it should be pointed out that of the vast number of small blood vessels that are present in the mature lung, the very large majority have arisen in late foetal and in postnatal life, rather than the embryonal / early foetal period, on which the literature on vasculogenesis has focused. Thus, the emergence of these very large numbers of small vessels occurs at a phase where there is no question of an immature mesenchyme, be it with or without "vascular lakes".

2.6 References

- Akeson AL, Wetzel B, Thompson FY, Brooks SK, Paradis H, Gendron RL, Greenberg JM. Embryonic vasculogenesis by endothelial precursor cells derived from lung mesenchyme. Dev Dyn 2000;217:11-23.
- Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964-967.
- Burri PH. Structural aspects of prenatal and postnatal development and growth of the lung. In: Lung Growth and Development, McDonald JA (ed), Marcel Dekker, Inc, New York: 1997;1-35.
- Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. Development 1998;125:725-732.
- Coffin JD, Harrison J, Schwartz S, Heimark R. Angioblast differentiation and morphogenesis of the vascular endothelium in the mouse embryo. Developmental Biol 1991;148:51-62.
- Colen KL, Crisera CA, Rose MI, Connelly PR, Longaker MT, Gittes GK. Vascular development in the mouse embryonic pancreas and lung. J Pediatr Surg 1999;34:781-785.
- Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, Bowen-Pope DF. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ Res 2000;87:728-730.
- DeMello DE, Sawyer D, Galvin N, Reid LM. Early fetal development of lung vasculature. Am J Respir Cell Mol Biol 1997;16:568-581
- DeMello DE, Reid LM. Embryonic and early fetal development of human lung vasculature and its functional implications. Pediatr Dev Pathol 2000;3:439-449.
- Dumont DJ, Fong GH, Puri MC, Gradwohl G, Alitalo K, Breitman ML. Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. Dev Dyn 1995;203:80-92.
- Gale NW, Yancopoulos GD. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. Genes and Development 1999;13:1055-1066.
- Gendron RL, Tsai F-Y, Paradis H, Arceci RJ.. Induction of embryonic vasculogenesis by bFGF and LIF in vitro and in vivo. Developmental Biol 1996;177:332-346.
- Goedbloed JF, Smits- van Prooije AE. Quantitative analysis of the temporal patterns of somite formation in the mouse and rat. Acta Anat 1986;125:76-82.

- Gutierrez C, Rodriguez A, Palenzula S, Forteza C, Rossello JL. Congnital misalignment of pulmonary veins with alveolar capillary dysplasia causing persistent neonatal pulmonary hypertension: report of two affected siblings. Pediatr Dev Pathol 2000;3:271-276.
- Hatzopoulos AK, Folkman J, Vasile E, Eiselen GK, Rosenberg RD. Isolation and characterization of endothelial progenitor cells from mouse embryos. Development 1998;125:1457-1468.
- Kresch MJ, Christian C, Wu F, Hussain N. Ontogeny of apoptosis during lung development. Pediatr Res 1998;43:426-431.
- McDonald DM, Munn L, Jain RK. Vasculogenic mimickry: How convincing, how novel, and how significant? Am J Pathol 2000;156:383-388.
- Murray PDF. The development in vitro of the blood of the early chick embryo. Proc Royal Soc (London) 1932;11:497-521.
- Pardanaud L, Yassine F, Dieterlen-Lievre F. Relationship between vasculogenesis, angiogenesis and haemopoiesis during avian ontogeny. Development 1989;105:473-485.
- Poole TJ, Coffin JD. Vasculogenesis and angiogenesis: two disctinct morphogenetic mechanisms establish embryonic vascular pattern. J Exp Zool 1989;251:21-28.
- Sabin F. Studies on the origin of blood vessels and of red bloodcorpuscles as seen in the living blastoderm of chicks during the second day of incubation. Contrib Embryol Carnegie Inst Washington 1920;9:214-62.
- Scavo LM, Ertsey R, Chapin CJ, Allen L, Kitterman JA. Apoptosis in the development of rat and human fetal lungs. Am J Respir Cell Mol Biol 1998;18:21-31.
- Schachtner SK, Wang YQ, Baldwin HS. Qualitative and quantitative analysis of embryonic pulmonary vessel formation. Am J Respir Cell Mol Biol 2000;22:157-165.
- Ten Have-Opbroek AAW. Lung development in the mouse embryo. Exp Lung Res 1991;17:111-130.
- Tibboel D, Hosgor M, Molenaar JC. Congenital diaphragmatic hernia: from a surgical defect to developmental biology of the lung. Gaslini 2000;32:306-312.
- Verbeek, F.J. Theory & Practice of 3D-reconstructions from serial sections. In: Image Processing, A Practical Approach. R.A. Baldock and J. Graham, eds. 1999, Oxford: Oxford Univ Press, pp. 153-195.
- Verbeek, F.J. and Huijsmans, D.P. A Graphical database for 3D reconstruction supporting 4 different Geometrical Representations. In Medical Image Databases. S.T.C. Wong, ed. 1998, Kluwer Academic Publ: Boston;117-144.
- Verbeek, F.J., Huijsmans, D.P., Baeten, R.W.A.M., Schoutsen, C.M., and Lamers, W.H. Design and implementation of a program for 3D-reconstruction from serial sections; a data-driven approach. Micr Res Techn 1995;30:496-512.
- Verbeek, F.J., Lawson, K.A., and Bard, J.B.L. Developmental BioInformatics: linking genetic data to virtual embryos. Int J Dev Biol 1999;43:761-771.

- Warburton D, Lee MK. Current concepts on lung development. Curr Opin Pediatr 1999;11:188-192.
- Wood HB, May G, Healy L, Enver T, Morriss-Kay GM. CD34 expression patterns during early mouse development are related to modes of blood vessel formation and reveal additional sites of hematopoiesis. Blood 1997;90:2300-2311.
- Zeng X, Wert SE, Federici R, Peters KG, Whitsett JA. VEGF enhances pulmonary vasculogenesis and disrupts lung morphogenesis in vivo. Dev Dyn 1998;211:215-227.

Chapter 3

Expression of angiogenesis-related factors during normal human fetal lung development

Based on the article:
Expression of angiogenesis-related factors during normal human fetal lung development
M. Hosgor, J.D. de Rooij, Y. IJzendoorn, R. Rottier, W.J. Mooi, D. Tibboel, R.R. de Krijger.
Submitted for publication

3.1 Abstract

In recent years, much attention has been focused on the embryonal development of epithelial structures in the lung and on epithelial-mesenchymal interactions. In contrast, little is known about the factors relevant for pulmonary vascular development. We have performed a longitudinal study of the expression pattern of a series of proteins of established importance for angiogenesis. Human fetal lung tissue between 9 weeks gestation and term was analyzed by immunohistochemistry for the expression of von Hippel-Lindau protein (VHL), hypoxia-inducible factor 1α, vascular endothelial growth factor A, fetal liver kinase 1, and endothelial and inducible nitric oxide synthase. All proteins were expressed in the endothelium of all types of blood vessels and in the arterial media, except VHL, which was only weakly expressed. Whereas all proteins, except HIF1α, were expressed in the epithelium of the airways, the adventitia was devoid of reactivity for all markers. The above-mentioned expression pattern was already present at 9 weeks gestation and did not change significantly during development. These findings imply a role for this pathway already from the glandular phase of lung development all throughout fetal development.

3.2 Introduction

The close alignment of the airways and pulmonary arteries indicates that during lung morphogenesis, cellular interactions between the epithelium and the mesenchyme must play a role in blood vessel formation (Akeson et al., 2000). The elucidation of the molecular interactions regulating normal pulmonary vascular development is essential for the understanding of neonatal pulmonary vascular disease, such as primary pulmonary hypertension, or pulmonary hypertension associated with congenital diaphragmatic hernia (CDH). The formation of the large central pulmonary blood vessels in early fetal development is the result of vascular outgrowth and sprouting, known as angiogenesis (Patan, 2000). During angiogenesis, new blood vessels form from pre-existing ones, by a complex process involving dissolution of matrix, endothelial cell migration, proliferation, organization into a network structure, and lumen formation [Risau, 1997]. Recently, the expression of molecules involved in blood vessel formation was reviewed (Oetgen, 2001).

Among the many factors with an established or potential role in this complex process vascular endothelial growth factor (VEGF) has been identified as an important mitogen for endothelial cells. This action of VEGF is transmitted by the tyrosine kinase receptor KDR/murine homologue fetal liver kinase Flk-1 (Bhatt et al., 2000). In addition, VEGF is

a promoter of nitric oxide (NO) release from endothelial cells by the induction of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) expression (Papapetropoulos et al., 1997). In previous studies, it has been shown that NO is an essential mediator of VEGF-induced angiogenesis (Fukumora et al., 2001). Also, it has been demonstrated that Flk-1 mediates NO-release from endothelial cells (Kroll et al., 1999).
The von Hippel-Lindau (VHL) tumor suppressor gene has been recently implicated in the regulation of VEGF gene expression through an effect on hypoxia-inducible factor 1α (HIF-1α) (Oetgen, 2001; Krek, 2000; Kondo et al., 2001). Apart from upregulation of VEGF under hypoxic conditions, there are numerous other genes whose expression is affected by HIF-1α, including flk-1, eNOS, and iNOS. Mutations in the VHL gene are responsible for highly vascularized tumors in various organs, including the cerebellum, kidney, and the adrenal medulla (Maher et al., 1990). These findings suggest that this VHL "pathway", mediated by HIF-1α, plays an important role in abnormal, but presumably also in normal angiogenesis.

A number of studies has described the immunohistochemical localization of the above-mentioned angiogenesis-related proteins, including VHL, VEGF, Flk-1, eNOS, and iNOS, in various animals but only occasionally in humans. However, most studies in human lung tissue were limited to only one or two of these molecules and/or reported only certain developmental stages (Kessler et al., 1995; Richards et al., 1996; Los et al., 1996; Sakashita et al., 1999; Sherman et al., 1999; Watkins et al., 1997). To date, no studies of the pulmonary developmental expression of HIF-1α, eNOS, or iNOS have been reported in humans.
Therefore, we investigated the tissue localization of VHL, HIF-1α, VEGF, Flk-1, eNOS, and iNOS in different compartments and wall layers of the pulmonary vasculature throughout human embryonic and fetal development, between 9 weeks gestation and term. Herewith, we aimed to provide a framework, that allows the elucidation of their fundamental roles and interactions during human pulmonary vascular development.

3.3 Materials and methods

Tissue specimens. The lung tissues used in this study were retrieved from the archives of the Department of Pathology, Erasmus Medical Center, Rotterdam, following approval of the experimental design and protocols by the Ethical Committee. The specimens (n = 45) were subdivided into four groups, according to the classical morphological stages of human embryonic and fetal lung development: pseudoglandular stage (6 - 16 weeks

gestation, n = 12), canalicular stage (17 - 28 weeks gestation, n = 11), saccular stage (29-36 weeks gestation, n = 10), and alveolar stage (37 weeks gestation to term, n = 12) (Merkus et al., 1996). All autopsies were performed within 24 hours of death of the fetus and there were no pulmonary abnormalities in any of the lung specimens, especially no signs of pulmonary hypoplasia.
The specimens were fixed by immersion in 4% buffered formalin and embedded in paraffin. Five µm-thick sections were mounted on 3-amino-propyl-trioxysilane (Sigma, St. Louis, MO) coated glass slides and processed for immunohistochemistry.

3.4 Immunohistochemistry

Immunohistochemistry was performed using a standard avidin-biotin complex method. In brief, after deparaffinization for 10 minutes in xylene and rinsing in alcohol 100%, slides were treated with 3% H_2O_2 in methanol for 20 minutes to block the endogenous peroxidase activity. Slides were then subjected to a 15 minute microwave treatment in citric acid buffer, pH 6.0. Slides were incubated for one hour at room temperature with a rabbit polyclonal antibody against VEGF (Santa Cruz, sc-152, dilution 1:200), or with a mouse monoclonal antibody against Flk-1 (Santa Cruz, sc-6251, dilution 1:200). Alternatively, they were incubated overnight at 4°C with a mouse monoclonal antibody against VHL (Oncogene, OP102, dilution 1:100), with a mouse monoclonal antibody against HIF-1α (a generous gift of Dr. H. Turley, Nuffield Dept. of Clinical Laboratory Sciences, UK, dilution 1:20), with a rabbit polyclonal antibody against eNOS (Transduction Laboratories, N30030, dilution 1:200), or with a mouse monoclonal antibody against iNOS (Transduction Laboratories, N32020-050, dilution 1:100). After rinsing with phosphate buffered saline (PBS), slides were incubated for 10 minutes with an undiluted biotinylated secondary antibody (Labvision, UP-999-BN). Slides were then rinsed again with PBS, and incubated for 10 minutes with undiluted peroxidase-conjugated streptavidin (Labvision, UP-999-HR). Peroxidase activity was detected by diamino-benzidine tetrahydrochloride (Fluka) with 0.3% H_2O_2 for 7 minutes. The slides were counterstained with hematoxylin, dehydrated through graded alcohol and xylene and mounted. For the VHL antibody, human adult cerebellum was used as a positive control tissue, for the VEGF antibody the positive control was human adult kidney, and for HIF-1α it was human placenta. All positive controls gave the expected results in all stainings. Negative controls were performed by omission of the primary antibodies.
Lung specimens at all developmental stages were evaluated by two independent observers (MH and RRdK). The expression of all markers was scored as positive, weakly positive, or negative for each of the following structures: endothelium of the pulmonary arteries,

veins and capillaries, vascular smooth muscle cells of the arterial medial layer, fibroblasts of the arterial adventitial layer, and epithelium of the proximal and distal airways.

3.5 Results

The immunohistochemical reactivity of the antibodies to VHL, HIF-1α, VEGF, Flk-1, eNOS, and iNOS was analyzed at each of the four stages of human lung development in endothelial cells of arteries, veins, and capillaries; smooth muscle cells of the media and fibroblasts of the adventitia of pulmonary arteries; and pulmonary epithelium. Whenever macrophages were present, usually at the saccular and alveolar stages, they were also scored. All proteins are expressed from the earliest time point studied (9 weeks of gestation). Furthermore, the pattern of expression of all proteins remains remarkably similar throughout the various developmental stages, which may be a reflection of the ongoing process of angiogenesis.

VHL. The main site of expression of the VHL protein is in the epithelium of the developing lung, including the proximal and distal airways (Fig. 1A, on page 92), and the alveolar epithelium (Fig 1B, on page 92). In addition, the endothelial cells and the medial smooth muscle cells of pulmonary arteries were weakly labeled (Fig. 1C, D, on page 92). Macrophages, when present, also expressed VHL.

HIF-1α. In contrast to VHL, HIF-1α is predominantly expressed in the endothelium of arteries and veins of all sizes, as well as in capillary endothelium. At the pseudoglandular stage, an extensive network of small vessels can be seen in the interstitium, with a predominance of vessels in the vicinity of the developing airways (Fig. 2A, on page 93). The endothelium of arteries and veins is also stained (Fig. 2A, B, on page 93). At term, the rich capillary network in the delicate interalveolar septa is also highlighted by HIF-1α staining. In almost all specimens, the smooth muscle cells of the pulmonary arteries also show HIF-1α staining (Fig. 2C, D, on page 93). Finally, if macrophages were present, they were HIF-1α-reactive.

VEGF. Similar to VHL, the epithelial cells are extensively positive for VEGF, both the larger and the smaller airways, as well as alveolar epithelium (Fig. 3A, B, on page 94). In many specimens, clear labeling of the subepithelial matrix could be observed. In addition, the medial smooth muscle cells of pulmonary arteries of all sizes are labeled (Fig. 3C, on page 94). Finally, in some, but not all, samples endothelial cells of arteries, veins, and

capillaries display VEGF staining (Fig. 3C and D, on page 94). Whenever alveolar macrophages are present, in the saccular and alveolar stages, they stain for VEGF.

Flk-1. The endothelial cells of all types of vessels, including arteries and veins of all sizes, and capillaries, are stained by Flk-1 (Fig. 4A-C, on page 95). In addition, the epithelium of the conducting airways, is also labeled by Flk-1 (Fig. 4D, on page 95), as well as the media of the pulmonary arteries, although this labeling is sometimes weak (Fig. 4A, on page 95). Also, intra-alveolar macrophages are positive for Flk-1.

eNOS. The expression pattern of eNOS is largely comparable with that of Flk-1. All endothelial cells are labeled (Fig. 5A-C, on page 96), although at the saccular and alveolar stages labeling is weak or absent in the capillaries. The medial smooth muscle cells are usually weakly positive (Fig. 5A, on page 96), whereas the epithelium of the conducting airways but not the alveolar epithelium is clearly labeled in all cases (Fig. 5D, on page 96). Macrophages, whenever present, are strongly eNOS positive.

iNOS. Although iNOS labeling is also seen in the endothelium, expression is predominant in the endothelial cells of the arteries (Fig. 6A, on page 97), whereas labeling of veins and capillaries is usually weak (Fig. 6B, C, on page 97). The expression pattern for the epithelium, medial smooth muscle of pulmonary arteries, and macrophages is identical to that of eNOS (Fig. 6D, on page 97).

3.6 Discussion

We have investigated the ontogenic expression pattern of a specific group of proteins, known to be involved in angiogenesis, in a longitudinal study of human embryonic and fetal lung development. We show that each of these proteins is present from the earliest time point analyzed, 9 weeks of gestation, throughout all morphological stages of lung development until term, with no major changes in expression pattern. This is compatible with the notion that angiogenesis in the developing lung is a continuous process, which has begun at an earlier stage (deMello et al., 2000) and continues postnatally.
This study is performed by immunohistochemistry on paraffin-embedded tissues. Although care has been taken to ensure that the delay between death and autopsy was minimized, and that fixation conditions and times were similar, it cannot entirely be excluded that these factors may have had an impact on the staining properties of the tissue specimens. Therefore, we have not attempted to quantify our results, except for the crude division of staining intensities into negative, weakly positive, and clearly positive

staining. In addition, we believe that there are other techniques than immunohistochemistry if quantification of results is necessary. However, none or only very limited frozen tissue samples are available from these autopsy cases.
The role of the VHL protein in angiogenesis has become evident after the elucidation of gene mutations in patients with the hereditary VHL syndrome, who develop a variety of highly vascularized tumors (Maher et al., 1990). This marked vascularity was shown to be caused by high VEGF expression. A direct link between VHL and VEGF was established following the unravelling of the role of VHL in ubiquitination of HIF1α (Krek, 2000). So far, few studies have reported the expression of the VHL protein in human lung tissue. Two groups of authors have found expression in the epithelium and in macrophages in the adult lung (Los et al., 1996; Sakashita et al., 1999). In addition, VHL mRNA was detected in human lung endoderm already between 4 and 10 weeks gestation (Richards et al., 1996). In our study, we confirm the epithelial expression of the VHL protein, which appears to become restricted to the type II pneumocytes in the saccular and alveolar stages, and the localization in macrophages. In addition, we report its expression in the majority of cases in the endothelium of pulmonary arteries and in a proportion of cases in the smooth muscle cells of the arterial media.

Interestingly, the HIF1α expression pattern, which has not been reported previously in human (lung) development, differed from that of VHL. While the expression in endothelium and smooth muscle cells of arteries concurred, HIF1α was not expressed in epithelial cells, but rather in the endothelium of veins and capillaries. This latter expression, apparently unopposed by VHL could be responsible for a strong inductive signal for the expression of VEGF and NOS isoforms (Ryan et al., 1998; Carmeliet et al., 1998; Palmer et al., 1998), resulting in angiogenesis. In the epithelium, VHL may have led to downregulation of HIF1α to a level which is undetectable by our techniques. In the arteries, finally, the relatively weak VHL expression might allow for HIF1α expression, although this is speculative and needs further confirmation. In addition, it must be noted that VHL is not the only protein regulating HIF1α expression (Brüne et al., 2001; Blancher et al., 2001).

The expression of VEGF has been reported previously in the epithelium and smooth muscle of the pulmonary arteries of the midgestational human fetus, between 16 and 22 weeks gestation (Shifren et al., 1994; Acarregui et al., 1999). In this study we confirm expression in these two cell compartments and extend this to the period of 9 weeks gestation until term. In addition, we observed VEGF immunoreactivity in endothelial cells, which have not been reported to express VEGF, except for one recent report where

it was found in endothelial cells of fetuses between 16 and 32 weeks gestation (Lassus et al., 2001). In one of the above-mentioned studies, another antibody, recognizing only the $VEGF_{165}$ isoform, was used (Shifren et al., 1994). The other study used the same antibody as we did, but it was applied on frozen sections of cultured lung tissue (Acarregui et al., 1999). Thus, these different methodologies may well have caused these apparent discrepancies. Alternatively, it cannot entirely be excluded that the observed VEGF staining, obtained with an antibody recognizing all isoforms of VEGF-A, results from binding of VEGF to its receptor and subsequent internalization into the cytoplasm of the endothelial cells. Further experiments, employing in situ hybridization, should be performed to resolve this issue.

Thus far, the expression of Flk-1 has only been reported in rodent lung development, where it was expressed in endothelial cells (Marszalek et al., 2001; Gebb et al., 2000; Bhatt et al., 2000). This was confirmed in our study for human lung development as well. In addition, Flk-1 expression was also observed in smooth muscle cells in the arterial media and in bronchial epithelium. Presently, it remains unclear what role Flk-1 could play at these sites.

Of the three isoforms of NOS, we have investigated iNOS (type II NOS) and eNOS (type III NOS). A large body of literature exists regarding the expression of these molecules, predominantly in various animal models. In addition, there have been a few studies in human lung epithelium, but these concern mainly cultured cells or adult tissue (Kobzik et al., 1993; Watkins et al., 1997; Shaul et al., 1994; Asano et al., 1994). In our study, the expression of both NOS isoforms is similar and the expression of both in endothelial cells parallels observations in rodents and sheep. In addition, we find extensive expression of both forms in the epithelium of airways, which is in accordance with some studies in animals (Sherman et al., 1999; Xue et al., 1996) and the previously mentioned human studies. Also, the arterial smooth muscle cells were stained by antibodies to both NOS isoforms. It is intriguing that this reactivity is present all throughout human lung development and suggests a role for NOS isoforms in various aspects of lung development. Very recently, a role for nitric oxide (NO) in branching morphogenesis of rat lungs has been postulated and this might very well explain the presence of NOS isoforms in early development (Young et al., 2002). Furthermore, there is ample evidence that NOS isoforms and NO contribute to VEGF-induced angiogenesis (Fukumura et al., 2001; Papapetropoulos et al., 1997). Such findings are further supported by the fact that HIF1α has been shown to be a target of NO (Brüne et al., 2001).

Taken together, we have shown that a series of proteins, belonging to what we tentatively call the VHL "pathway", with known interactions and a known role in angiogenesis are expressed throughout human lung development from 9 weeks gestation until term. The

expression of HIF1α, Flk-1, and the NOS isoforms had not been reported before in the human fetal lung and such comprehensive analysis had not been performed earlier. We speculate that this pathway plays an important role in normal angiogenesis and perturbations may lead to abnormal vascular development, such as can be seen in cases of serious neonatal respiratory insufficiency complicated by pulmonary hypertension such as congenital diaphragmatic hernia.

3.7 References

- Acarregui, M.J., Penisten, S.T., Goss, K.L., Ramirez, K., Snyder, J.M. Vascular endothelial growth factor gene expression in human fetal lung in vitro. Am J Resp Cell Mol Biol 1999;20:14-23.
- Akeson, A.L., Wetzel, B., Thompson, F.L., Brooks, S., Paradis, H., Gendron, R.L. and Greenberg, J.M. Embryonic vasculogenesis by endothelial cell precursor cells derived from lung mesenchyme. Dev Dyn 2000;217:11-23.
- Asano, K., Chee, C.B.E., Gaston, B., Lilly, C.M., Gerard, C., Drazen, J.M., Stamler, J.S. Constitutive and inducible nitric oxide synthase gene expression, regulation, and activity in human lung epithelial cells. PNAS 1994;91:10089-10093.
- Bhatt, A.J., Amin, S.B., Chess, P.R., Watkins, R.H. and Maniscalco, W.M. Expression of vascular endothelial growth factor and Flk-1 in developing and glucocorticoid-treated mouse lung. Pediatr Res 2000;47:606-613.
- Blancher, C., Moore, J.W., Robertson, N., Harris, A.L. Effects of ras and von Hippel-Lindau (VHL) gene mutations on hypoxia-inducible factor (HIF)-1α, HIF-2α, and vascular endothelial growth factor expression and their regulation by the phophatidylinositol 3'-kinase/Akt signaling pathway. Cancer Res 2001;61:7349-7355.
- Brüne, B., von Knethen, A., Sandau, K.B. Transcription factors p53 and HIF-1α as targets of nitric oxide. Cellular Signalling 2001;13:525-533.
- Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono F., Abramovitch, R., Maxwell, P., Koch, C.J., Ratcliffe, P., Moons, L., Jain, R.K., Collen, D., Keshet E. Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation, and tumour angiogenesis. Nature 1998;394:485-490.
- Demello, D.E., Reid, L.M. Embryonic and early fetal development of human lung vasculature and its functional implications. Pediatr Dev Pathol 2000;3:439-449.
- Fukumura, D., Gohongi, T., Kadambi, A., Izumi, Y., Ang, J., Yun, C.O., Buerk, D.G., Huang, P.L. and Jain, R.K. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. PNAS 2001;98:2604-2609.
- Gebb, S.A., Shannon, J.M. Tissue interactions mediate early events in pulmonary vasculogenesis. Dev Dyn 1994;217:159-169.
- Kessler, P.M., Vasavada, S.P., Rackley, R.R., Stackhouse, T., Duh, F.M., Latif, F., Lerman, M.I., Zbar, B. and Williams, B.R.G. Expression of the von Hippel-Lindau tumor suppressor gene, VHL, in human fetal kidney and during mouse embryogenesis. Mol Med 1995;1:457-466.

- Kobzik, L., Bredt, D.S., Lowenstein, C.J., Drazen, J., Gaston, B., Sugarbaker, D. and Stamler, J.S. Nitric oxide synthase in human rat lung: immunocytochemical and histochemical localization. Am J Respir Cell Mol Biol 1993;9:371-377.
- Kondo, K. and Kaelin, W.G. The von Hippel-Lindau tumor suppressor gene. Exp Cell Res 2001;264:117-125.
- Krek, W. VHL takes HIF's breath away. Nature Cell Biol 2000;2:E121-123.
- Kroll, J. and Waltenberger, J. A novel function of VEGF receptor-2 (KDR): rapid release of nitric oxide in response to VEGF-A stimulation in endothelial cells. Biochem Biophys Res Commun 1999;265:636-639.
- Lassus, P., Turanlahti, M., Heikkilä, P., Andersson, L.C., Nupponen, I., Sarnesto, A., Andersson, S. Pulmonary vascular endothelial growth factor and Flt-1 in fetuses, in acute and chronic lung disease, and in persistent pulmonary hypertension of the newborn. Am J Resp Crit Care Med 2001;164:1981-1987.
- Los, M., Jansen, G.H., Kaelin, W.G., Lips, C.J.M., Blijham, G.H. and Voest, E.E. Expression pattern of the von Hippel-lindau protein in human tissues. Lab Invest 1996;75:231-238.
- Maher, E.R., Yates, J.R., Harries, R., Benjamin, C., Harris, R., Moore, A.T. and Ferguson-Smith, M.A. Clinical features and natural history of von Hippel-Lindau disease. Quart. J. Med. 1990;7:1151-1163.
- Marszalek, A., Daa, T., Kashima, K., Nakayama, I., Yokoyama, S. Expression of vascular endothelial growth factor and its receptors in the developing rat lung. Jpn J Physiol 2001;51:313-318.
- Merkus, P.J.F.M., ten Have-Opbroek, A.A.W. and Quanjer, P.H. Human lung growth: a review. Pediatr Pulmonol 1996;21:383-397.
- Oetgen, P. Transcriptional regulation of vascular development. Circ Res 2001;89:380-388.
- Palmer, L.A., Semenza, G.L., Stoler, M.H., Johns, R.A. Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. Am J Physiol 1998;274:L212-219.
- Papapetropoulos, A., Garcia-Cardena, G., Madri, J.A. and Sessa, W.S. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J Clin Invest 1997;100:3131-3139.
- Patan, S. Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. J Neurooncol 2000;50:1-15.
- Richards, F.M., Schofield, P.N., Fleming, S. and Maher, E.R. Expression of the von Hippel-Lindau disease tumour suppressor gene during human ambryogenesis. Hum Mol Genet 1996;5:639-644.
- Risau, W. Mechanisms of angiogenesis. Nature 1997;86:671-674.
- Ryan, H.E., Lo, J., Johnson, R.S. HIF-1α is required for solid tumor formation and embryonic vascularization. EMBO J 1998;17:3005-3015.

- Sakashita, N., Takeya, M., Kishida, T., Stackhouse, T.M., Zbar, B. and Takahashi, K. Expression of the von Hippel-Lindau protein in normal en pathological human tissues. Histochem J 1999;31:133-144.
- Shaul, P.W., North, A.J., Wu, L.C, Wells, L.B., Brannon, T.S., Lau, K.S., Michel, T., Margraf, L.R., Star, R.A. Endothelial nitric oxide synthase is expressed in cultured human bronchiolar epithelium. J Clin Invest 1994;94:2231-2236.
- Sherman, T.S., Chen, Z., Yuhanna, I.S., Lau, K.S., Margraf, L.R. and Shaul, P.W. Nitric oxide synthase isoform expression in the developing lung epithelium. Am J Physiol 1999;276:L383-L390.
- Shifren, J.L., Doldi, N., Ferrara, N., Mesiano, S., Jaffe, R.B. In the human fetus, vascular endothelial growth factor is expressed in epithelial cells and myocytes, but not vascular endothelium: implications for mode of action. J Clin Endocrinol Metab 1994;79:316-322.
- Watkins, D.H., Peroni, D.J., Basclain, K.A., Garlepp, M.J. and Thompson, P.J. Expression and activity of nitric oxide synthases in human airway epithelium. Am J Respir Cell Mol Biol 1997;16:629-639.
- Xue, C., Reynolds, P.R., Johns, R.A. Developmental expression of NOS isoforms in fetal rat lung: implications for transitional circulation and pulmonary angiogenesis. Am J Physiol 1996;270:L88-100.
- Young, S.L., Evans, K., Eu, J.P. Pre- and postnatal lung development, maturation, and plasticity. Nitric oxide modulates branching morphogenesis in fetal rat lung explants. Am J Physiol Lung Cell Mol Physiol 2002;282:L379-385.

Chapter 4

Expression of angiogenic factors in human congenital diaphragmatic hernia compared to age-matched normal lungs

Based on the article:
Expression of angiogenic factors in human congenital diaphragmatic hernia compared to age-matched normal lungs
Hösgör M, De Rooij JD, IJzendoorn Y, De Krijger RR, Tibboel D.
Submitted for publication

4.1 Abstract

Introduction
Pulmonary hypertension in patients with congenital diaphragmatic hernia (CDH) remains one of the pivotal factors determining outcome. Although the morphology of the abnormal pulmonary vasculature in CDH is well documented, no detailed information is available on the expression of angiogenic factors such as those of the VHL (von Hippel-Lindau) pathway, including VHL, HIF-1α,VEGF, Flk-1, eNOS, and iNOS. Although few studies have reported the immunohistochemical localization of these proteins in humans, no comprehensive analysis of cell type-specific expression, comparing the differences between normal and CDH lungs, has been performed yet.
Aim: To compare the immunohistochemical expression pattern of the angiogenic factors VHL, HIF-1α, VEGF, its receptor Flk-1, endothelial and inducible nitric oxide synthase (eNOS, iNOS), in specific pulmonary cell types in hypoplastic lungs of human CDH and age- matched controls.

Materials and methods
We studied 40 human lung specimens, including 22 normal term lung specimens and 16 lung specimens of term high-risk CDH patients who died < 24 hours after birth. Immunohistochemical analysis was performed on paraffin sections using antibodies against VHL, HIF-1α, VEGF, its receptor Flk-1, as well as iNOS and eNOS.

Results
In both groups, all proteins were strongly expressed in the endothelium of pulmonary arteries of all sizes, with the exception of VHL and HIF-1α, which were negative in CDH cases. VHL expression was not detected in the medial layer of controls, but in CDH lungs there was a significant staining. VEGF, Flk-1, and iNOS expression was present, but eNOS expression was weak although without differences between controls and CDH cases. In the adventitia of the arteries as well as in the epithelium of smaller and larger airways identical staining patterns were observed.

Conclusion
The positive VHL protein expression in the media of CDH arteries combined with a negative VHL and HIF-1α staining in the endothelium suggests a disturbed endothelium-media interaction in CDH interfering with normal vascular remodelling and growth.

4.2 Introduction

For many decades the classical morphological anomalies in congenital diaphragmatic hernia (CDH) have been documented, consisting of abnormal pulmonary development resulting in a variable amount of hypoplasia and pulmonary vascular abnormalities. Both abnormalities have only been described from a morphological point of view. The vascular abnormalities consist of medial hyperplasia , decreased numbers of vascular branches per unit lung as well as adventitional thickening.[1-3] In contrast, the developmental process resulting into these morphological features and its consequences such as an aberrant pulmonary vascular reaction are largely unknown. For instance, well-known vasodilators used in the treatment of other forms of pulmonary hypertension, such as inhaled nitric oxide, have no proven benefit in CDH. Studies in animal models have suggested that other pathways might be involved such as overexpression of the endothelin A-receptor[5] or other ways of intracellular trafficking.[6,7]
Up till now, factors involved in pulmonary vascular development have been described in a non-systematic manner, both in animal models of CDH (iNOS in rat and sheep) as well as in humans.[8-11]

Recently, vascular biologists reviewed (early) vascular development and transcription factors involved in this process leading to a well defined temporal sequence of molecules relevant for vascular development from embryonic vascular stem cells towards a mature vessel architecture.[12] Also pathways regulating the expression of vascular endothelial derived growth factor (VEGF) and its receptor Flk-1, as well as inducible and endothelial nitric oxide synthase (iNOS and eNOS) have been described in more detail. In this context the Von Hippel Lindau (VHL) -pathway is known to be relevant in vascular development.[13] It is especially important that under normoxic and relatively hypoxic circumstances the downstream genes are regulated differently.[13] As the hypoxic environment is the normal environment of the developing human fetus, we conducted a study in which we investigated immunohistochemically the presence and spatio- temporal distribution of the VHL-pathway in CDH patients and age-matched controls.

4.3 Materials and methods

In total we used 40 lung specimens. Lung tissue was obtained from 16 high-risk CDH-patients who died within 24 hours after birth, including 5 who died within 1 hour after birth. All CDH-cases were treated according to a standard protocol including the concepts

of delayed surgery, prevention of hyperventilation in which conventional ventilation was used as the primary ventilatory mode.
As vasodilators, inhaled NO was used as the primary drug of choice while systemic blood pressure was supported by normovolemia, dopamine, and in occasional cases norepinephrine. The presence of pulmonary hypertension in these patients was documented by repeated cardiac ultrasound as reported before by our group.[14]
None of the patients included in this study was subjected to ECMO-therapy. As controls, 22 age- macthed normal term lungs, which were derived from autopsies performed within 24 hours after death from non-lung-related causes were evaluated. No pulmonary hypoplasia was present in these cases as documented by the lung/body weight ratio.

Immunohistochemistry.
Immunohistochemistry was performed using a standard avidin-biotin complex method. In brief, after deparaffinization in xylene and rehydration through graded alcohol, slides were treated with 0.33% H_2O_2 in methanol to block the endogenous peroxidase activity. Slides were then subjected to a 15 minute microwave treatment in citric acid buffer, pH 6.0. After a blocking according to manufacturers instruction (Biogenex, MO, San Ramon, US) slides were incubated overnight at 4°C with a mouse monoclonal antibody against VHL (Oncogene, USA), dilution 1:1000, a mouse monoclonal antibody against hypoxia inducible factor (HIF-1α) (a generous gift of Dr. H. Turley, Nuffield, Dept. of Clin. Lab Sciences, UK) dilution 1:20. Alternatively, slides were incubated for 1 hour at room temperature with a rabbit polyclonal antibody against VEGF (Santa Cruz Biotechnology, Ca), dilution 1:200, a mouse monoclonal antibody against Flk-1 (Santa Cruz Biotechnology, Ca), dilution 1:200, a rabbit polyclonal antibody against eNOS (Transduction Laboratories, Lexington, US), dilution 1:200, and a mouse monoclonal antibody against iNOS (Transduction Laboratories, Lexington, US), dilution 1:100 (15-19). After rinsing with phosphate buffered saline (PBS), slides were incubated for 10 minutes with a biotinylated secondary antibody (Biogenex, San Ramon, MO, US). Slides were rinsed again with PBS, incubated for 10 minutes with peroxidase-conjugated streptavidin, undiluted (Biogenex, San Ramon, MO, US). Peroxidase activity was then detected by diamino-benzidine tetrahydrochloride (Fluka, Neu-Ulm, Germany) with 0.3% H_2O_2 for 7 minutes and counterstained with hematoxylin. Negative controls were processed by omission of the primary antibodies.
All specimens were scored by two independent blinded investigators.
The following structures were scored in all our analyses: arteries (endothelium, media, and adventitia), veins, capillaries and epithelium.

4.4 Results

The most relevant differences in staining pattern in both groups are documented in table 1 and presented in figure A – F (on page 98-99). In both groups, all proteins were strongly expressed in the endothelium of pulmonary arteries of all sizes, with the exception of VHL and HIF-1α, which were negative in CDH cases. In the medial layer of pulmonary arteries, VHL expression was not detected in normal lungs, but in CDH lungs there was a significant staining. HIF-1α, VEGF, Flk-1, and iNOS expression was present, but eNOS expression was weak, showing only gradual differences without significance. In the adventitia of the arteries as well as in the airways identical staining patterns were observed in both groups.

No differences were obtained in the staining patterns between patients who died within 1 hour after birth with documented severe pulmonary hypoplasia and children who lived up till 24 hours after birth.

Table 1

Location	Molecules	Controls	CDH
Endothelium	VHL	-	-
	HIF-1α	+	-
Media	VHL	-	+
	HIF-1α	+	+
Adventitia	VHL	no differences	
	HIF-1α		

The most relevant differences in arterial staining pattern are represented.

4.5 Discussion

This is the first report describing a comprehensive immunohistochemical evaluation of the VHL-pathway in hypoplastic lungs of human CDH-patients and age-matched controls. The negative medial staining of VHL in control specimen together with the positive staining of HIF-1α in the media possibly reflects a block in the transcription of downstream molecules in CDH. In CDH also the VHL protein was found to be positive. Intriguing is the observation of negative endothelial staining of HIF-1α in CDH which

might explain the observed morphology.
Although the morphology of the pulmonary vascular bed has been documented already 25 years ago,[1,2] the question remains whether these vascular abnormalities should be considered as a final common pathway of abnormal pulmonary development or as a result of a specific (in time and location) developmental arrest. As remodeling is an important process in the late phases of prenatal development to prepare the fetus for the significant increase in pulmonary vascular flow directly after birth, a failure in this process can not be excluded.
As an alternative the high oxygen content in the alveolar space and activation of oxygen free radicals as a consequence of artificial ventilation used in the immediate newborn period in patients with high risk CDH with high inspiratory oxygen could also trigger the VHL-pathway.
In this context it is important to note that no difference was observed in the staining pattern of cases who died within 1 hour after birth, including 3 who died within 10 minutes, and the potential negative effect of vigorous attempts to overcome the ongoing arterial hypoxia in other patients (for maximally 24 hours).
As we used autopsy material for our evaluation, no attempt was made for a more quantitative approach such as Northern blot analysis and/or real time PCR because a variable amount of time between death of the child and tissue fixation occurred for obvious reasons (although always between 12 and 24 hours). In ideal circumstances open lung biopsies immediately after demise of the newborn are warranted to reach standardisation.

Apart from our observations in normal and abnormal lung development, the VHL-pathway is known to be related to vascular tumor formation.[15] In another study we recently proved that many of the molecules are already detectable in embryonic lung specimens from week 9 of gestation onwards and as such the VHL-pathway appears to have a significant place in normal pulmonary vascular development. The importance of the VHL-pathway for vascular development is underlined by the fact that mutants of molecules involved in the VHL-pathway such as VEGF knockout mice result in abnormal pulmonary vascular development while eNOS mutants have documented pulmonary hypertension.[20-24]
A more detailed study evaluating the VHL-pathway at different gestational ages is essential to understand the actual timing of occurrence of abnormal protein expression. It is known that normal regulation of the interaction between the different layers of the arterial wall is essential in the process of remodeling. In this way a better insight can be obtained of the molecular mechanisms resulting into abnormal pulmonary vasculature such as in case of CDH.

Still other options are open which might explain the abnormal architecture such as a dysbalance between proliferation and apoptosis of the developing muscular coat in the pulmonary vasculature as well as factors involved in degradation of the extracellular matrix.[25,26]

4.6 Conclusion

Significant differences in staining pattern of molecules involved in the VHL-pathway (VHL, HIF-1α) especially in the media and endothelium of the pulmonary arteries were found in CDH cases and age matched controls. These observations will guide our future investigations unraveling the molecular background of pulmonary hypertension in CDH.

4.7 References

1. Kitagawa M, Hislop A, Boyden EA, Reid L. Lung hypoplasia in congenital diaphragmatic hernia: a quantitative study of airway, artery, and alveolar development. Brit J Surg 1971;58:342-346.
2. Naeya RL, Sochat SJ, Whitman V. Unsuspected pulmonary vascular abnormalities associated with diaphragmatic hernia. Pediatrics 1976;58:902-904.
3. Taira Y, Yamataka T, and Miyazaki E, Puri P. Adventitial changes in pulmonary vasculature in congenital diaphragmatic hernia complicated by pulmonary hypertension. J Pediatr Surg 1998;33:382-387.
4. Clark RH, Kueser TJ, Walker MW, Southgate WM et al. Low-dose nitric oxide therapy for persistent pulmonary hypertension of the newborn. Clinical Inhaled Nitric Oxide Research Group. N Engl J Med 2000;342:469-474.
5. Okazaki T, Sharma HS, McCune SK, Tibboel D. Pulmonary vascular balance in congenital diaphragmatic hernia. Enhanced endothelin-1 gene expression as a possible cause of pulmonary vasoconstriction. J Pediatr Surg 1998;33:81-84.
6. Shekerdemian LS, Ravn HB, Penny DJ. Intravenous Sildenafil lowers pulmonary vascular resistance in a model of neonatal pulmonary hypertension. Am J Respir Crit Care Med 2002;165:1098-1102.
7. Hoeper MM, Galie N, Simonneau G, Rubin LJ. New treatments for pulmonary arterial hypertension. Am J Respir Crit Care Med 2002;165:1209-1216.
8. Shehata SMK, Mooi WJ, Okazaki T, El-Banna IA, Sharma HS, Tibboel D. Enhanced expression of vascular endothelial growth factor in lungs of newborns with congenital diaphragmatic hernia and pulmonary hypertension. Thorax 1999;54(4):427-431.
9. Karamanoukian HL, Peay T, Love JE, Abdel-Rahman E, Dandonna P, Azizkhan RK, Glick PL. Decreased pulmonary nitric oxide synthase activity in the rat model of congenital diaphragmatic hernia. J Pediatr Surg 1996;31:1016-1019.
10. Chinoy MR, Graybill MM, Miller SA et al. Angiopoietin-1 and VEGF in vascular development and angiogenesis in hypoplastic lungs. Am J Physiol Lung Cell Moll Physiol 2002;283:L60-L66.
11. Lassus P, Turanlahti M, Heikkila P, Andersson LC et al. Pulmonary vascular endothelial growth factor and Flt-1 in fetuses, in acute and chronic lung disease, and in persistent pulmonary hypertension of the newborn. Am J Respir Crit Care Med 2001;164:1981-1987.
12. Oetgen P. Transcriptional regulation of vascular development. Circul Res 2001;89:380-388.
13. Krek W. VHL takes HIF's breath away. Nature Cell Biol 2 2000;E121-123.
14. Shehata SMK, Tibboel D, Sharma HS, Mooi WJ. Impaired structural remodeling of pulmonary arteries in newborns with congenital diaphragmatic hernia: a histological study of 29 cases. J Path 1999;189(1):112-118.
15. Kondo K, Kaelin WG. The von Hippel-Lindau tumor suppressor gene. Exp Cell res 2001;264:117-125.

16. Kessler PM, Vasavada SP, Rackley RR, Stackhouse T et al. Expression of the von Hippel-Landau tumor suppressor gene, VHL, in human fetal kidney and during mouse embryogenesis. Mol Med 1995;1:457-466.
17. Los M, Jansen GH, Kaelin WG, Lips CJM, Blijham GH, Voest EE. Expression pattern of the von Hippel-Landau protein in human tissues. Lab Invest 1996;75:231-238.
18. Ferrara N. Role of vascular endothelial growth factor in the regulation of angiogenesis. Kidney Int 1999;794:814.
19. Shweiki D, Itin A, Sofer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may indicate hypoxia-initiated angiogenesis. Nature 1992;359:843-845.
20. Carmeliet P, Ferreira A, Breier G, Pollefeyt S, Kieckens L, Gertenstein M, Fahrig M et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996;380:435-439.
21. Ferrara N, Carver-Moore K, Dowd M et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996;380:439-442.
22. Steudel W, Schrerre-Crosbie M, Bloch KD et al. Sustained pulmonary hypertension and right ventricular hypertrophy after chronic hypoxia in mice with congenital deficiency of nitric oxide synthase 3. J Clin Invest 1998;101(11):2468-2477.
23. Zhao L, Long L. Morrell NW, Wilkins MR. NPR-A-Deficient mice show increased susceptibility to hypoxia-induced pulmonary hypertension. Circulation 1999;99(5):605-607.
24. Fagan KA, Fouty BW, Tyler RC, Morris KG Jr et al. The pulmonary circulation of homozygous or heterozygous eNOS-null mice is hyperresponsive to mild hypoxia. J Clin Invest 1999;103(2):291-299.
25. Fukuda Y, Ishizaki M, Okada Y et al. Matrix metalloproteinases and tissue inhibitor of metalloproteinase-2 in fetal rabbit lung. Am J Physiol Lung Cell Mol Physiol 2000;279:L555-561.
26. Hayashi T, Stetler-Stevenson WG, Gleming MV et al. Immunohistochemical study of metalloproteinases and their tissue inhibitors in the lungs of patients with diffuse alveolar damage and idiopathic pulmonary fibrosis. Am J Pathol 1997;149:1241-1256.

Chapter 5

Thyroid transcription factor-1 expression during normal human lung development and in congenital diaphragmatic hernia patients

Based on the article:
Thyroid transcription factor-1 expression during normal human lung development and in congenital diaphragmatic hernia patients
M. Hösgör, Y. IJzendoorn, W. J. Mooi , D. Tibboel , R. R. de Krijger
J Pediatr Surgery 2002;37:1258-1262.

5.1 Abstract

Background/ Purpose
Thyroid transcription factor-1 (TTF-1) was detected in human respiratory epithelial cells from 11 weeks of gestation. TTF-1 is involved in both lung morphogenesis and in the regulation of surfactant proteins. Recently, low expression of TTF-1 in the nitrofen rat model of congenital diaphragmatic hernia (CDH) was demonstrated and restoration of this down-regulation by antenatal glucocorticolds (CS) was reported. Our aim was to investigate the expression of TTF-1 as a marker of lung morphogenesis in normal human lung development, and in age-matched controls of human lung specimen in hypoplastic lungs of human CDH and other forms of lung hypoplasia.

Methods
Immunohistochemistry by a monoclonal TTF-1 antibody was performed on paraffin sections of human fetal and neonatal lung tissues. The so-called developmental group (12 weeks of gestation till term) included 47 lung specimens. The congenital hypoplasia group included 8 term CDH patients who died within 12 hours after birth, 3 term CDH patients, who had antenatal CS therapy and 4 term CDH patients following ECMO therapy. For comparison, 6 term born patients, who died of other forms of pulmonary hypoplasia, were used as comparative specimen. Immunohistochemical localization of TTF-1 was evaluated by light microscopy for three different areas of the airways including intrapulmonary bronchi, intermediate airways so-called terminal bronchioles, distal airways and later sacculi and alveoli.

Results
Nuclear TTF-1 staining was observed in the progenitor cells of the developing bronchiolar cells early in the human lung developmental series. At term, TTF-1 was expressed in both Type II epithelial cells and in subsets of respiratory nonciliated bronchiolar epithelial cells in a pattern similar in all studied groups. No TTF-1 expression was detected at the level of the intrapulmonary bronchi.

Conclusions
No difference in TTF-1 expression was observed in the developing early fetal and term normal, neither in hypoplastic human lungs. This expression did not change with antenatal CS and postnatal ECMO treatment. Although TTF-1 appears to play an important role in lung, morphogenesis, a pivotal role in human lung development is not likely.

5.2 Introduction

Recent progress in developmental biology has revealed a variety of genes and growth factors to play an important role in lung development.[1] Amongst these, thyroid transcription factor-1, (TTF-1) a 38 kDa nuclear protein, was identified as a member of the Nkx2 family of homeodomain transcription factors.[2] Deletion of TTF in transgenic mice causes severe thyroid and lung hypoplasia suggesting an essential role of TTF-1 in the early differentiation of the foregut epithelium during fetal lung development.[3] In the human, TTF protein was detected by immunohistochemistry in nuclei of the respiratory epithelium in lungs as early as 11 weeks of gestation.[2] TTF-1 is expressed in subsets of respiratory epithelial cells in the developing lung, including nonciliated bronchiolar and rarely in nonciliated bronchial respiratory epithelial cells .[4] At the time of birth and postnatally, TTF expression is detected primarily in Type II epithelial cells and in subsets of bronchiolar epithelial cells at least in anomalies of the lung such as cystic adenomatoid malformations.[5] TTF- 1 binds to and activates the transcription of pulmonary surfactant proteins (SP-A, SP-B, and SP-C) and Clara cell secretory protein (CCSP).[5]

Pulmonary hypoplasia associated with congenital diaphragmatic hernia (CDH) remains a major therapeutic problem. Abnormal growth and differentiation of the lungs in infants with CDH have been documented as retarded development of the pulmonary acinus, fewer alveoli in the ipsilateral and the contralateral lung, markedly thickened alveolar walls, increased interstitial tissue, markedly diminished alveolar air space, and gas-exchange surface area.[6]
Lung, hypoplasia and immaturity observed in the fetal rat model of CDH, induced by prenatal exposure to nitrofen, are very similar to those observed in the human malformation.[7] Using the above-mentioned animal model, recently, low expression of TTF-1 was found in hypoplastic lungs suggesting that TTF-1 is an essential transcription factor in lung formation and down-regulation of this transcription factor influences both differentiation of respiratory epithelial cells and the expression of target genes, including surfactant proteins and Clara cell secretory protein.[8,9]
Antenatal glucocorticoid (CS) treatment in premature babies and in the nitrofen rat model showed that CS enhances the maturation of the alveolar portion of the respiratory epithelium, increasing Type II cell differentiation and results in thinning of the pulmonary mesenchyme.[10]
Recently, it was reported that antenatal CS restores the low expression of TTF-1 in hypoplastic lungs of the nitrofen rat model resulting in marked improvements in lung maturity and surfactant production.[11]
Because of the potential importance of TTF-1 in the early differentiation of respiratory

epithelium and its suggested important role in lung development with resulting hypoplasia due to alterations of its expression, we assessed the immunohistochemical localization of TTF-1, as a marker of lung morphogenesis in normal human lung development, and in age-matched controls of human lung specimen in hypoplastic lungs of human CDH and other forms of lung hypoplasia.

5.3 Materials and methods

Tissue Specimens

Following approval by the University Ethical Committee of the experimental design and Protocols, lung tissue specimens used in this study were retrieved from the archives of the Department of Pathology, Erasmus University Medical Center, Rotterdam. The so-called developmental group, (12 weeks gestation till term) included 47 lung specimens. As published before, they are derived of a series of normal human embryos (69 in total).[12] The congenital hypoplasia group included 8 term CDH patients who died within 12 hours after birth. including 5 cases who died within one hour. Moreover, 3 term CDH patients, who had one or more courses of 12.5 mg betamethasone according to a protocol proposed by the CDH international study group (principal investigator Dr. K. Lally, Houston, USA) and 4 term born CDH patients following ECMO therapy were evaluated. The age of the lung samples in this group was 2 days in 2 patients who died of intracranial hemorrhage during ECMO for which ECMO-therapy was stopped and 4 days in the other 2 cases due to isoelectric EEG (brain death) due to prolonged pre-ECMO periods of severe asphyxia. For comparison, 6 term patients, who died of other form of pulmonary hypoplasia, served as comparative specimen. This group of patients had a mean of 40 weeks post-conceptional age (39 – 41) and their diagnosis were arthrogryposis 2, bilateral renal agenesis 2, multiple congenital abnormalities and Pena-Shokeir syndrome in one patient each.

All autopsy lung specimens were fixed by routine immersion in 4% buffered formalin and embedded in paraffin. Five µm-thick sections were mounted on 3-amino-propyl-trioxysilane (Sigma, St Louis, MO, USA) coated glass slides and processed for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed using a standard avidin-biotin complex method. In brief, after deparaffinization in xylene and rehydration through graded alcohol, slides were treated with 0.3% H_2O_2 in methanol to block the endogenous peroxidase activity. Slides were then subjected to a 15 min. microwave treatment in citric acid buffer, pH 6.0.

After a blocking step with 10% normal goat serum (Dako, Glostrup, Denmark) for 15 min., slides were incubated for 30 min. at room temperature with mouse monoclonal antibody against thyroid transcription factor-1 (Neomarkers, CA, USA), dilution 1:100. After rinsing with phosphate buffered saline (PBS), slides were incubated for 30 min. with a biotinylated secondary antibody (Biogenex, San Ramon, MO, USA) in a 1:50 dilution. Slides were rinsed again with PBS, incubated for 30 min. with peroxidase-conjugated streptavidin using a dilution of 1:50 (Biogenex, San Ramon, MO, USA). Peroxidase activity was then detected by diaminobenzidine tetrahydrochloride (Fluka, Neu-Ulm, Germany) with 0.3% H_2O_2 , for 7 min, and counterstained with hematoxylin. Negative controls were processed by omission of the primary antibody.

5.4 Results

The immunohistochemical TTF-1 nuclear staining was evaluated by light Microscopy for three different areas of the airways including:

1. intrapulmonary bronchi with a pseudostratified columnar ciliated epithelium and a partial or complete cartilaginous ring,
2. intermediate airways so-called terminal bronchioles lined with cuboidal ciliated epithelium and the nonciliated epithelium,
3. Distal airways: nonciliated cuboidal epithelium, and later sacculi and alveoli lined by Type 1 and Type H pneumocytes.

In the developmental group, nuclear staining for TTF-1 was observed in columnar and cuboidal nonciliated cells of the bronchiolar epithelia and in respiratory epithelial cells lining the distal air spaces from 12 weeks gestational age onward (Fig. 1, on page 100). By mid-gestation, nuclei of many epithelial cells of the terminal airways were labeled intensely, and TTF-1 staining was consistently present in distal bronchiolar cells. Beginning from 12 weeks gestational age, staining in nuclei of columnar cells in more proximal airways was absent or only a few scattered cells were labeled. At term, TTF-1 expression persisted in subsets of nonciliated distal bronchiolar epithelia and in alveolar Type 11 cells, whereas Type I cells remained negative (Fig. 2, on page 100). Because TTF-1 staining was restricted to the respiratory epithelium at all stages of normal human lung development, no staining of the surrounding mesenchyme was detected.
In different forms of pulmonary hypoplasia, the expression pattern of TTF-1 staining, was similar in the hypoplastic lungs (Fig. 3,4, on page 101) with the exception of a few samples that showed strong TTF-1 staining in both proximal and distal airway epithelial cells in CDH cases. The staining was restricted to nonciliated distal bronchiolar epithelia

and alveolar Type II cells as we observed also in lung hypoplasia due to other causes and in normal term human lung specimens (Fig. 3,4, on page 101). The three cases evaluated following prenatal CS treatment showed no significant differences in staining pattern either.

5.5 Discussion

Previous studies supported the concept that TTF-1 plays a critical role in cell differentiation during lung morphogenesis and enhances gene expression of surfactant proteins of the postnatal lung, at least in an experimental CDH model.[8,9] However, the precise role of TTF-1 in the multistep process of morphogenesis and differentiation of various pulmonary cell types remains unknown. In the human, TTF-1 expression was detected at a high level in the distal tips of the developing airways, and in both nonciliated respiratory epithelial cells of the distal bronchioles and the alveolar Type 11 epithelia] cells at term and postnatally.[13] This temporo-spatial expression of TTF-1 is in line with our findings in the normal human lung developmental series ranging from 12 weeks of gestation till term which served as reference series.

Previously, Coleman et al. demonstrated a diminished immunolabeling of TTF-1 in the peripheral pulmonary epithelium in normal rat lungs, TTF-1 expression was restricted to Type II pneumocytes. while loss of expression in all other peripheral epithelial cell types was found.[8] In nitrofen-induced hypoplastic lungs, this spatial distribution of TTF-1 staining was considered to reflect a delay in peripheral acinar development.[8,9] However, in the surgically created fetal lamb model of CDH, Benachi et al.[18] showed contradictory findings being the expression pattern of TTF-1 staining was the same both in normal and CDH lungs. They also detected an increase in the number of stained Type H cells (personal communication). In our study we observed no differences in the intensity or location of TTF-1 staining in hypoplastic lungs of human term born cases of lung hypoplasia and CDH cases compared with normal human lung specimen at term. In a recent publication of Zhou et al.[20] evaluating cases of human pulmonary hypoplasia including 2 CDH cases similar results using the same technique was reported. This suggests, although TTF-1 appears to have a potential important role in pulmonary morphogenesis, that at least in the human a pivotal role in the pathogenesis of lung hypoplasia is not very likely.
We are aware that the immunohistochemical staining used in our study can not be used to determine the level of expression nor the activity of the protein studied.

As intensity of staining is highly influenced by the quality of the material under investigation, the duration of the staining period, and the variability in immunohistochemistry we decided to stay away from any attempt. To quantify our results we are well aware of the fact that semiquantitative assessment of immunohistochemical staining is suggested in the literature published in high ranked journals like the Lancet.[14] and also used by us[15]. In case of fresh material it is ideal to combine both mRNA levels using Northern blot analysis or RT-PCR on tissue specimen.

Furthermore, the distribution of cells expressing TTF-1 was reported to be consistent with the overlapping distribution patterns of surfactant proteins A, B, C, and CCSP in the normal human lung, supporting its importance in the modulation of surfactant protein gene expression. Unchanged expression of TTF-1 both in normal and hypoplastic human lungs suggested to us that, transcriptional control of surfactant protein synthesis may not be mediated by TTF-1 alone, and that distinct members of other transcription factor families such as hepatocyte nuclear growth factor-3 (HNF-3) and GATA may act in combination to regulate TTF-1 expression.[16,17]
Especially intriguing are the findings that, antenatal CS therapy has been shown to improve differentiation of Type II cells. Recently, prenatal exposure to CS has been reported to restore down-regulation of TTF-1 gene expression in lung hypoplasia, at least in the nitrofen rat.[11] Considering, the potential beneficial effects of antenatal CS and postnatal ECMO treatment on CDH hypoplastic lungs, we also evaluated TTF-1 expression in these groups of CDH. The distribution of TTF-1 staining in both groups was similar to the expression pattern in the untreated croup of CDH. This finding is in accordance with the relative importance of TTF-1 in the pathogenesis of lung hypoplasia.

Another method which is nowadays evaluated to enhance pulmonary growth is tracheal occlusion.[18] Both antenatal CS as well as tracheal obstruction are subject to critical evaluation, especially concerning effects on epithelial cell differentiation, as recently reviewed by us.[19]
In the present study, we showed that there was no difference in TTF-1 expression in the developing early fetal and term normal and hypoplastic human lungs. Moreover this expression did not change with antenatal CS or postnatal ECMO treatment. Although TTF-1 appears to have a potential importance in lung morphogenesis, elucidation of other transcription factors regulating TTF-1 gene expression and function, may lead to better understanding of the role of TTF-1 in human pulmonary development.

5.6 References

1. Warburton D, Schwarz M, Tefft D, et al: The molecular basis of lung morphogenesis. Mech Dev 2000;15:55-81.
2. Ikeda K, Clark JC, Shaw-White JR, et al: Gene structure and expression of human thyroid transcription factor- 1 in respiratory epithelial cells. J Biol Chem 1995;270:8108-8114.
3. Oguchi H, Kimura S: Transcription factors and thyroid disease. Mol Med 1995;32:518-526.
4. Morotti RA, Gutierrez MC, Askin F, et al: Expression of thyroid transcription factor-1 in congenital cystic adenomatold malformation of the lung. Pediatr Dev Pathol 2000;3:455-461.
5. Bruno MD, Bohinski RJ, Huelsman KM, et al: Lung cell-specific expression of the murine surfactant protein A (SP-A) gene is mediated by interactions between the SP-A promoter and thyroid transcription factor-1. J Biol Chem 1995;270:6531-6536.
6. Kluth D, Tenbrick R, von Ekesparre M, et al: The natural history of congenital diaphragmatic hernia and pulmonary hypoplasia in the embryo. J Pediatr Surg 1993;28:456463.
7. Guilbert TW, Gebb SA, Shannon JM: Lung hypoplasia in the nitrofen model of congenital diaphragmatic hernia occurs early in development. Am J Physiol Lung Cell Mol Physiol 2000;279:L I 159-L I 171.
8. Coleman C, Zhao J, Gupta M, et al: Inhibition of vascular and epithelial differentiation in murine nitrofen-induced diaphragmatic hernia. Am J Physiol 1998;274:L636-L646.
9. Losada A. Xia H, Migliazza L, et al: Lung hypoplasia caused by nitrofen is mediated by down-regulation of thyroid transcription factor TTF-1. Pediatr Surg Int 1999;15:188-191.
10. Whitsett JA, Stahlman MT: Impact of advances in physiology, biochemistry, and molecular biology on pulmonary disease in neonates. Am J Respir Crit Care Med 1998;157:S67-S71.
11. Losada A. Tovar JA, Xia HM, et al: Down-regulation of thyroid transcription factor-1 gene expression in fetal lung, hypoplasia is restored by lucocorticoids. Endocrinology 2000;141:2166-2173.
12. van der Harten HJ, Brons JTJ, Schipper NW, et al: The prenatal development of the normal human skeleton: a combined ultrasonographic and postmortem radiographic study. Pediatr Radiol 1990;21:52-56.
13. Stahlman MT, Gray ME, Whitsett JA: Expression of thyroid transcription factor-1 (TTF-1) in fetal and neonatal human lung. J Histochem Cytochem 1996;44:673-678.
14. Giaid A, Michel RP, Stewart DJ, Sheppard M, Corrin B, Hamid Q. Expression of endothelin-1 in lungs of patients with cryptogenic fibrosing alveolitis. Lancet 1993;341:1550-1554
15. Shehata SMK, Sharma HS, Mooi WJ, Tibboel D. Expression Patterns of Heat Shock Protein in Lungs of Neonates with Congenital Diaphragmatic Hernia. Arch Surg 1999;134: (11):1248-53.
16. Stahlman MT, Gary ME, Whitsett JA: Temporal-spatial distribution of hepatocyte nuclear factor-3β in developing human lung and other foregut derivatives. J Histochem Cytochem 1998;46:955-962.

17. Shaw-White JR, Bruno MD, Whitsett JA: GATA-6 activates transcription of thyroid transcription factor-1. J Biol Chem 1999;274:2658-2664.
18. Benachi A, Chailley-Heu B, Delezoide AL, et al: Lung growth and maturation after tracheal occlusion in diaphragmatic hernia. Am J Resp Crit Care Med 1998;157:921-927.
19. van Tuyl M, Hosgor M, Tibboel D: Tracheal ligation and corticosteroids in congenital diaphragmatic hernia: for better for worse? Pediatr Res Vol. 2001;50:No. 4.
20. Zhou H, Morotti RA, Profitt SH et al. Expression of Thyroid Transcription Factor-1, Surfactant Proteins, Type I Cell-associated Antigen, and Clara Cell Secretory Protein in Pulmonary Hypoplasia. Pediatr Dev Pathol 2001;4:364-371.

Chapter 6

Tracheal ligation and corticosteroids in congenital diaphragmatic hernia: for better for worse?

Based on the article:
Tracheal ligation and corticoisteroids in congenital diaphragmatic hernia: for better for worse?
M. van Tuyl, M. Hösgör, D. Tibboel
Ped Res 2001;50:441-444.

6.1 Introduction

Congenital diaphragmatic hernia (CDH) continues to frustrate clinicians for several reasons: it is not possible to predict accurately the extent of pulmonary hypoplasia in the individual fetus and the postnatal response to treatment modalities such as inhalational nitric oxide (NO) is variable. Moreover, the use of extra corporeal membrane oxygenation (ECMO) carries a high morbidity and sometimes profound long-term sequelae.

Analysis of the literature reveals a myriad of treatment modalities proposed as "solutions" for the problem pulmonary hypoplasia in CDH. They range from termination of pregnancy following prenatal ultrasound to gentle handling of the lung postnatally in order to diminish iatrogenic damage of the fragile hypoplastic lungs.[1] The fetal sheep model of CDH opened the way to fetal intervention and endoscopic fetoscopic procedures (FETENDO) such as tracheal ligation/occlusion in the human.[2-7] Others have studied the basic mechanisms of pulmonary growth in a drug-induced CDH model (the Nitrofen rodent model) with or without the evaluation of additional treatment modalities such as corticosteroids and/or TSH releasing hormone (TRH).[8-14]

The supposed "lung growth" resulting from tracheal ligation is not founded on clean concepts of underlying mechanisms, although a variety of factors has been suggested.[15-17]

Intriguing are recent observations from different laboratories of the negative effects of tracheal ligation, in fetal sheep, on type II cell differentiation, as nicely evaluated by Kay *et al.* in this issue.[18] The question of whether we will end up with a combined approach of tracheal ligation/occlusion and maternal betamethasone therapy to rescue type II cell differentiation as optimal treatment of CDH in humans is therefore still open.
We have to bear in mind that the experiments described in the paper of Kay *et al.* were not conducted in a CDH model. As a consequence, we can only assume that the response will be the same in the hypoplastic lungs of CDH. However, no research data are available to support this assumption. Other questions are unanswered too, because the authors only assessed certain structural features of Type II cell density and markers of mRNA for two surfactant proteins. Whether steroids do more than up-regulate mRNA for the surfactant proteins or enhance function remains controversial. The study by Kay *et al.* involved no physiologic assessment of lung function, gas exchange or the development of pulmonary hypertension.

Before we apply the results of the experimental approach of Kay *et al.* in a clinical setting, we have to analyze the arguments for the use of corticosteroids to enhance lung

development in prenatally diagnosed CDH, because the few reports on the use of corticosteroids in human CDH consist of personal communications, individual case reports, and anecdotal small series. However prenatal steroids are used to enhance lung development in premature infants.

A meta-analysis of published studies on prenatal glucocorticoids in threatened premature labor of fetuses without CDH consistently demonstrated beneficial effects on neonatal outcome for those infants born at 24 to 34 weeks of gestation.[19] Therefore, the National Institutes of Health consensus recommends antenatal administration of corticosteroids at least for 24 h, but if possible for 48 h, to all fetuses between 24 and 34 weeks of gestation at risk of preterm delivery.[20] However, as often suggested, it is still contradictory whether lungs of animal and human infants with CDH are surfactant deficient and morphologic immature like lungs from premature infants with surfactant deficient lung disease that do benefit from antenatal corticosteroid therapy.[21] As a consequence it is hard to predict the significance of antenatal corticosteroids for the individual CDH patient. Moreover, we still do not know the long-term effects of antenatal corticosteroid treatment and there is growing evidence that these drugs may have adverse perinatal and longer term effects.[22,23] Profound effects on postnatal alveolar septation have already been documented.[24,25] In this light another important issue, recently raised by Smith *et al.*,[22] is whether to use multiple or single antenatal courses of corticosteroids which is currently in use.

Considering the high incidence of chronic lung disease in CDH infants[26] the same concern is warranted for postnatal use of corticosteroids as recently eluded upon by Stark *et al.* in another group of patients: extremely-low-birth-weight infants.[27]

We have to await the result of a recently started randomized controlled trial evaluating the use of betamethasone in prenatally diagnosed CDH, guided by the International CDH studygroup (principal investigator: Dr K. Lally, Houston, U.S.A.)

After demonstration of accelerated lung maturation in premature sheep by Liggins,[28,29] several experimental studies in the Nitrofen CDH rodent model as well as in the surgically created sheep model have shown the beneficial effects of antenatal corticosteroid therapy including acceleration of the synthesis and release of surfactant, reduction of alveolar septal thickness, increase in maximum lung volume and compliance, and improvement in the antioxidant defence mechanisms.[8-14] Prenatal use of corticosteroids in the Nitrofen model supposedly changes the pulmonary vascular architecture, although there are no reports of the expression of the glucocorticoid receptor in small pulmonary arteries. In contrast, expression of the thyroid hormone receptor has

been described.[30] The administration of glucocorticoids and TRH to Nitrofen-treated pregnant rats increased di-saturated phosphatidylcholine levels in the fetal offspring, reduced lung glycogen levels and significantly improved lung compliance and morphology.[8-11] In addition, in our CDH rat model the combination of dexamethasone and TRH treatment of pregnant rats did not affect survival during ventilation in the pups and decreased glutathione reductase.[14] We, therefore, concluded that antenatal administration of dexamethasone as a monotherapy would offer better prospects for randomized trials in prenatally diagnosed children with CDH than would the combination of dexamethasone and TRH.

6.2 Mechanisms of glucocorticoid action

During fetal and postnatal development, glucocorticoids function as signaling molecules to modulate the orderly sequence of differentiation in most tissues. From midgestation onwards, the fetus is exposed to increasing levels of cortisol of primarily fetal origin. Many studies, in humans as well as in animals, have provided strong evidence that the administration of glucocorticoids to the immature fetus accelerates lung maturation. Even "physiological stressors", such as infection and premature rupture of the membranes, have been shown to accelerate fetal lung maturation, indicating that endogenous fetal glucocorticoids are instrumental in the normal course of lung maturation too. Endogenous hormones, however, do not initiate alveolar epithelial maturation, but are only involved in the modulation of genes responsible for surfactant production (for review see ref. 31).

Corticosteroids are known to induce several components of surfactant and to increase saturated phosphatidylcholine by stimulating key enzymes involved in phospholipid synthesis, such as fatty acid synthetase, choline phosphate cytidylyltransferase, and lysophosphatidylcholine acyl CoA acyl transferase. In addition, they stimulate lamellar body development in type II cells, and increase both tissue and alveolar content of surfactant.[32] Finally, they also increase levels of the surfactant-associated proteins A, B, C and D. In addition to these positive effects on surfactant production, glucocorticoids also stimulate antioxidant enzyme activity.[33] Although still not completely defined, some of these effects appear to result largely from increased production of fibroblast-pneumonocyte factor by the fetal lung fibroblasts.[34]
In contrast to these positive effects of corticosteroids, a variety of negative effects have been documented in the literature. In cultured lymphocytes, glucocorticoids caused apoptosis and an arrest in the G1 phase of the cell cycle thereby affecting proliferation which may finally lead to a reduction in cell number.[35]

Also, prenatal dexamethasone treatment reduced overall DNA, but not the collagen content in lung tissue of neonatal rats.[36] Moreover, a recent study showed that prenatal administration of dexamethasone to premature rats exposed to prolonged hyperoxia resulted in increased fibrosis in the dexamethasone treated lungs compared with the lungs from untreated animals.[37]

In embryonic rat lung studies, corticosteroid treatment causes distorted branching, tubular dilatation, suppression of lung growth and epithelial cell proliferation, attenuation of mesenchymal tissue and compression of mesenchyme between adjacent epithelial tubules which represent the features of both distorted and accelerated maturation.[38,39]

6.3 Glucocorticoid-receptor interaction

Glucocorticoids exert their effects *via* a nuclear receptor of the steroid hormone receptor superfamily. This superfamily includes a number of ligand-responsive transcriptional enhanced proteins, including the glucocorticoid and thyroid hormone receptors. All members of the family share a highly conserved modular structure, with discrete functional domains for hormone binding, DNA binding, and transactivation.[40] Concomitant with a rise in glucocorticoid plasma levels near term, enhancement of glucocorticoid receptor gene expression has been shown by Sweezy *et al.* in the fetal rat.[41] Autoradiographic localization studies demonstrated increased glucocorticoid receptor gene expression in the mesenchyme, and more specifically in those mesenchymal cells adjacent to the terminal saccular epithelium (the cell population responsible for fibroblast-pneumonocyte factor production).[42,43] To enhance our understanding of the potential effects of corticosteroids on the hypoplastic lung, we studied the glucocorticoid (GC)-receptor in hypoplastic CDH rat lungs and age-matched controls. No significant differences were observed in the tissue distribution or time of appearance of the GC-receptor under these experimental conditions.[30]

6.4 Modulation of pulmonary growth in CDH

Findings presented at the 16th annual ECMO meeting in Keystone (March 2000) by investigators from Boston (J. Wilson, J. Schnitzer) and Liverpool (P. Losty) made it likely that members of the fibroblast growth factor family play important roles in an organotypic culture system of hypoplastic lungs in Nitrofen-induced CDH. During that same meeting Ch. Stolar and colleagues advocated a pivotal impact of other well known growth factors,

such as vascular endothelial derived growth factor (VEGF), on lung growth following tracheal ligation in the sheep CDH model as well as in an organotypic culture system including both the embryonic rat heart and lung buds. This is especially intriguing because human CDH lungs showed abnormal expression of VEGF, even in the endothelium of pulmonary arteries less than 75 μm.[44]

In the light of the international corticosteroid treatment protocol of prenatally diagnosed CDH, including over 300 patients, and the National Institutes of Health sponsored tracheal ligation/occlusion study of prenatally diagnosed CDH patients with the highest risk ("liver up" patients), we must bear in mind that our knowledge of the optimal way to modulate prenatal pulmonary growth and differentiation is far from complete.

Although corticosteroids are known to induce apoptosis, they exert intriguing effects, not yet evaluated in humans, on VEGF expression and platelet-derived growth factor (PDGF), and on the PDGF-A receptor and pulmonary fibroblasts.[45-47]
The presumed negative effects of tracheal ligation on type II cell differentiation in experimental CDH models are reason to carefully evaluate the use of corticosteroids in human cases. Not only the overall outcome should be assessed but also the more fundamental cell-biologic changes occurring during the transition from the saccular phase of pulmonary development to the alveolar phase, which takes place late in gestation in humans. The application of new technology such as the use of micro-arrays will help us to understand pulmonary development at the molecular level. The time dependent expression of a number of genes relevant for the progression in lung development in general[48,49] should be taken into account, both in spontaneous as well as in experimental induced CDH. In this way we will be able to pinpoint the exact mechanisms resulting into pulmonary hypoplasia in CDH as well as the effect of modulating "agents" such as corticosteroids. We can not run the risk that tracheal ligation with or without corticosteroids although "again" suggested as magic bullets for the improvement of the survival rate in newborns with CDH, turns out to be a new chapter in the book of unanswered questions in congenital diaphragmatic hernia.

6.5 References

1. IJsselstijn H, Tibboel D. The lungs in congenital diaphragmatic hernia: do we understand? Pediatr Pulmonol 1998;26:204-218.
2. Hashim E, Laberge JM, Chen MF, Quillen EW Jr. Reversible tracheal obstruction in the fetal sheep: effects on tracheal fluid pressure and lung growth. J Pediatr Surg 1995;30:1172-1177.
3. Wilson JM, DiFiore JW, Peters CA. Experimental fetal tracheal ligation prevents the pulmonary hypoplasia associated with fetal nephrectomy: possible application for congenital diaphragmatic hernia. J Pediatr Surg 1993;28:1433-1440.
4. Piedboeuf B, Laberge JM, Ghitulescu G, Gamache M, Petrov P, Belanger S, Chen MF, Hashim E, Possmayer F. Deleterious effect of tracheal obstruction on type II pneumocytes in fetal sheep. Pediatr Res 1997;41:473-479.
5. Benachi A, Chailley-Heu B, Delezoide AL, Dommergues M, Brunelle F, Dumez Y, Bourbon JR. Lung growth and maturation after tracheal occlusion in diaphragmatic hernia. Am J Respir Crit Care Med 1998;157:921-927.
6. Flageole H, Evrard VA, Piedboeuf B, Laberge JM, Lerut TE, Deprest JA. The plug-unplug sequence: an important step to achieve type II pneumocyte maturation in the fetal lamb model. J Pediatr Surg 1998;33:299-303.
7. Harrison MR, Mychaliska GB, Albanese CT, Jennings RW, Farrell JA, Hawgood S, Sandberg P, Levine AH, Lobo E, Filly RA. Correction of congenital diaphragmatic hernia in utero IX: fetuses with poor prognosis (liver herniation and low lung-to-head ratio) can be saved by fetoscopic temporary tracheal occlusion. J Pediatr Surg 1998;33:1017-1023.
8. Suen HC, Bloch KD, Donahoe PK. Antenatal glucocorticoid corrects pulmonary immaturity in experimentally induced congenital diaphragmatic hernia in rats. Pediatr Res 1994;35:523-529.
9. Suen HC, Losty PD, Donahoe PK, Schnitzer JJ. Combined antenatal thyrotropin-releasing hormone and low-dose glucocorticoid therapy improves the pulmonary biochemical immaturity in congenital diaphragmatic hernia. J Pediatr Surg 1994;29:359-363.
10. Losty PD, Suen HC, Manganaro TF, Donahoe PK, Schnitzer JJ. Prenatal hormonal therapy improves pulmonary compliance in the nitrofen-induced CDH rat model. J Pediatr Surg 1995;30:420-426.
11. Losty PD, Pacheco BA, Manganaro TF, Donahoe PK, Jones RC, Schnitzer JJ. Prenatal hormonal therapy improves pulmonary morphology in rats with congenital diaphragmatic hernia. J Surg Res 1996;65:42-52.
12. Schnitzer JJ, Hedrick HL, Pacheco BA, Losty PD, Ryan DP, Doody DP, Donahoe PK. Prenatal glucocorticoid therapy reverses pulmonary immaturity in congenital diaphragmatic hernia in fetal sheep. Ann Surg 1996;224:430-439.

13. Hedrick HL, Kaban JM, Pacheco BA, Losty PD, Doody DP, Ryan DP, Manganaro TF, Donahoe PK, Schnitzer JJ. Prenatal glucocorticoids improve pulmonary morphometrics in fetal sheep with congenital diaphragmatic hernia. J Pediatr Surg 1997;32:217-222.
14. IJsselstijn H, Pacheco BA, Albert A, Sluiter W, Donahoe PK, De Jongste JC, Schnitzer JJ, Tibboel D. Prenatal hormones alter antioxidant enzymes and lung histology in rats with congenital diaphragmatic hernia. Am J Physiol 1997;272:L1059-L1065.
15. Kitano Y, Yang EY, von Allmen D, Quinn TM, Adzick NS, Flake AW. Tracheal occlusion in the fetal rat: a new experimental model for the study of accelerated lung growth. J Pediatr Surg 1998;33:1741-1744.
16. Kitano Y, Adzick NS. New developments in fetal lung surgery. Curr Opin Pulm Med 1999;5:383-389
17. Kitano Y, Davies P, von Allmen D, Adzick NS, Flake AW. Fetal tracheal occlusion in the rat model of nitrofen-induced congenital diaphragmatic hernia. J Appl Physiol 1999;87:769-775.
18. Kay S, Laberge J-M, Flageole H, Richardson S, Belanger S, Piedboeuf B. Use of antenatal steroids to counteract the negative effects of tracheal occlusion in the fetal lamb model. Pediatr Res 2001;50:495-501.
19. Crowley PA. Antenatal corticosteroid therapy: a meta-analysis of the randomized trials, 1972 to 1994 Am J Obstet Gynecol 1995;173:322-335.
20. Editorial. Effect of corticosteroids for fetal maturation on perinatal outcomes. NIH Consensus Development Panel on the Effect of Corticosteroids for Fetal Maturation on Perinatal Outcomes. JAMA 1995;273:413-418.
21. IJsselstijn H, Zimmermann LJ, Bunt JE, de Jongste JC, Tibboel D. Prospective evaluation of surfactant composition in bronchoalveolar lavage fluid of infants with congenital diaphragmatic hernia and of age-matched controls. Crit Care Med 1998;26:573-580.
22. Smith GN, Kingdom JC, Penning DH, Matthews SG. Antenatal corticosteroids: is more better? Lancet 2000;355:251-252.
23. Kay HH, Bird IM, Coe CL, Dudley DJ. Antenatal steroid treatment and adverse fetal effects: what is the evidence? J Soc Gynecol Investig 2000;7:269-278.
24. Tschanz SA, Burri PH. Postnatal lung development and its impairment by glucocorticoids Pediatr Pulmonol Suppl 1997;16:247-249.
25. Ikegami M, Jobe AH, Newnham J, Polk DH, Willet KE, Sly P. Repetitive prenatal glucocorticoids improve lung function and decrease growth in preterm lambs. Am J Respir Crit Care Med 1997;156:178-184.
26. Bos AP, Hussain SM, Hazebroek FW, Tibboel D, Meradji M, Molenaar JC. Radiographic evidence of bronchopulmonary dysplasia in high-risk congenital diaphragmatic hernia survivors. Pediatr Pulmonol 1993;15:231-234.

27. Stark AR, Carlo WA, Tyson JE, Papile LA, Wright LL, Shankaran S, Donovan EF, Oh W, Bauer CR, Saha S, Poole WK, Stoll BJ; National Institute of Child Health and Human Development Neonatal Research Network. Adverse effects of early dexamethasone treatment in extremely-low-birth-weight infants. N Eng J Med 2001;344:95-101.
28. Liggins GC. Premature delivery of foetal lambs infused with glucocorticoids. J Endocrinol 1969;45:515-523.
29. Liggins GC. Premature parturition after infusion of corticotrophin or cortisol into foetal lambs. J Endocrinol 1968;42:323-329.
30. Keijzer R, Blommaart PJE, Lamers WH, Tibboel D. An ontogenic study of steroid hormone receptor expression in lungs of congenital diaphragmatic hernia rats and age matched controls. Am J Respir Crit Care Med 1998;157:A174.
31. Ballard PL. Glucocorticoid regulation of lung maturation. Mead Johnson Symp Perinat Dev Med 1997;30:22-27.
32. Ballard PL. Hormonal regulation of pulmonary surfactant. Endocr Rev 1989;10:165-181.
33. Walther FJ, Ikegami M, Warburton D, Polk DH. Corticosteroids, thyrotropin-releasing hormone, and antioxidant enzymes in preterm lamb lungs. Pediatr Res 1991;30:518-521.
34. Smith BT, Post M. Fibroblast-pneumonocyte factor. Am J Physiol 1989;257:L174-L178.
35. King KL, Cidlowski JA. Cell cycle regulation and apoptosis. Annu Rev Physiol 1998;60:601-617.
36. Schellenberg JC, Liggins GC, Stewart AW. Growth, elastin concentration, and collagen concentration of perinatal rat lung: effects of dexamethasone. Pediatr Res 1987;21:603-607.
37. Chen Y, Martinez MA, Frank L. Prenatal dexamethasone administration to premature rats exposed to prolonged hyperoxia: a new rat model of pulmonary fibrosis (bronchopulmonary dysplasia). J Pediatr 1997;130:409-416.
38. Oshika E, Liu S, Singh G, Michalopoulos GK, Shinozuka H, Katyal SL. Antagonistic effects of dexamethasone and retinoic acid on rat lung morphogenesis. Pediatr Res 1998;43:315-324.
39. Oshika E, Liu S, Ung LP, Singh G, Shinozuka H, Michalopoulos GK, Katyal SL. Glucocorticoid-induced effects on pattern formation and epithelial cell differentiation in early embryonic rat lungs. Pediatr Res 1998;43:305-314.
40. Evans RM. The steroid and thyroid hormone receptor superfamily. Science 1988;240:889–895.
41. Sweezey NB, Ghibu F, Gagnon S, Schotman E, Hamid Q. Glucocorticoid receptor mRNA and protein in fetal rat lung in vivo: modulation by glucocorticoid and androgen. Am J Physiol 1998;275:L103-L109.
42. Beer DG, Butley MS, Cunha GR, Malkinson AM. Autoradiographic localization of specific [3H]dexamethasone binding in fetal lung. Dev Biol 1984;105:351-364.
43. Caniggia I, Tseu I, Han RN, Smith BT, Tanswell K, Post M. Spatial and temporal differences in fibroblast behavior in fetal rat lung. Am J Physiol 1991;261:L424-L433.

44. Shehata SM, Mooi WJ, Okazaki T, El-Banna I, Sharma HS, Tibboel D. Enhanced expression of vascular endothelial growth factor in lungs of newborn infants with congenital diaphragmatic hernia and pulmonary hypertension. Thorax 1999;54:427-431.
45. Nauck M, Roth M, Tamm M, Eickelberg O, Wieland H, Stulz P, Perruchoud AP. Induction of vascular endothelial growth factor by platelet-activating factor and platelet-derived growth factor is downregulated by corticosteroids. Am J Respir Cell Mol Biol 1997;16:398-406.
46. Warshamana GS, Martinez S, Lasky JA, Corti M, Brody AR. Dexamethasone activates expression of the PDGF-alpha receptor and induces lung fibroblast proliferation. Am J Physiol 1998;274:L499-L507.
47. Bruce MC, Honaker CE, Cross RJ. Lung fibroblasts undergo apoptosis following alveolarization. Am J Respir Cell Mol Biol 1999;20:228-236.
48. Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, Yagishita N, Matsui D, Koga Y, Itoh N, Kato S. FGF10 is essential for limb and lung formation. Nat Genet 1999;21:138-141.
49. Bellusci S, Furuta Y, Rush MG, Henderson R, Winnier G, Hogan BL. Involvement of Sonic Hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. Development 1997;124:53-63.

Chapter 7

General discussion

Congenital Diaphragmatic Hernia, many questions few answers

Based on the article:
Congenital Diaphragmatic Hernia, many questions few answers
M. Hösgör, D. Tibboel
Pediatric Pulmonology, accepted for publication

7.1 Introduction

Congenital diaphragmatic hernia remains one of the major challenges in the neonatal and pediatric surgical ICU for various reasons: the wide variability in clinical presentation, the severe respiratory insufficiency, and the high pulmonary hypertension, either continuously or intermittently.[1,2]
It is the combination of pulmonary hypoplasia together with the unpredictable response to a variety of treatment modalities applied to decrease the pulmonary vascular resistance that mainly determines the outcome in the individual newborn.[3] Overall mortality, is reported to range from 20 to 60%, but these rates are hardly comparable because of differences in case selection.[4,5,6] Likewise, it is hard to compare treatment results, especially because there are no standards for documenting patients with congenital diaphragmatic hernia. Although the condition has derived its name from the primary anomaly, the hole in the diaphragm, in the last two decades clinicians have come to realize that the diaphragmatic defect does not determine survival. Animal studies and evaluation of serial sections of early human embryo's, have convincingly shown that disturbed pulmonary development during the embryonic phase should be considered the primary defect.[7]

7.2 Etiological aspects

It is now generally accepted that major structural abnormalities result from specific genetic abnormalities in selected cases such as Smith Lemli Opitz syndrome and DiGeorge syndrome (chromosome 22q11). In many instances however clinicians remain empty handed when trying to explain to the parents the cause of their offspring's anomaly.
They than tend to fall back on the term "multifactorial," which is synonymous with "I don't know", because it is very hard to identify specific environmental factors including dietary components, medication or environmental pollutants as the causes of major congenital anomalies.[8] CDH is not an exception to this rule. Considering the wide variability in phenotypic appearance of children with CDH; the real incidence including cases with intra-uterine fetal death as well as terminations of pregnancies following prenatal chromosomal evaluation (Trisomy 13,18,21) and syndromic cases (Fryn's syndrome) without a clear underlying genetic background, CDH should be considered a disease rather than a single congenital anomaly. In this way it is hard to identify "the patient" with CDH. Both the genetic background and the effect of environmental factors may vary considerably in the individual patient leading to abnormal early lung

development and closure of the diaphragm. No candidate genes have been identified so far, although chromosome 15 anomalies and a variety of other chromosomal abnormalities have been suggested in the literature. CDH is well known to occur as part of major numerical chromosomal anomalies such as the trisomies 18, 13 and 21.[4] Routine chromosomal analysis with sensitive methods to identify even point mutations or small deletions, together with availability of DNA data bases of patients and parents might provide important information to elucidate the etiology in individual cases.

7.3 Developmental aspects

Pediatric surgeons and obstetricians such as Michael Harrison, Scott Adzick and Jay Wilson, Jan Deprest and Bruno Piedboeuf performed a wonderful series of experiments especially in the sheep and rabbit, to induce congenital diaphragmatic hernia at different developmental stages. Much of our knowledge is based on these animal models in which the primary development of the lungs and diaphragm is normal and both insult to the developing lung and the hole of the diaphragm are created surgically.[9-13]
These models has provided valuable data with regard to type II cells differentiation, pulmonary vascular developmental abnormalities, and ventilatory strategies in the newborn lambs with CDH, including the effects of surfactant therapy and liquid ventilation. Other investigators have used this approach in rodent animal models (rats and mice), which provided information about the closure process of the diaphragm and the early stages of (ab)normal lung development. In these models the defect is induced at the stage when the foregut has just separated into the esophagus and the trachea, which in rodents is usually at 9 to 10 days of gestation. In rats the diaphragm does not close until day 17 (term day 21).[14-18]
These models even enable to isolate the lung buds and to culture them with or without the heart, so that branching morphogenesis in detail and the effect of mutational deletions can be investigated.[19]

A different model is the Nitrofen-induced rodent model. This, however, has the disadvantage that the toxic substance applied does not only effect the developing diaphragm and lungs but also has a teratogenic effect on other organ systems, such as the central nervous system, skeleton and heart. What in the near future will turn out to be the ideal model for unrevelling the etiology and pathogenesis of CDH is still unknown. The classical hypothesis in the rodent model declaring the diaphragm the primary defect and the pulmonary hypoplasia a secondary phenomenom caused by compression of the developing lung by the bowel loop, has become more and more disputed. Recent studies

have shown that the pulmonary hypoplasia occurs independently of the presence of the defect in diaphragm and is already present for several days before the normal closure process of the diaphragm takes place during ontogeny.[7]
In the rodent model competition for space between the developing lungs and the liver has been proven by longitudinal underwater dissection. Although all these studies provide some information about the pathogenesis of CDH, it is still unclear what the etiology is in the majority of cases at least in humans.

7.4 Prenatal modulation of lung growth

Prenatal modulation of lung growth has been investigated, through both surgical correction and hormone treatment, in animal models as well as in selected cases of human CDH.
The disappointing effects of postnatal treatment together with the unpredictable responses to new treatment modalities such as high frequency oscillation (HFO), extra-corporeal membrane oxygenation (ECMO) and finally liquid ventilation, have stimulated studies aimed at correcting the defect intra-uterine and subsequent catch up growth, or at modulating differentiation of the gas exchange area by hormones like corticosteroids. The San Francisco group headed by Michael Harrison as well as the research groups in Boston (Jay Wilson) and Philadelphia (Scott Adzick) have shown that either intra-uterine surgery, open or closed is a relatively safe event even in the human fetus.[20] An NIH-sponsored trial failed to show the benefit of intra-uterine surgical correction in unselected patients following prenatal diagnoses compared with postnatal treatment. This is why identification of the individual patient suitable for prenatal lung growth modulating procedures, such as temporary obstruction of the trachea, has become one of the major goals of the ongoing NIH-sponsored trial. Only patients with a predicted high mortality as revealed by a low lung head ratio in case of so-called "liver up patients" are eligible for inclusion in studies using a so-called "closed procedure" with a detachable balloon to obstruct the trachea. Experimental studies evaluating the effect of tracheal obstruction of the fetal lung have provided data evidencing that lung growth really takes place, i.e. increased DNA\ protein levels and cell counts. Even the vascular abnormalities are reported to diminish following tracheal obstruction. The effects on the type- II cell differentiation remain to be documented in more detail, especially when combined therapies such as tracheal obstruction with prenatal corticosteroids are considered. Intra-uterine fetal surgical manipulation of pulmonary growth performed in selected centers should yield an adequate number of patients for randomized controlled trials. Otherwise we will only have anecdotal reports and thus remain in uncertainty about patient selection.

In view of the well documented positive effects of prenatal corticosteroids in threatened premature labor and those of additional corticosteroids following prenatal induction of CDH in the Nitrofen rodent model, corticosteroids have been used sporadically, with varying results. The use of corticosteroids in prenatally diagnosed CDH in humans is partly based on the assumed surfactant deficiency in association with CDH. Very few studies in fact have documented a primary surfactant deficiency in human CDH.[21] It is recommended, therefore, that the use of corticosteroids should be restricted to randomized controlled trials.[22] Such a trial is now being performed under guidance of the international CDH study group, rendering 150 patients in both groups to be potentially conclusive.

Until the results of trials like those became known it is impossible to decide in favor or against the use of corticosteroids in prenatally diagnosed CDH.
Intriguing is the observation that not only thyroid hormone, given prenatally in the Nitrofen model, diminishes the immaturity of the lungs, it appears that the addition of retinoic acid in this model decreased the number of diaphragmatic defects as well as the amount of pulmonary hypoplasia.[23] Whether vitamin A can be used as an appropriate modulator of lung growth, given its documented effects on pulmonary epithelial cells, remains to be investigated in more detail. Other known growth factors, with the exception of fibroblast growth factor (FGF) 10, have proven to be unable to enhance pulmonary growth following isolation of lung buds in Nitrofen-induced pulmonary hypoplasia under in-vitro circumstances. As a so-called dual hit sequence determines the situation at birth,[7] we should be aware that after the initiation of abnormal lung development, the intensity of competition for space between the developing lungs and intra-thoracic organs such as the liver and/or bowel loops, determines the defect's "natural history" in the second and third trimesters of gestation. This interference with fetal breathing movements determines

1. the extent of pulmonary hypoplasia,
2. the size of the defect,
3. the maturational delay of vascular remodeling, and
4. the differentiation of the gas exchange area.

In fact, neither surgically or hormonal modulation in the second or third trimester of gestation will effect the primary defect, but most probably only change the "natural history" of the defect.

It is obvious that after prenatal diagnosis parents may decide not to continue the pregnancy. This decision should only be made following peer review by a so-called perinatal treatment team, including all specialists potentially involved in the care of both the parents and the fetus with a CDH. It must never be based on the opinion of an

individual consultant. As long as solid prenatal determinants for survival of the individual patient are not available, individual counseling is very difficult.

7.5 Postnatal treatment

Most hospitals now have treatment protocols for the standard care of the child with CDH. Treatment, however, shows a large variety, and enough each modality has been considered the "optimal" treatment, none has ever been tested in a randomized controlled trial, except for the use of nitric oxide (NO) and ECMO. None of the trials testing NO and ECMO however, was primarily developed to evaluate the effect of NO or ECMO on cases with CDH. By inclusion of CDH cases ECMO and NO in the trials neither proved to have a benefit or even resulted a worse outcome. We have come to realize that aggressive ventilatory support many destruct the vulnerable lung, thus inducing secondary surfactant deficiency on inactivation, accumulation of inflammatory cells and release of a whole variety of cytokines. The concept of "gentle handling" is one that has gained more acceptance nowadays. Another is the use of high frequency oscillation in an attempt to diminish shear forces and volutrauma.[24] This has resulted in even up to 90% survival rates as reported by centers in Canada and the USA (Toronto, Boston).
Iatrogenic lung damage, which sometimes even results into progressive respiratory insufficiency and death, or in other cases chronic lung disease with the need for supplementary oxygen, diuretics and potentially nocturnal pulmonary hypertensive crises has consequently drawn more attention.

The use of surfactant in the treatment of CDH is still insufficiently evaluated, both with regard to dosage and timing. A number of clinics will give surfactant to every patient following prenatal diagnosis, which is bound to produce an unknown number of over-treated babies. There is still space and a need for a properly design trial in this respect. Delayed surgery following stabilization of the patient, has now become the accepted mode of surgical repair. However clinics do not show conformity about optimal timing, which they may range from several hours in patients with very modest ventilatory needs to several weeks in patients who had been subjected to ECMO therapy and were weaned off successfully.

With the introduction of new therapies it is essential to outweigh the balance between decreasing mortality and increasing morbidity. Attention is now being focussed on the long-term morbidity, which is not only related to the abnormal prenatal lung growth potentially superimposed by iatrogenic ventilatory damage, but also to gastrointestinal

problems such as severe gastro-esophageal reflux with requiring operative correction in selected cases. There is a strong need for prospective long-term follow-up programs taking into account the broad spectrum of morbidity with a special interest in

1. somatic growth and neurodevelopmental outcome;
2. lung function, as it is known that the lung perfusion scan will not significantly improve, while alveoli will form extensively after birth;
3. gastrointestinal dysmotility and gastro-esophageal reflux, and
4. late death caused by continuous pulmonary hypertension and decreased level of activities in daily life.

7.6 Conclusion

Congenital diaphragmatic hernia remains one of the diseases in which many questions remain unanswered. International collaboration both at the level of etiology (DNA data bases) and that of pre- and postnatal treatment modalities, should be peformed in the form of properly designed randomized controlled trials, if feasible. The international CDH study group provides an important "tool" in this respect.[27]
Such trials should go hand in hand with the evaluation of developmental biological aspects as studied in various animal models[28-30] thereby circumventing the "trial and error" approach that many clinicians still adopt with regards to the individual patient with congenital diaphragmatic hernia.[31]

Table I summarizes the different aspects of congenital diaphragmatic hernia ranging from etiology to postnatal treatment as well as the level of evidence based on personal experience and literature data.

Table 1 CDH, many questions; few answers

Table 1a

Understanding congenital diaphragmatic hernia: developmental aspects

	Subject	Level of knowledge
Primary defect		Absent
	Genetic	Occasional
	Environmental	Suggestions
Lung growth		
	Signalling molecules	Fractional
	Receptors	" "
	Transcription factors	" "
Diaphragmatic development		
	Induction	Suggestive
	Relation with lung	"Neighbours"
	Genes	Fractional
Variability of the defect and pulmonary hypoplasia		
	Cause	Absent
	Timing	Suggestions
	Relation with position of liver	Ultrasound
		Clinical observations
Pulmonary parenchyma and vascular development		
	Common pathways	Suggestions
	Protein-sharing	Immunohistochemistry
		In Situ hybridization
	Proliferation-apoptosis index	Preliminary data
Modulation of pulmonary growth		
	Vitamin A	Established
	Thyroid hormone	Few data available
	Corticosteroids	Contradictionary findings
Tracheal occlusion:		
	Mechanisms	Speculative
	Human application	NIH sponsored trial
Vascular abnormalities		
	Morphology	Well described
	Function	"Surprise"
	Developmental arrest	Descriptive
	Remodelling	Extrapolated

Table 1b

Understanding congenital diaphragmatic hernia: animal models

Animal models	Research focus
Sheep CDH model	Modulation of lung growth
Rodent model	Developmental biology
Rabbit model	Biology and function

Table 1c

Understanding congenital diaphragmatic hernia: the clinical situation

Treatment	Evidence of benefit
Artificial ventilation, including gentle air	Clearly beneficial
HFO	Emerging data: (non)-believers
NO	Unpredictable
ECMO	One trial, no effect
Surfactant	Case reports, no RCT available
Liquid ventilation	Case reports, neonatal trials emerging
Antenatal corticosteroids	"For better, for worse"
Fetal surgery	Under investigation

7.7 References

1. Beresford, M. W., and N. J. Shaw. Outcome of congenital diaphragmatic hernia. Pediatr Pulmonol 2000;30(3):249-56.
2. Tibboel, D., Shehata, S.M.K., Guldemeester, A.H. The pulmonary vasculature in congenital diaphragmatic hernia. In E. K. Weir, Archer, S.L., Reeves, J.T., editor. The fetal and neonatal pulmonary circulations. Armank, NY: Futura publishing company, Inc, New York 1999.
3. Thebaud, B., J. C. Mercier, and A. T. Dinh-Xuan. Congenital diaphragmatic hernia. A cause of persistent pulmonary hypertension of the newborn which lacks an effective therapy. Biol Neonate 1998;74(5):323-36.
4. Torfs, C. P., C. J. Curry, T. F. Bateson, and L. H. Honore. A population-based study of congenital diaphragmatic hernia. Teratology 1992;46(6):555-65.
5. Benjamin, D. R., S. Juul, and J. R. Siebert. Congenital posterolateral diaphragmatic hernia: associated malformations. J Pediatr Surg 1988;23(10):899-903.
6. Fauza, D. O., and J. M. Wilson. Congenital diaphragmatic hernia and associated anomalies: their incidence, identification, and impact on prognosis. J Pediatr Surg 1994;29(8):1113-7.
7. Keijzer R., Liu J., Tibboel D. and Post M. Dual-hit hypothesis explains pulmonary hypoplasia in the nitrofen model of congenital diaphragmatic hernia. Ameri J Path 2000;156:1299-1306.
8. Tibboel, D., and A. V. Gaag. Etiologic and genetic factors in congenital diaphragmatic hernia. Clin Perinatol 1996;23(4):689-99.
9. Harrison M.R., Ross N.A. and A.A. de Lorimier. Correction of congenital diaphragmatic hernia in utero III. Development of a successful surgical technique using abdominoplasty to avoid compromise of umbilical blood flow. J Pediatr Surg 1981;16(6): 934-942.
10. Harrison M.R. et al. Correction of congenital diaphragmatic hernia in utero IX: fetuses with poor prognosis (liver herniation and low lung-to-head ratio) can be saved by fetoscopie temporary tracheal occlusion. J Pediatr Surg 1998;33(7):1017-22; discussion 1002-3.
11. Flake A.W., Crombleholme T.M., Johnson M.P. et al. Treatment of severe congenital diaphragmatic hernia by fetal tracheal occlusion: Clinical experience with fifteen cases. Am J Obstet Gynaecol 2000;183:1059-1066.
12. Piedboeuf B. et al. Deleterious effect of tracheal obstruction on type II pneumocytes in fetal sheep. Pediatr Res 1997;41(4 Pt 1)473-479.
13. De Paepe M.E., Johnson B.D., Papakadus K. et al. Lung growth response after tracheal occlusion in fetal rabbits is gestational age-dependent. Am J Respir Cell Mol Biol 1999;21:65-76.
14. Kitano Y, Yang E.Y., von Allmen D. et al. Tracheal occlusion in the fetal rat: a new experimental model for the study of accelerated lung growth. J Pediatr Surg 1998;33:1741-1744.

15. Kitano Y., Kanai M., Davies P. et al. Lung growth induced by prenatal occlusion and its modifying factors: A study in the rat model of congenital diaphragmatic hernia. J Pediatr Surg 2001;36:251-259.
16. Cilley R.E. et al. Nitrofen dose-dependent gestational day-specific murine lung hypoplasia and left-sided diaphragmatic hernia. 1997;272(2 Pt 1):362-371.
17. Allan D.W. and Greer J.J. Pathogenesis of nitrofen-induced congenital diaphragmatic hernia in fetal rats [in Process Citation]. J Appl Physiol 1997;83(2):338-347.
18. Tenbrinck R. et al. Experimentally induced congenital diaphragmatic hernia in rats. J. Pediatr Surg 1990;25(4):426-429.
19. Mendelsohn C., Lohnes D., Decimo D. et al. Function of retinoic acid receptors during development. II. Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development 1994;120:2749-71.
20. Harrison M.R., Mychaliska G.B., Albanese C.T. et al. Correction of congenital diaphragmatic hernia in fetoscopic temporary tracheal occlusion. J Pediatr Surg 1998;33:1017-1022.
21. Ijsselstijn, H., L. J. Zimmermann, J. E. Bunt, J. C. de Jongste, and D. Tibboel. Prospective evaluation of surfactant composition in bronchoalveolar lavage fluid of infants with congenital diaphragmatic hernia and of age- matched controls. Crit Care Med 1998;26(3):573-80.
22. Van Tuyl, M., M. Hosgor, and D. Tibboel. Tracheal ligation and corticosteroids in congenital diaphragmatic hernia: for better for worse? Pediatr Res 2001;50(4):441-4.
23. Thebaud, B., A. M. Barlier-Mur, B. Chailley-Heu, A. Henrion-Caude, D. Tibboel, A. T. Dinh-Xuan, and J. R. Bourbon. Restoring effects of vitamin a on surfactant synthesis in nitrofen- induced congenital diaphragmatic hernia in rats. Am J Respir Crit Care Med 2001;164(6):1083-9
24. Wung J.T., Sahni R., Moffitt S.T., Lipsitz E., Stolar C.J. Congenital diaphragmatic hernia: survival treated with very delayed surgery, spontaneous respiration, and no chest tube. J Pediatr Surg 1995;30:406-409.
25. Kays D.W., Langham M.R. Jr., Ledbetter D.J., Talbert J.L. Detrimental effects of standard medical therapy in congenital diaphragmatic hernia. Ann Surg 1999;32:395-400.
26. Sakurai Y., Azarow K., Cutz E., Messineo A., Pearl R., Bohn D. Pulmonary barotrauma in congenital diaphragmatic hernia: a clinicopathological correlation. J Pediatr Surg 1999;34:1813-1817.
27. Clark R.H., Hardwin W.D., Jr. Hirschl R.B. et al. Current Surgical Management of congenital diaphragmatic hernia: a report from the Congenital Diaphragmatic Hernia Study Group. J Pediatr Surg 1998;33:1004-1009.
28. Warburton D., Schwarz M., Tefft D., Flores-Delgado G., Anderson K.D., and Cardoso W.V. The molecular basis of lung morphogenesis. Mech dev 2000;92:55-81.
29. Van Tuyl M. and Post M. From Fruit Flies to Mammals: Mechanisms of Signalling via The Sonic Hedgehog pathway in Lung Development. Respiration Research 2000;1:30-35.
30. Cardoso W.V. Lung morphogenesis revisited: old facts, current ideas. Dev Dyn 2000;219:121-130.

31. Bohn D. Congenital Diaphragmatic Hernia. Am J Respir Crit Care Med 2002;166:911-915.

Chapter 8

Summary / Samenvatting

8.1 Summary

Congenital diaphragmatic hernia (CDH) is a serious congenital anomaly with an incidence of one in 2000 to 3000 births. Given its variability in clinical expression and ongoing high mortality, CDH still is a major challenge to pediatric surgeons and neonatologists. The best way of treatment for the individual patient is very hard to determine, and treatment of so-called high-risk patients is still a matter of 'trial and error'.

The morphological abnormalities are well documented, showing abnormal growth of the fetal lungs in the prenatal period with resulting pulmonary hypoplasia in variable amounts. It is the structural abnormalities of the pulmonary vasculature, however, that greatly determine the outcome for the individual patient. The morphological changes of the pulmonary vasculature consist of medial hyperplasia, adventitional thickening and peripheral extension of the muscular coat of the pulmonary arteries.

Chapter 1 discusses various aspects of prenatal modulation of lung growth and the possibilities for postnatal treatment; finally the aims of the studies are described.

Chapter 2 gives the results of an experimental study investigating different forms of pulmonary vascular development in mouse embryos, with special attention to the role of vasculogenesis in early pulmonary vascular development.
In a series of mouse embryos we made computer-generated 3-D reconstructions of the pulmonary vasculature. In contrast to the generally accepted way of early pulmonary vascular development we found no signs of lack of continuity between the stem of the pulmonary artery and the peripheral extension in the lung bed. We could not prove de novo development of vascular lacks, a phenomenon suggested previously in extensive studies in serial sections of human embryos.

Chapter 3 describes the immunohistochemical evaluation of a series of human embryonic lung specimens from 9 weeks onwards till term , focussing on the expression of various so-called angiogenesis-related factors. The Von Hippel Lindau (VHL) pathway has been identified as highly important to early vascular development. However, serial documentation of the different molecules involved in the VHL pathway was still lacking. This is why we investigated the expression patterns both in the lung parenchyma and in the pulmonary vasculature for Von Hippel Lindau factor (VHL), hypoxia inducible factor 1α (HIF-1α), vascular endothelial derived growth factor (VEGF), the isoforms of nitric oxide synthase being endothelial and inducible nitric oxide synthase (e-NOS; i-NOS), as well as the receptor for VEGF being Flk-1.

As early as 9 weeks gestation the different components of the VHL pathway were clearly visible in a time specific manner. All proteins except VHL were expressed in the endothelium of all types of blood vessels and in the arterial media . While all proteins except HIF-1α were expressed in the epithelium of the airways, the adventitia was devoid of activity of all markers.
These findings imply a role for the pathway as early as the pseudo glandular phase of lung development all throughout fetal development.

These results were combined with the data described in **chapter 4**. In a series of 40 human lung specimens, consisting of 22 normal term specimens and 16 specimens of term high-risk CDH patients, the VHL pathway was documented by immunohistochemistry. Most importantly, the positive VHL protein expression in the media of CDH arteries combined with the negative VHL and HIF-1α staining in the endothelium, suggests a disturbed endothelium-media interaction in CDH, interfering with normal vascular remodeling and growth. This observation triggered new ideas about the optimal way of modulating pulmonary vascular growth, or eventually interference with protein expression in the future..

Pulmonary hypoplasia is an important factor in the pathology of congenital diaphragmatic hernia. This is why many authors have suggested retarded epithelial differentiation in these patients.
Because data in humans are still lacking we conducted a study described in **chapter 5**. We evaluated the nuclear expression of thyroid transcription factor 1 (TTF-1) in a series of 47 lung specimens of patients treated with either conventional ventilation or ECMO (n = 4) in the postnatal period, or with prenatal corticosteroids (n = 3). Specimens of six term born patients who had died of other forms of pulmonary hypoplasia were used as comparative specimens. In contrast to the experimental data, TTF-1 expression did not differ between the developing early fetal and term normal lungs, and between the hypoplastic lungs. This expression did not differ between patients treated with antenatal corticosteroids and those who underwent postnatal ECMO-treatment.
For this reason we concluded that although TTF-1 appears to play an important role in lung morphogenesis, a pivotal role in human lung development is unlikely.
As the possible effects of antenatal corticosteroids on lung maturation have been extensively documented in threatened premature labor, an ongoing debate is focussing on the role of tracheal ligation and corticosteroids in CDH. Based on what we found evaluating the effects of corticosteroids on human CDH lungs, i.e. decreased proliferation and increased apoptosis in the lung specimens, a critical review of the significance of tracheal obstruction and/or corticosteroids was undertaken (**chapter 6**). On the basis of

our own observations in combination with the variable results in animal models, we could not find a firm argument to support the hypothesis that corticosteroids might significantly contribute to the outcome in prenatally diagnosed human CDH cases. The role of tracheal ligation, which has a negative effect on type-II cell differentiation, is still under investigation in randomized controlled trials supported by the National Institute of Health (USA).

In **chapter 7** the general discussion provides both the pros and cons and the levels of evidence documented for the different ontogenetic features in CDH, as well as the ways in which numerous authors advocated antenatal modulation of pulmonary growth . Although a wide variety of treatment modalities are used in CDH, almost none of them have reached a level I evidence. Firstly because many clinics have only limited numbers of CDH patients, secondly because international collaboration is lacking, and thirdly because it is difficult to perform randomized controlled trials for an anomaly with a relatively low incidence.
The state of art of our knowledge about congenital diaphragmatic hernia is still at best described, even today, as "many questions – few answers".

8.2 Samenvatting

Congenitale hernia diafragmatica (CDH) is een ernstige aangeboren afwijking die voorkomt bij één tot twee op de 5000 pasgeborenen. Gezien het wisselend klinische beeld en de blijvende hoge mortaliteit blijft de behandeling van CDH een van de grootste uitdagingen voor kinderchirurgen en neonatologen.
De beste behandelingsmethode voor de individuele patiënt is moeilijk te bepalen. De behandeling van zogenaamde 'high-risk' patiënten is nog steeds in een proefondervindelijk stadium.
De morfologische afwijkingen zijn goed gedocumenteerd. Er is sprake van abnormale groei van de foetale longen in de prenatale periode, met als gevolg een wisselende hoeveelheid pulmonale hypoplasie. Niettemin zijn het de structurele afwijkingen van het pulmonale vaatstelsel die grotendeels de prognose voor de individuele patiënt bepalen. De morfologische veranderingen in het pulmonale vaatstelsel bestaan uit hyperplasie van de media, verdikking van de adventitia, en perifere extensie van de spierlaag van de longslagaders.

In **hoofdstuk 1** worden verschillende aspecten van prenatale modulatie van longgroei besproken, alsmede de mogelijkheden voor postnatale behandeling. Dit hoofdstuk besluit met een beschrijving van de doelstellingen van de verschillende onderzoeken.

In **hoofdstuk 2** zijn de resultaten beschreven van een experimenteel onderzoek naar de rol van verschillende mechanismen van pulmonale-vasculaire ontwikkeling in muizen-embryo's. Hierin werd speciale aandacht besteed aan de rol van vasculogenese bij de vroege vaatontwikkeling in de long.
In een serie muizenembryo's werden met behulp van een computer 3-D reconstructies van het pulmonale vaatbed gemaakt. In tegenstelling tot de algemeen aanvaarde interpretatie van de vroege pulmonale vaatontwikkeling, werden geen aanwijzingen voor het ontbreken van de continuïteit tussen de stam van de longslagader en de perifere extensie in het longbed vastgesteld. De novo ontwikkeling van vasculaire lacunes, iets dat eerder na breed onderzoek in series menselijke embryo's werd gesuggereerd, kon niet worden bewezen.

Hoofdstuk 3 beschrijft de immunohistochemische evaluatie van een serie longpreparaten van menselijke embryo's vanaf 9 weken tot de geboorte, met name wat betreft de expressie van een serie zogeheten angiogenese-gerelateerde factoren. De Von Hippel Lindau 'pathway' (VHL pathway) blijkt als zeer belangrijk voor de vroege vasculaire ontwikkeling. Tot op heden was er echter nog geen serieonderzoek beschreven van de

verschillende moleculen die betrokken zijn bij de VHL pathway. Daarom onderzochten wij de expressiepatronen in het longparenchym en in het pulmonale vaatstelsel voor de Von Hippel Lindau factor (VHL), de hypoxie-inducerende factor 1α (HIF-1α), de vasculaire-endotheliale groeifactor (VEGF), de isovormen van stikstofoxide synthase, d.w.z. endotheliale en inducerende stikstofoxide synthase (e-NOS; i-NOS) alsmede de receptor voor VEGF, namelijk Flk-1.
Al bij 9 weken zwangerschap waren de verschillende componenten van de VHL pathway duidelijk zichtbaar op een tijdspecifieke wijze. De eiwitten werden aangetoond in het endotheel van alle typen bloedvaten en in de media, met uitzondering van VHL. Alle eiwitten behalve HIF-1α kwamen tot expressie in het epitheel van de luchtwegen, de adventitia toonde voor geen van de merkers enige activiteit.
Deze bevindingen impliceren een rol voor de pathway al vanaf de pseudo glandulaire fase van de longontwikkeling gedurende de gehele foetale ontwikkeling.

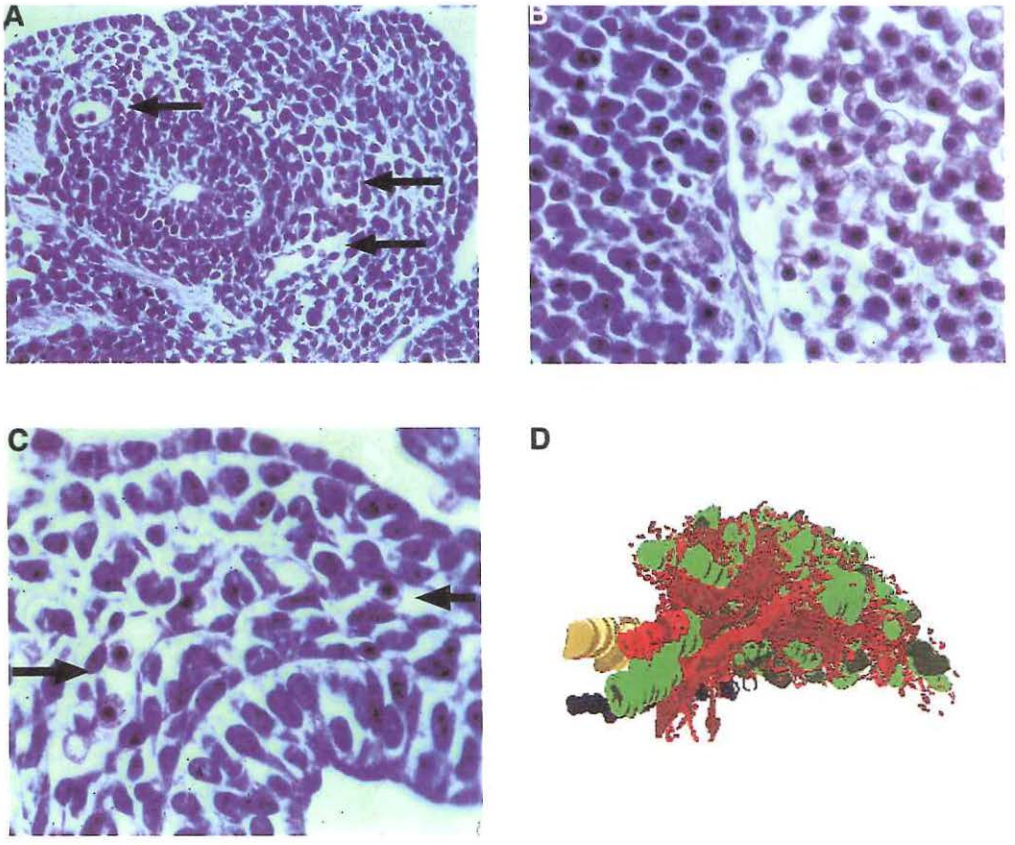
Deze resultaten werden gecombineerd met de gegevens beschreven in **hoofdstuk 4**. In een serie van 40 menselijke longpreparaten, 22 van normale a terme longen en 16 a terme 'high-risk' CDH patiënten, werd de expressie van de VHL pathway onderzocht door middel van immuunhistochemie.
De belangrijkste conclusie: de positieve VHL eiwitexpressie in de media van CDH arteriën, in combinatie met een negatieve VHL- en HIF-1α-kleuring in het endotheel, suggereert dat in CDH een verstoorde endotheel-media interactie de normale vasculaire 'remodeling' en groei belemmert. Deze observatie leidde tot nieuwe ideeën over de optimale wijze om pulmonale vasculaire groei in de toekomst te moduleren.

Pulmonale hypoplasie is een belangrijke factor bij de pathologie van CDH. Daardoor is vaak gesuggereerd dat vertraagde epitheliale differentiatie bij deze patiënten een rol speelt.
Omdat hierover geen gegevens bij de mens beschikbaar zijn, deden we een onderzoek – beschreven in **hoofdstuk 5** – naar de nucleaire expressie van de thyroid transcriptiefactor 1 (TTF-1). Dit deden we in een serie van 47 longpreparaten van patiënten die waren behandeld met conventionele beademing of met ECMO (n = 4) in de postnatale periode, of met prenatale corticosteroïden (n = 3). Preparaten van zes a terme geboren patiënten die aan andere vormen van pulmonale hypoplasie waren overleden dienden als vergelijkingsmateriaal. In tegenstelling tot de experimentele gegevens, werd geen verschil in TTF-1 expressie gezien tussen de zich ontwikkelende vroege foetale en a terme normale long, en de hypoplastische menselijke longen. Er was geen verandering van expressie bij prenatale corticosteroïden gebruik en postnatale ECMO-behandeling.

Hieruit concludeerden we dat alhoewel TTF-1 een belangrijke rol blijkt te spelen bij de morfogenese van de long, deze factor waarschijnlijk geen essentiële kernrol vervult bij de ontwikkeling van de long.
Aangezien prenatale corticosteroïden invloed zouden kunnen uitoefenen op de rijping van de long, zoals uitgebreid is gedocumenteerd bij dreigende premature bevalling, richt men zich op de rol van de trachea obstructie en corticosteroïden bij CDH. Op basis van onze bevindingen bij de evaluatie van het effect van corticosteroïden op menselijke CDH longen, d.w.z. verminderde proliferatie en verhoogde apoptose in de onderzochte longpreparaten, deden we een kritische review naar het belang van obstructie van de luchtpijp en/of het belang van corticosteroïden (**hoofdstuk 6**). Op basis van onze eigen observaties en de wisselende resultaten in diermodellen, vonden we geen sterk argument voor de hypothese dat corticosteroïden sterk bijdragen aan de uitkomst bij prenataal gediagnosticeerde CDH bij de mens. De rol van de trachea obstructie, welk een negatief effect heeft op de type-II cel differentiatie, is nog steeds onderwerp van onderzoek en maakt deel uit van lopende gerandomiseerde en gecontroleerde trials gesubsidieerd door de National Institute of Health in de Verenigde Staten

In **hoofdstuk 7** worden de voor en tegens en het niveau van bewijs van de verschillende ontogenetische kenmerken van CDH besproken. Tevens wordt aandacht besteed aan de wijze waarop talrijke auteurs prenatale modulatie van pulmonale groei bepleiten.
Alhoewel er vele verschillende behandelingsmethoden voor CDH worden toegepast, heeft nog geen daarvan een bewijsniveau I behaald. Hiervoor zijn diverse oorzaken: veel klinieken behandelen weinig van deze patiënten, er is gebrek aan internationale samenwerking, en het is moeilijk om gerandomiseerde gecontroleerde trials uit te voeren voor een afwijking met een relatieve lage incidentie.
Tot op heden is onze kennis van aangeboren hernia diafragmatica het best te beschrijven als: "veel vragen, weinig antwoorden".

Color figures



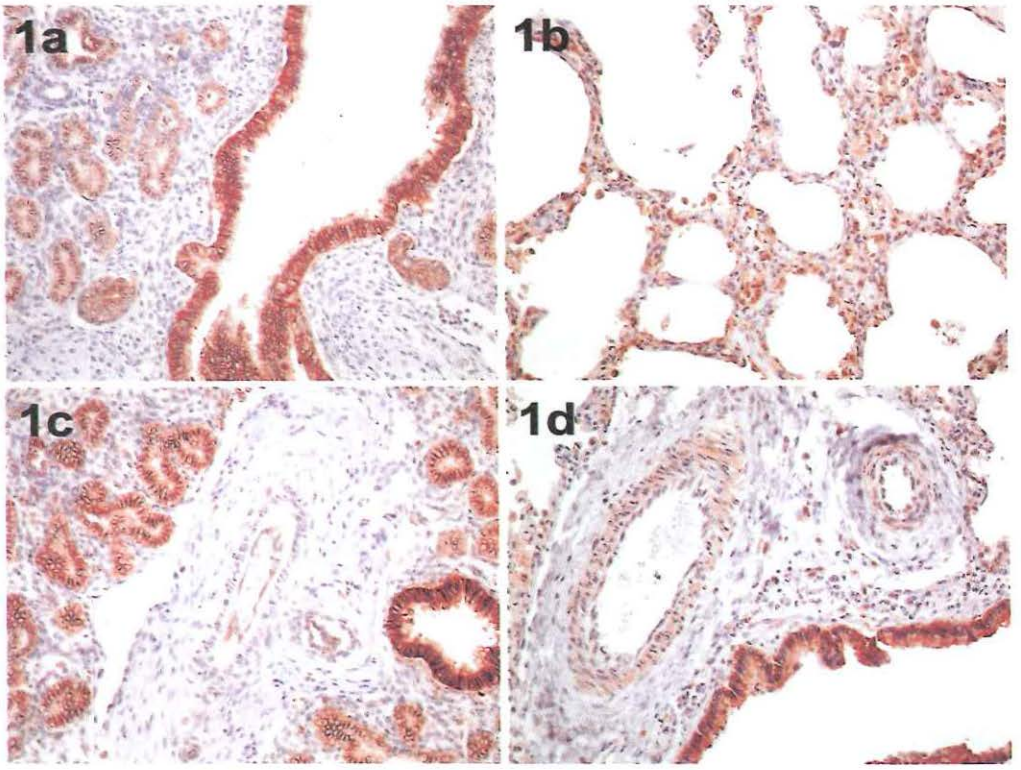
Chapter 2 figure 1 page 18-19

A Lung at ED 12. Note two small vessels containing erythrocytes on each side of the developing airway.

B At high power, a small vessel is seen to contain erythrocytes but no haematopoietic progenitor cells. Many small irregular spaces are seen between mesenchymal cells, but there is no morphological evidence that these relate in any way to developing blood vessels (compare with D).

C Haematopoiesis in liver at ED 12. The large vessel lumen (left) contains the circulating erythrocytes, which contrast with the mixture of blood precursor cells seen in the tissue (right) indicative of local haematopoiesis.

D 3D reconstruction of vessels in murine lung at ED 13. Red indicates blood vessels; green is parenchyme. Note continuous vascular profiles (red) extending to the periphery of the lung tissue (green). Apparent discontinuities at the far periphery are explained by limitations of the resolution of the technique as well as by ambiguity of identification of some small empty spaces as vessels.



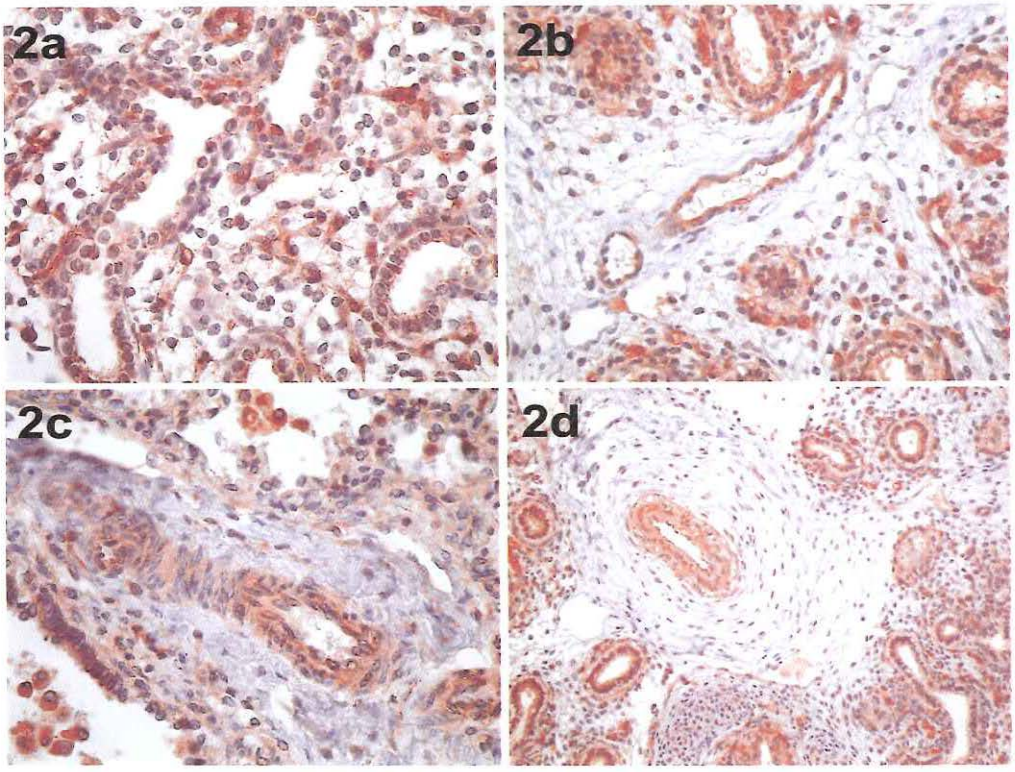
Chapter 3 figure 1. VHL page 31

A At 23 weeks of gestation, strong labelling of proximal airway epithelial cells can be observed, whereas the more distal epithelium is also clearly stained.

B At term, many epithelial cells in the distal airways are labelled; at this stage, also the proximal airways are still strongly stained (not shown).

C In the majority of specimens, the endothelial cells of arteries are immunopositive, like in this fetal lung at 23 weeks of gestation.

D In this term lung, the media of this pulmonary arterial branch shows clear labelling of the smooth muscle cells; the endothelial cells are also slightly stained.

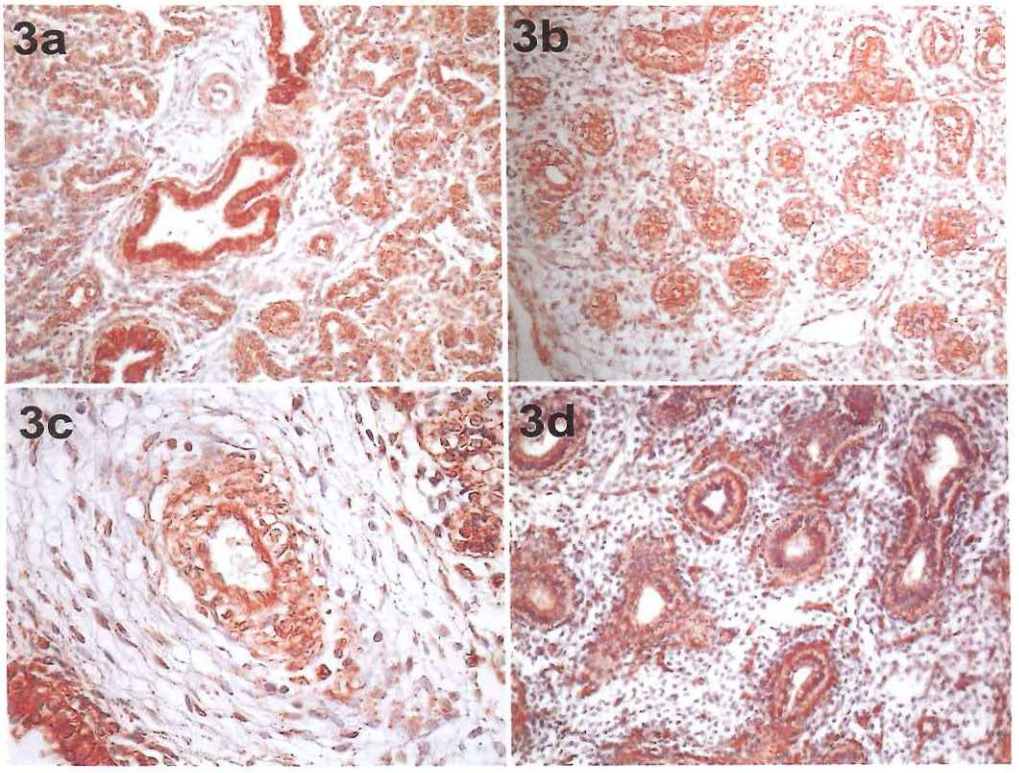


Chapter 3 figure 2. HIF-1α page 31

A At 19 weeks gestation, HIF-1α stains many small vascular structures in the primitive lung interstitium.

B At the same stage of development, in an 18-week-old lung, the venous endothelium is also immunopositive.

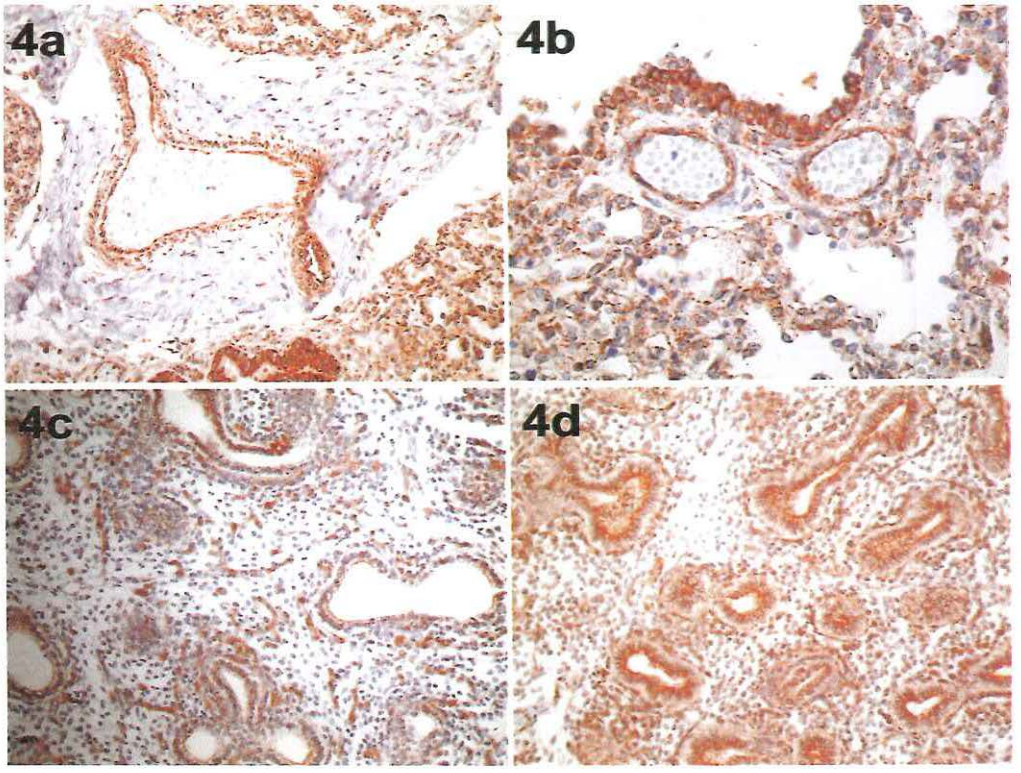
C-D Both at term (C), and at 18 weeks gestation (D), the endothelial cells and the medial smooth muscle cells are stained.



Chapter 3 figure 3. VEGF page 31-32

A-C The epithelial cells of large airways (A), the smaller airways (A and B), as well as the endothelium and the smooth muscle cells in the wall of pulmonary arteries (C) are labelled by VEGF at 18 weeks gestation.

D Especially in early gestation, as in this 14-week-old lung, the small capillary-type vessels are highlighted by positive immunostaining for VEGF.



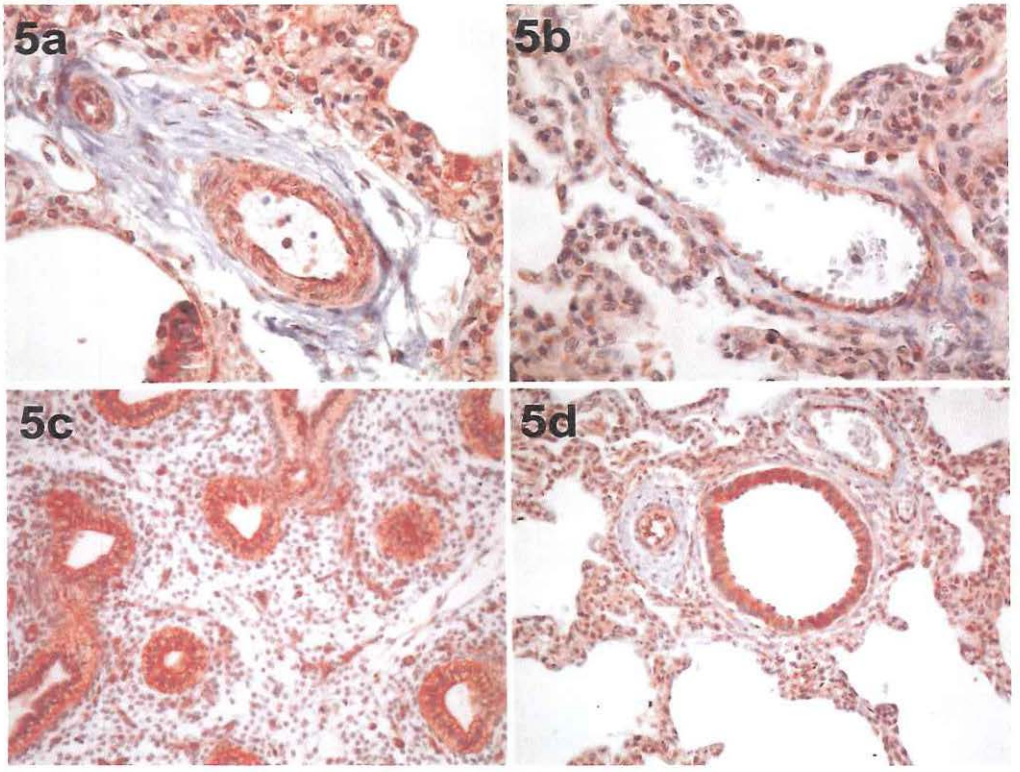
Chapter 3 figure 4. Flk-1 page 32

A At 34 weeks gestation, Flk-1 labelling is evident in the endothelium, but also the smooth muscle of pulmonary arteries.

B At the same gestational age, Flk-1 labelling is also seen in the endothelium of veins and of the developing capillary network, between the sacculi and alveoli.

C At 14 weeks gestation, as in the VEGF staining, the small vessels in the interstitium are highlighted.

D This representative slide, at 17 weeks of gestation, shows Flk-1 staining of the epithelium of the conducting airways.



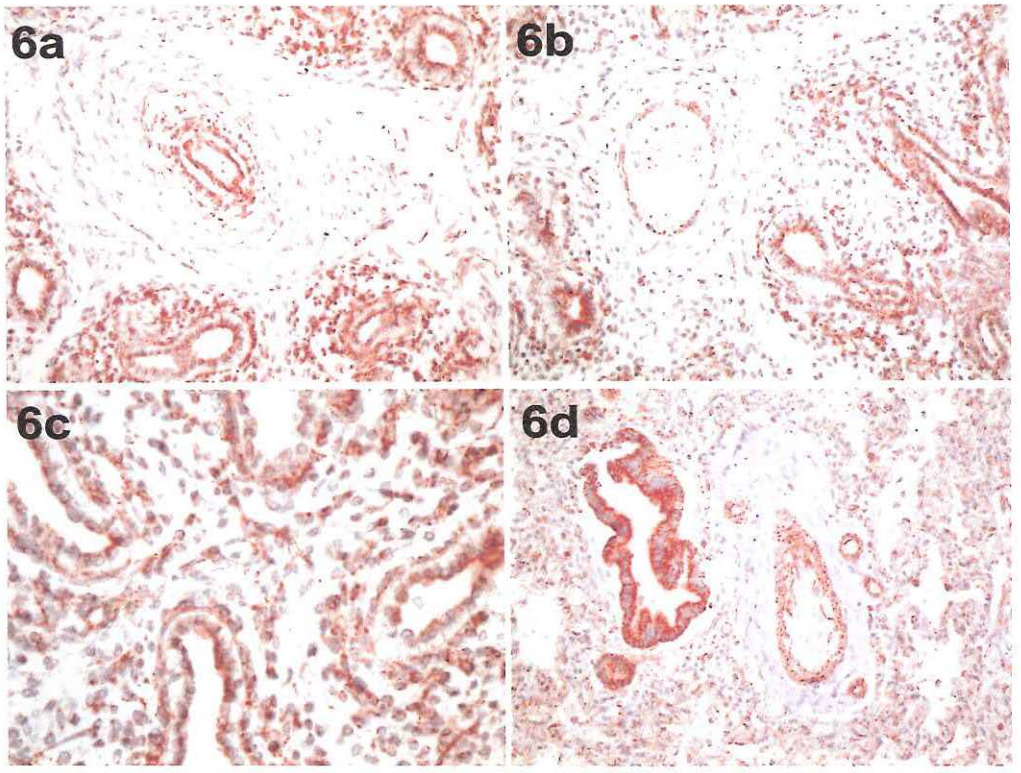
Chapter 3 figure 5. eNOS page 32

A The endothelium and smooth muscle of pulmonary arteries are labelled at 35 weeks of gestation.

B In this term lung, the venous endothelium is positive for eNOS.

C As with Flk-1 and VEGF, eNOS labels the small capillary-type vessels in the interstitium at 13 weeks gestation.

D This slide shows eNOS labelling of the epithelium of conducting airways at term.



Chapter 3 figure 6. iNOS page 32

A-C iNOS labelling of endothelial cells in pulmonary artery (A), vein (B), and interstitial capillaries (C), at 18 weeks of gestation. The arterial media in A is also positive.

D At term, strong iNOS staining is seen in the epithelium of a bronchiole, as well as in the endothelium and smooth muscle in the wall of the adjacent pulmonary arterial branch.

Chapter 4 figure 1 page 43 ⟶

A Detail of normal term lung tissue stained with an antibody to VHL, showing virtual absence of immunoreactivity in the smooth muscle cells of the arterial media. To the left, there are some immunoreactive epithelial cells which have detached from the basement membrane. In the rest of the picture, isolated immunopositive epithelial cells can be seen.

B Lung tissue from term patient with CDH, stained with an antibody to VHL, showing clear staining of the smooth muscle cells in the arterial media. Numerous immunopositive epithelial cells can be seen as well.

C HIF1α-staining of lung tissue from normal term baby. Strong staining in the arterial media and in the endothelial cells of the arterial intima can be seen. In addition, some intra-alveolar macrophages and endothelial cells of capillaries are stained.

D HIF1α-staining of lung tissue from term CDH patient. Marked staining of the arterial media can be seen, whereas all other cell types are negative.

E HIF1α-staining of lung tissue from normal term baby. The endothelial cells of a pulmonary vein are stained, in addition to intra-alveolar macrophages and endothelial cells of capillaries.

F HIF1α-staining of lung tissue from term CDH patient. Absence of staining can be appreciated in small veins, as well as in the capillaries in the interalveolar septa.

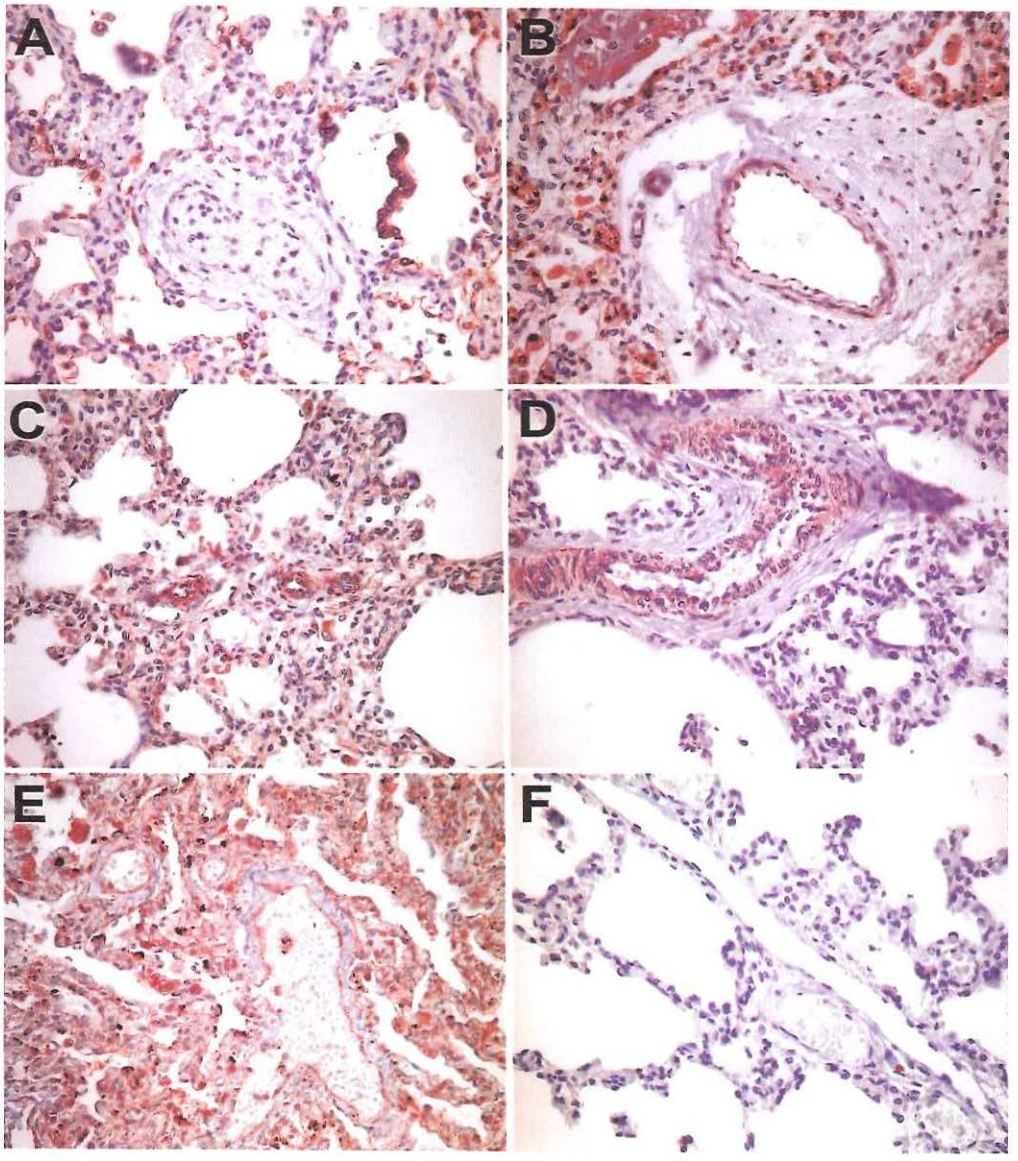

A
B
C
D
E
F

Figure 1

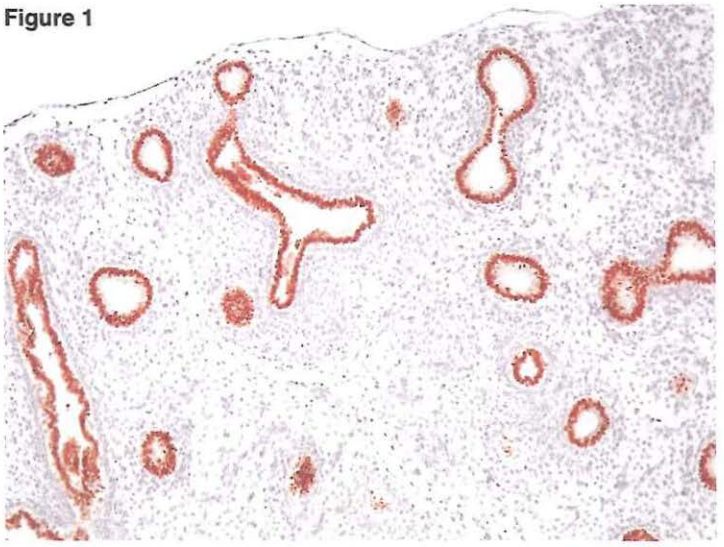
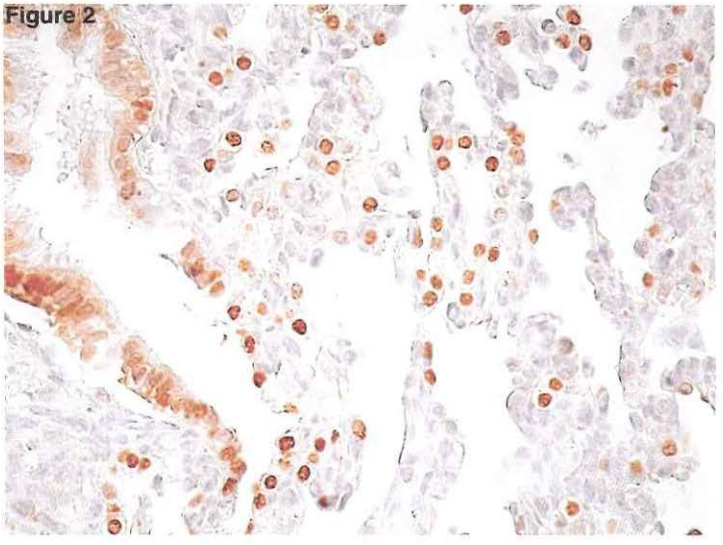


Figure 2



Chapter 5 figure 1 page 53

Nuclear staining for TTF-1 in columnar and cuboidal nonciliated cells of the bronchiolar epithelia and in respiratory epithelial cells lining the distal air spaces in 13 weeks gestation human lung

Chapter 5 figure 2 page 53

TTF-1 expression in subsets of nonciliated distal bronchiolar epithelia and in alveolar Type 11 cells in normal human lung at term

Figure 3

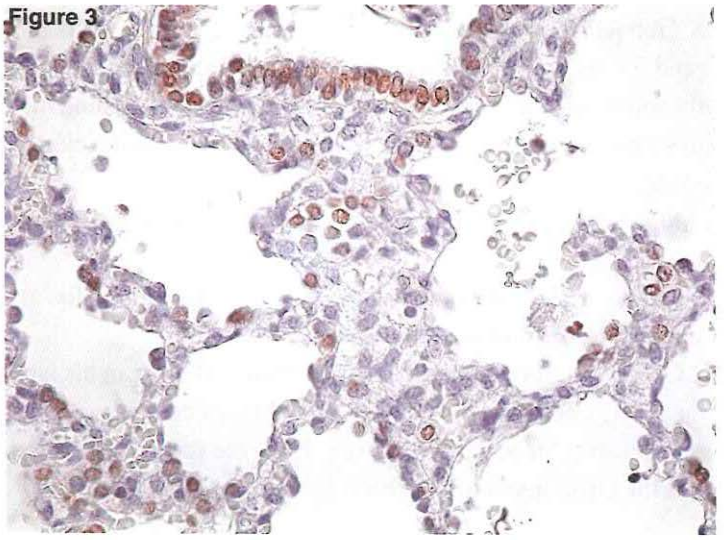
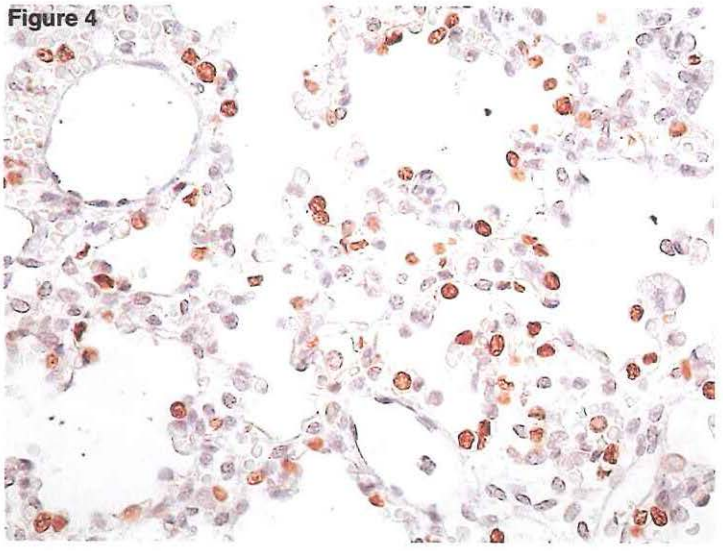


Figure 4



Chapter 5 figure 3 page 53 and 54

The expression pattern of TTF-1 staining in term born CDH patient is restricted to nonciliated distal bronchiolar epithelia and alveolar Type II cells

Chapter 5 figure 4 page 53 and 54

TTF-1 expression in nonciliated distal bronchiolar epithelia and alveolar Type II cells in antenatally CS-treated term born CDH patient

Acknowledgements

I'm so grateful to Prof.dr. D. Tibboel for his tremendous support throughout the years in order to complete this work and for his kind help, supervision and encouragement in guiding my research. I greatly appreciate all his efforts and contributions for teaching me how to approach research questions relevant for pediatric surgical diseases and develop a different point of view in pediatric surgery.
I like to thank Prof.dr. W.J. Mooi for his guidance and helpful criticism during the period of this work.
My co-promoter, Dr. R.R. de Krijger, many thanks for your kind help especially in the last phase of completion of the manuscript and mental support.
I would like to thank Dr. J.H.C. Meijers, for giving me the opportunity to work in his lab, for his kind friendship and for all his efforts to teach me the principles of cell biology.
I thank Frans, Sylvia, Richard, Vincent, Manousos, Ton, Ann, John, the present members of lab 785 and all the people in the Department of Cell Biology for a friendly and helpful working atmosphere.
In addition, I wish to thank Prof.dr. J.C. Molenaar, Prof.dr. F.W.J. Hazebroek and their beloved wives for their hospitality in the Erasmus Medical Center-Sophia and their social events in the city of Rotterdam.
Special thanks to Ynske IJzendoorn, Jessica de Rooij and the present members of the immunohistochemistry lab of the Department of Pathology, for enabling me to perform immunohistochemical techniques and their nice friendship.
I like to thank Annemarie Illsley for her arrangements in the preparation of this thesis and Margo Terlouw-Willebrand for her editorial advises.
Thanks to Marieke and Rita, both secretary at the Cell Biology Department, for their patience and continuous help.

Curriculum Vitae

1959, June 30th	Born in Izmir, Turkey
1978 - 1983	Medical Degree Ege University Medical Faculty, Izmir
1989 - 1994	Resident, Department of Pediatric Surgery Dokuz Eylul University Medical Faculty, Izmir
1995 – 1998	Chief Resident, Department of Pediatric Surgery Dr. Behcet Uz Children's Hospital, Izmir
1998	Awarded Ministry of Health Grant
1998 - 2000	Research Fellow Department of Pediatric Surgery and Department of Cell Biology and Genetics Sophia Children's Hospital, Erasmus Medical Center, Rotterdam, The Netherlands
2000	European Board Certificate in Pediatric Surgery, U.E.M.S.
2001 - present	Chief Assistant, Department of Pediatric Surgery Dr. Behcet Uz Children's Hospital, Izmir