MECHANISMS OF NORMAL AND ABNORMAL PULMONARY DEVELOPMENT

MECHANISMEN VAN NORMALE EN ABNORMALE LONGONTWIKKELING

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INTRODUCTION

Chapter 1

Normal pulmonary development

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Keijzer, R., van Dooren, M., Meijers, C., and Tibboel, D. Genetics of foregut-malformations; lessons from mice relevant for pediatric surgeons. (submitted)

1.1 Introduction

One of the prerequisites of life is the process of respiration in which an organism exchanges carbon dioxide for oxygen in order to obtain energy through the oxygenation of molecules containing carbon. The interfaces used by different organisms for gas exchange vary from simple diffusion between prokaryotic bacteria and the medium in which they reside, to a much more complex diffusible interface, namely a gas-exchanging organ also known as the lung used by higher eukaryotes, such as humans. The human lungs have an estimated 300.000.000 alveoli with a surface area of approximately 70 m^2 by 0.1 μm in early adulthood (Comroe, 1965; Weibel, 1963) and is comprised of at least 40 differentiated cell types. The alveolar surface of the lung is surrounded by a capillary network that develops in close apposition. This enables gas exchange to occur with the blood that delivers the oxygen to the tissues. Therefore a 70 kg adult human can use 14.5 L oxygen per hour or 10^{20} molecules per second at rest. The oxygen demand can rise to approximately 330 L per hour during exercise (Comroe, 1965; Weibel, 1963). To establish such a highly complex organ, well-orchestrated cell interactions are required during development to generate a functional lung.

A disturbance at any part of the respiratory chain may lead to respiratory insufficiency which can eventually end in lethality. One of the main clinical respiratory problems leading to a disturbance of this chain is pulmonary hypoplasia, which is an underdevelopment of the lung due to a decreased cell number. It is associated with different clinical conditions, such as congenital diaphragmatic hernia (CDH), oligohydramnios based on renal malformations or leakage of amniotic fluid, and anencephaly. In order to better understand pulmonary hypoplasia and possible means to reverse this condition, a further understanding of the fundamental basis of normal pulmonary development is necessary. Herein, several aspects of normal pulmonary development are reviewed. First, the respiratory structures of several organisms are described and compared, since successful developmental processes are conserved through evolution. This will provide a background for the description of the developmental anatomy and embryology of normal mammalian pulmonary development. Subsequently, the molecular mediators involved in pulmonary development, such as genes, transcription factors and growth factors will be described.

1.2 Evolution of respiratory structures

In order to assimilate oxygen and use it for the production of energy, organisms use different respiratory structures ranging from a diffusing membrane, to tracheae, gills or lungs. Which respiratory structure is used depends mainly on the size of the organism. The very small protozoans use their entire surface to exchange carbon dioxide for oxygen from the environment. The larger multicellular organisms use different strategies. Aquatic worms lengthen and flatten their bodies to refresh their external surfaces with new oxygen. Sessile sponges rely on the ebb and flow of ambient water. When organisms became too large and dense for such simple external exchange, special respiratory structures were implemented.

Insects use a trachea for gas exchange. The trachea consists of branching tubes that deliver oxygen to and remove carbon dioxide from the tissues, without an intermediate circulatory system. The tubular system opens to the outside through pores, called spiracles. There are two spiracles in the thorax and eight in the abdomen. The limitations of the tracheal system are slow gas diffusion in long and narrow tubes, and effective gas transport can only occur if the tubes do not exceed a certain length. Recently, an extensive study of *Drosophila* using screens to find genes involved in tracheal development has yielded new insights into mammalian pulmonary development. The FGF signaling pathway used for branching morphogenesis in *Drosophila* tracheal development is also used in mammalian pulmonary development [for review see (Metzger and Krasnow, 1999)].

Most fish use gills as a means for respiration. Gills are evaginations of the body surface, in contrast to lungs, which are invaginations of the body surface. Interestingly, both gills and lungs are formed from outpouchings of the gut wall during embryogenesis. Usually gills are used for water breathing and lungs for air breathing. As in lungs, gills usually have a large surface area in relation to their mass and both are thin-walled structures, which are well supplied with blood vessels. In gills deoxygenated blood from the heart flows in a direction opposite to that of the water flow across the exchange surfaces, which makes gas exchange efficient.

A transitional form between water- and air breathing organisms are lungfishes (Dipnoi), which are remnants of the Devonian period (Jesse et al., 1967). They have both gills and lungs, so that in a period of drought they can rely on their lungs for oxygenation of their blood. Amphibians are another example of the transition between aquatic and terrestrial life. Amphibians begin life under water with gas exchange occurring through

the thin, gas-permeable skin and gills. As amphibian larvae develop, the gills degenerate and paired lungs develop. Finally, mammalian species do not use their skin for gas exchange. An exception, that has been found recently is the Julia Creek dunnart (*Sminthopsis douglasi*), a marsupial mouse, which has normal lungs, but completely depends on gas exchange through the skin during the first days after birth. (Mortola et al., 1999). (See Figure 1)

In contrast to the simple saclike lungs of lungfishes and amphibians, higher vertebrates have achieved an increasing subdivision of the airways and a greater lung surface area. This complexity is necessary to support the elevated metabolic rates of mammals. An important property of these lungs is elasticity. A surface coating (surfactant) composed of lipids and proteins prevents the collapse of the small alveoli on expiration. The maximum capacity of human lungs is about six litres. During normal respiration, the tidal volume is about 500 millilitres. Subsequent to expiration, an additional 1200 millilitres can be expelled with additional effort, and then another 1200 millilitres remains inside the lungs as a residual volume. With extra effort an additional 3000 millilitres can be drawn into the lungs at the end of inspiration.

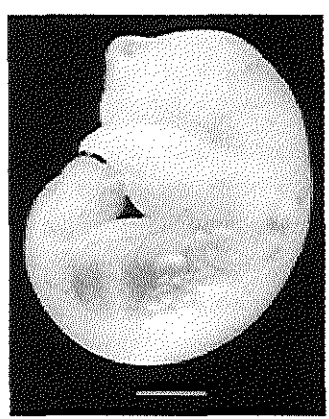

Figure 1 *Sminthopsis douglasi* (Julia Creek dunnart, a marsupial mouse) embryo of one day old. Note the fine air bubbles at the side of the thorax. Despite the visible presence of lungs in the thorax, at this time no breathing movements can be observed.
(Taken from *Nature* (1999) 397:660)

1.3 Anatomy and embryology

Mammalian lung development starts with the outgrowth from the foregut endoderm of two lung buds into the surrounding splanchnic mesenchyme. Prior to this, a certain area of the foregut is specified to become the area where the lung (and not, the thyroid or pancreas) will develop. The origin and fate of the cells forming the lung anlage within the primitive foregut are as yet unknown. In human, the lung originates as an outpouch of the ventral wall of the posterior end of the laryngotracheal tube and divides into two bronchial buds at 3-4 weeks of gestation (Hopper and Hart, 1985). In the mouse and rat at 10 and 11.5 days of gestation, respectively (term mouse = 19 days; term rat = 22 days), the respiratory system develops from paired endodermal buds in the primitive foregut, just proximal to the developing stomach (Ten Have-Opbroek, 1981; Ten Have-Opbroek, 1991). As the two buds elongate, the primitive tubular foregut tube begins to pinch into two tubes, namely, the dorsal esophagus and the ventral trachea (Spooner and Wessells, 1970). Further outgrowth of the lung buds produce the secondary bronchi. In the mouse, the right lung characteristically has four major lobes (dorsal, caudal, medial and cranial lobes) whereas the left lung consists of only one small lobe. This arrangement also holds in the rat except the right lung lobes are termed cranial, medial, accessory and caudal. In human, the right lung has three lobes whereas the left lung is composed of two lobes (upper, middle and lower on the right; upper and lower on the left). The early branching of the primary bronchial buds tends to be monopodial. Each secondary bronchus subsequently undergoes progressive dichotomous branching as each branch bifurcates repeatedly. Reproducible branching is complete at 16 generations by 16 weeks of gestation in humans (Boyden, 1972). The final seven generations of airways (for a total of 23) in human are completed during the latter part of gestation. Alveolization begins after about 28-30 weeks in humans and is completed postnatally. This branching process will ultimately yield a functional lung with its very large surface area for gas exchange after birth. Already in 1967 Reid nicely summarized this process in her Laws of Development of the Human Lung (Reid, 1967):

(a) The bronchial tree is developed by the 16^{th} week of intrauterine life.

(b) Alveoli, as commonly understood, develop after birth, increasing in number until the age of eight years and in size until growth of the chest wall is finished.

(c) Blood vessels are remodeled and increase, certainly while new alveoli are forming and probably until the growth of the chest is complete.

During pulmonary development, four histological stages can be distinguished: the pseudoglandular stage, in which the bronchial tree develops and an undifferentiated primordial system forms; the canalicular stage, terminal sacs develop and vascularization occurs during this period; the saccular or terminal sac stage, in which the number of terminal sacs increases, vascularization proceeds and differentiation of type I and type II cells occurs; and the alveolar stage, in which there is a huge multiplication of the alveoli establishing the extensive surface area (see Table 1 for comparison of gestational stages between human, mouse and rat). In humans (as in guinea pigs and sheep), alveolization begins in utero and is completed after birth. In contrast, in rats and mice, alveolization occurs predominantly postnatally.

Table 1. Stages of lung development

Stage of pulmonary development	Human age (weeks of gestation)	Mouse age (days of gestation)	Rat age (days of gestation)
Pseudoglandular	3-16	10-16.5	11.5-18.5
Canalicular	16-26	16.5-17.5	18.5-19.5
Saccular	24-38	17.5-19 to 5 days	19.5-22 to 1 week
Alveolar	28 to 7 years	Days 5-30	Week 1 to 5

Comparison of stages of pulmonary development and gestational ages between human, mouse and rat.

Epithelial cell differentiation is initiated in the saccular stage and occurs in a proximo-distal pattern. The trachea and the upper airways are lined with pseudostratified ciliated columnar epithelium with scattered mucus secreting goblet cells. In between the epithelial cells, small foci of pulmonary neuroendocrine cells can be found. The lower airways are lined with ciliated columnar epithelium and Clara cells. The alveoli are lined with alveolar type I cells, which are very thin for an efficient gas exchange and type II cells, which produce the surface tension reducing surfactant.

Surrounding the epithelium lies the pulmonary interstitial tissue, which contains fibroblasts, myofibroblasts and smooth muscle cells. In addition, the pulmonary

interstitium comprises the pulmonary vasculature, consisting of arteries, veins and a large capillary network in close apposition to the alveoli. It also includes the lymphatic system and the nervous system. In the following sections the molecular mechanisms involved in the different phases of pulmonary development will be reviewed. The review will focus on mouse development, since this is the most studied species. Subjects such as lung neovascularization and lung cell differentiation in pulmonary development will not be covered in great detail, since they are beyond the scope of this thesis.

1.4 Endoderm specification

Even before the lung bud outgrowth occurs, endodermal cells from the primitive foregut are specified to become committed to respiratory epithelial cells. It is generally thought that HNF-3β, TTF-1 and GATA6 transcription factors play an important role in this process (Whitsett and Tichelaar, 1999) (see Figure 2). Interestingly, these factors are mammalian homologues of transcription factors that are involved in organogenesis of the pharynx in *C. elegans* (Kalb et al., 1998).

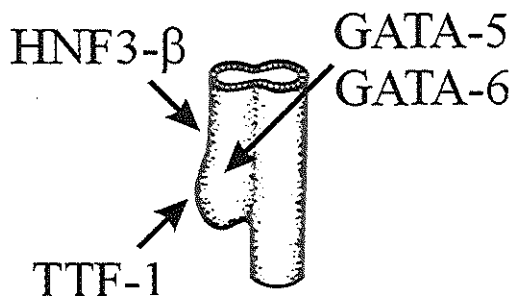

Figure 2 Factors assumed to be involved in pulmonary endoderm specification of the primitive murine foregut.

HNF-3β belongs to the winged-helix HNF (hepatocyte nuclear factor) family of transcription factors which is related to the forkhead family in *Drosophila* and plays a critical role in the formation and specification of foregut endoderm (Ang et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1993; Sasaki and Hogan, 1994). HNF-3β is expressed in the pulmonary epithelium starting from 10 days of gestation throughout development and HNF-3β null mutants die early in embryonic development because of a disturbance in the formation of foregut endoderm and its derivatives (Ang and Rossant, 1994; Monaghan et al., 1993; Weinstein et al., 1994; Zhou et al., 1996b). In addition,

HNF binding sites have been identified in the promoter regions of lung specific genes, such as the surfactant proteins (SP)-A, -B, -C and -D and the Clara cell marker-10 (CC-10), suggesting an important role for this factor in lung development [for review see (Hackett et al., 1996)].

TTF-1 (thyroid transcription factor), also known as Nkx2.1, belongs to a family of homeodomain transcription factors and are homologous to the *Drosophila* NK-2 gene (Guazzi et al., 1990; Kim and Nirenberg, 1989; Oguchi et al., 1995). Expression of TTF-1 is confined to the thyroid, areas of the forebrain, the pituitary, and lung endoderm and epithelium throughout lung development starting from 10 days of gestation (Damante and Di Lauro, 1994; Guazzi et al., 1990; Lazzaro et al., 1991; Mizuno et al., 1991). Null mutation of TTF-1 results in a failure of tracheo-esophageal separation and branching morphogenesis resulting in pulmonary hypoplasia (Kimura et al., 1996; Minoo et al., 1999). Moreover, the epithelial cells of these hypoplastic lungs have not undergone proper differentiation. In addition, the thyroid, pituitary and parts of the forebrain are absent. Like HNF, TTF-1 functions as a regulator of transcription of lung specific genes such as SP-B and -C and CC-10 [for review see (Hackett et al., 1996)]. Despite the described roles for TTF-1 in tracheo-esophageal separation and pulmonary development, there is no functional data available on the role of TTF-1 in endoderm formation and specification.

Recently, GATA6 has been implicated in pulmonary endoderm specification (Morrisey et al., 1998; Whitsett and Tichelaar, 1999). GATA6 belongs to a family of zinc finger containing transcription factors which bind to a DNA consensus sequence A/TGATA/G (Charron and Nemer, 1999; Orkin, 2000). Expression of GATA6 is localized in smooth muscle cells, the developing bronchi, the urogenital ridge and the bladder (Morrisey et al., 1996). Genetic ablation of GATA6 results in early embryonic lethality. Defects in extraembryonic development suggest that GATA6 plays a role in visceral endoderm formation (Koutsourakis et al., 1999; Morrisey et al., 1998). One experiment using GATA6-/- chimeric mouse embryos suggests that GATA6 is essential for the formation of endodermally derived respiratory epithelium (Morrisey et al., 1998). Again, in several lung specific promoter regions GATA6 binding sites have been recognized, and transcriptional regulation of some of these genes (SP-A, –C, and TTF-1) has been demonstrated *in vitro* (Bruno et al., 2000; Shaw-White et al., 1999).

1.5 Lung bud outgrowth and establishment of left-right asymmetry

The exact mechanisms controlling lung bud outgrowth are not completely understood. However, evidence is accumulating that fibroblast growth factor (FGF)-10 is critical for this process (see also Figure 3). First, FGF-10 is expressed in the splanchnic mesenchyme surrounding the evaginating lung buds (Bellusci et al., 1997b). Second, FGF-10 null mutant mice have no lung buds and limbs, whereas the trachea is normally formed in these mice (Min et al., 1998; Sekine et al., 1999). And third, FGF-10 has been demonstrated to have chemotactic effects on lung epithelium *in vitro* (Park et al., 1998; Weaver et al., 2000). FGF receptor (R)2-IIIb, which is expressed in foregut and lung endoderm mediates the FGF-10 signal (Peters et al., 1992). Mice null mutant for this receptor display similar pulmonary phenotypes as FGF-10 knockout mice (De Moerlooze et al., 2000). Taken together, these data provide a nice example of conserved gene function between *Drosophila* and the mouse. *Drosophila branchless* (FGF ligand homologue) (Sutherland et al., 1996) and *breathless* (FGFR homologue) (Glazer and Shilo, 1991) are genes that have been shown to be essential for tracheal cell migration and thus branching of the *Drosophila* trachea (Lee et al., 1996).

Despite preliminary data suggesting a possible interaction between the FGF- and sonic hedgehog (Shh) pathway (Bellusci et al., 1997b; Lebeche et al., 1999), there is not much known about the downstream target genes of the FGF signaling pathway. However, because FGF-10 may be involved in setting up a spatial master plan for the future lung buds, it is attractive to speculate about an interaction between FGF-10 and Homeobox-containing (Hox) genes. Hox genes have been implicated in regional conditional specification of morphogenetic progenitor fields, which are defined as regions in an embryo where cells give rise to a morphological structure (Davidson, 1993). Hox genes are characteristically organized in four clusters on different chromosomes and the 3' to 5' position of each gene within a cluster corresponds with the expression along the anterior-posterior axis of the developing body (Kappen, 1996; Krumlauf, 1994). Several Hox genes, specifically genes of the 3' regions of the Hox clusters a and b, have been shown to be expressed in the lung (Kappen, 1996). Also knockout studies suggest an essential role for Hoxa5 in pulmonary development (Aubin et al., 1997) although a precise role has not been established and no interaction between Hox- and FGF-signaling has yet been demonstrated.

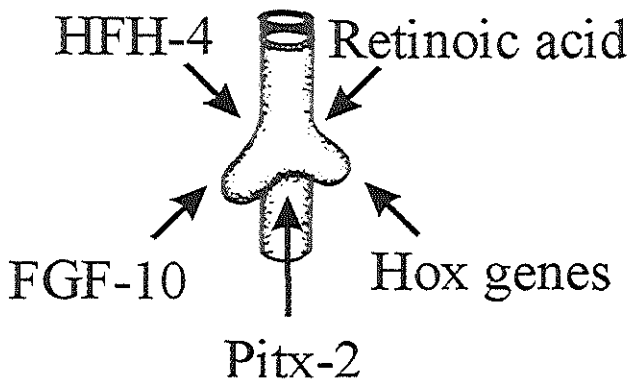

Figure 3 Factors involved in lung bud outgrowth and establishment of left-/right-asymmetry.

Interestingly, the hormone retinoic acid, whose role in pulmonary development and embryonic development is known since the early fifties (Wilson et al., 1953), might function as the mediator between Hox- and FGF-signaling. Retinoic acid has been shown to induce Hoxa5, b5 and b6 gene expression in lungs (Bogue et al., 1994). Furthermore, vitamin A deficiency during pregnancy results in varying anomalies, including the FGF-10 -/- phenotype of no lung buds and a blunt ending trachea (Dickman et al., 1997). Recently, both retinoic acid production and expression of retinoic acid receptors (RARs) have been shown to coincide with the early events of lung bud evagination from the primitive foregut (Malpel et al., 2000; Niederreither et al., 1999; Zhao et al., 1996a). In addition, evidence for a direct interaction between retinoic acid signaling and FGF-10 signaling has been observed (Bellusci et al., 1997b; Malpel et al., 2000).

Subsequent to the initial symmetrical lung bud outgrowth, an asymmetry in the left-right axis of the lung is established. In general, the right bronchus is slightly larger than the left one and is oriented more vertically. The right lung bud gives rise to four secondary buds and the left bud to only one (in human there are three secondary buds on the right and two on the left). From then on left-right asymmetry is established in the lungs. Only recently, some light has been shed on this subject. Data from a bone morphogenetic protein (BMP)-4/LacZ reporter suggest that even before the lung buds have grown out of the primitive foregut, left-right asymmetry is already established. The group of Hogan has shown that BMP-4/LacZ is asymmetrically expressed in the ventral mesoderm overlying the outgrowing lung buds until 11.5 days of gestation (Weaver et al., 1999). Factors involved in the specification of the left-right axis are hepatocyte nuclear

factor/forkhead homologue (HFH)-4, lefty-1, lefty-2, nodal, Pitx2 and Growth/differentiation factor-1 (Gdf-1) (Chen et al., 1998; Gage et al., 1999; Lu et al., 1999; Rankin et al., 2000; Supp et al., 1997). All of these genes are normally expressed on the left side of the body and therefore determine "left-ness" of the body. Interestingly, null mutation of one of these genes Pitx2 (a homeobox gene) results in right pulmonary isomerism. Pitx2 is the gene responsible for situs inversus in Rieger syndrome (Lu et al., 1999)

Another syndrome associated with situs inversus (and additionally ciliary dyskinesia) is Kartagener syndrome. The candidate gene for this syndrome is HFH-4 which is involved in the formation of ciliated cells. Targeted mutation of HFH-4 results in ciliary dyskinesia and random left-right laterality of the visceral organs (Chen et al., 1998).

1.6 Tracheo-esophageal separation

As stated above, the formation of a separate trachea and esophagus occurs differently in mice and man. In mice, two paired endodermal buds grow out of the primitive foregut and as the two buds elongate, the primitive foregut tube begins to pinch into two tubes, namely a dorsal esophagus and a ventral trachea (Ten Have-Opbroek, 1981). In human however, the trachea is first separated from the esophagus by a laryngotracheal groove. After elongation of the tracheal outgrowth, the end of it bifurcates and forms two bronchial lung buds (Sutliff and Hutchins, 1994).

Several genes that are involved in pulmonary development have also been implicated in tracheo-esophageal separation. Based on knockout studies the most important genes are Shh-, Gli2/Gli3- and TTF-1. Shh is one of the mammalian homologues of the *Drosophila* hedgehog gene, discovered in a screen for genes involved in segmentation of *Drosophila* embryos (Nusslein-Volhard and Wieschaus, 1980). Shh is expressed in respiratory epithelium, with high levels at the tips of the branching lung buds (Bellusci et al., 1997a). Besides its role in branching morphogenesis, Shh is involved in tracheo-esophageal separation. In mice null for Shh, the trachea and esophagus remain fused and two rudimentary sacs attached to this tube form the lungs (Litingtung et al., 1998; Pepicelli et al., 1998).

Gli1, 2 and 3 are the murine homologues of *Drosophila cubitus interruptus* (Hui et al., 1994). All three are expressed in pulmonary mesoderm and they have been implicated in transducing the Shh signal (Grindley et al., 1997). Gli1-/- mice do not display a lung

phenotype. In contrast, Gli2 and Gli3 have been demonstrated to be essential for normal foregut and pulmonary morphogenesis (Motoyama et al., 1998). Gli2-/- mice have unilobar lungs on the left and right side. Gli3-/- mice have hypoplastic lungs. Interestingly, in Gli2-/-;Gli3+/- compound mice, the situation is different, because this genetic background results in esophageal atresia with tracheo-esophageal fistula. A severe lung phenotype, namely fusion of the left and right lung in the midline was observed in these mice (Motoyama et al., 1998).

The final gene implicated in separation of the esophagus and trachea is TTF-1. As described before, genetic ablation of TTF-1 results in absence of the thyroid, pituitary, parts of the forebrain, and inhibition of branching morphogenesis of two rudimentary sac-like structures which are attached to a single tube esophago-trachea (Kimura et al., 1996; Minoo et al., 1999).

Similar anomalies have been observed in embryos exposed to severe vitamin A deficiency (Wilson et al., 1953) and in RARα and RARβ2 double knockout mice (Mendelsohn et al., 1994). However, it is not clear whether these malformations are the result of direct effects of disturbed vitamin A metabolism or are due to the effects of vitamin A on Hox gene expression. Moreover, seeing the above described phenotypes of Shh, Gli2/Gli3 and TTF-1 knockout mice, it is tempting to speculate about the effects of vitamin A on the Shh/Gli pathway and TTF-1 expression.

1.7 Branching morphogenesis

The morphogenesis of many organs such as the gut, kidney and mammary gland is highly dependent on controlled interactions between two tissue layers, epithelium and mesenchyme. The process in pulmonary development, known as branching morphogenesis is also dependent on interactions between epithelium and mesenchyme. The highly characteristic branching pattern of the bronchial epithelial buds of embryonic lungs has long been known to depend on a specific interaction with bronchial mesenchyme (Alescio and Cassini, 1962; Rudnick, 1933). The effect of bronchial mesenchyme on epithelial branching is organ- and species-specific and is temporally regulated (Masters, 1976; Spooner and Wessells, 1970; Wessells, 1970). In addition, proximal (trachea and main bronchi) and distal (lung bud) mesenchyme differ in their ability to support branching morphogenesis (Wessells, 1970). Moreover, ectopically induced buds on the trachea express specific distal lung genes (Shannon, 1994). These grafting experiments led to the assumption that epithelial-mesenchymal interactions must

be mediated by well-orchestrated signals between the mesenchyme and the epithelium. Recently, several growth factors, their receptors and other signaling molecules have been shown to act as inductive signals mediating these epithelial-mesenchymal interactions and regulating pulmonary morphogenesis (see Figure 4).

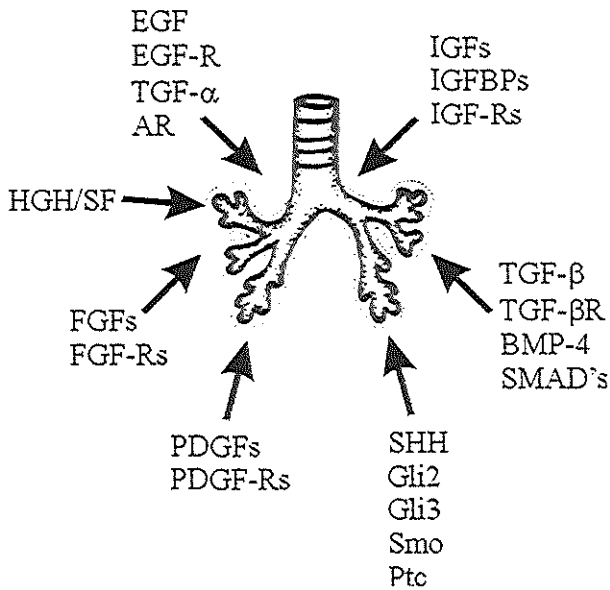

Figure 4 Schematic representation of several growth factors and receptors involved in branching morphogenesis.

1.7.1. Fibroblast growth factor family

Fibroblast growth factors (FGFs) are known to play key roles in embryonic growth and morphogenesis. As earlier mentioned, the FGF system has been shown to play an important role in primary tracheal branching of *Drosophila*. Loss-of-function of either *breathless* (Glazer and Shilo, 1991), an FGF receptor gene, or *branchless* (Sutherland et al., 1996), an FGF gene, leads to abnormal tracheal branch formation. The mammalian FGF family consists of at least 18 ligands and their signals are mediated through four tyrosine kinase FGF receptors. The most important ligands for pulmonary development (FGF-7 and FGF-10) are expressed in the developing mesenchyme (Bellusci et al., 1997b; Post et al., 1996), whereas the associated receptor (FGFR-2) is expressed in the pulmonary epithelium (Peters et al., 1992).

The importance of FGF signaling was nicely demonstrated by the complete inhibition of branching morphogenesis in the lung tubes of transgenic mice expressing dominant negative forms of FGFR-2 (Peters et al., 1992). Both FGFR-1 and FGFR-2 gene ablation result in early embryonic lethality (Arman et al., 1998; Deng et al., 1994; Yamaguchi et al., 1994). Studies using chimeric FGFR-2-/- embryos indicate that this receptor is essential for limb bud outgrowth and lung branching morphogenesis, which corroborates with the FGF-10 knockout mice (Arman et al., 1999). The ligands for the FGFR-2 receptor are both FGF-7 and FGF-10. Mice null for FGFR-3 (Colvin et al., 1996; Deng et al., 1996) or FGFR-4 (Weinstein et al., 1998) exhibit no pulmonary phenotype. However, FGFR-3 and FGFR-4 compound mutant mice display pulmonary emphysema and deficient alveolization (Weinstein et al., 1998). Previously, the chemotactic role of FGF-10 for pulmonary epithelium has been mentioned (Park et al., 1998; Weaver et al., 2000). Based on the dynamic expression of FGF-10 in the mesenchyme overlying the newly formed lung buds, an important role for FGF-10 in controlling lung bud outgrowth and elongation is now generally accepted. This role for FGF-10 is underscored by the blunt ending trachea and complete absence of lung buds in mice null mutant for FGF-10 (Min et al., 1998; Sekine et al., 1999). FGF-7 (also know as keratinocyte growth factor, KGF) is also expressed in pulmonary mesenchyme, and shares sequence homology with FGF-10 (Post et al., 1996; Yamasaki et al., 1996). However, in contrast to FGF-10, FGF-7 has no chemotactic effects on pulmonary epithelium, but is involved in patterning, proliferation and differentiation of the epithelium (Cardoso et al., 1997; Park et al., 1998; Shiratori et al., 1996; Simonet et al., 1995). Surprisingly, mice bearing a null mutation of the FGF-7 gene have no obvious lung abnormalities (Guo et al., 1996), suggesting that FGF-7 can be replaced by other factors, such as FGF-1 and FGF-10. FGF-1 is also expressed in pulmonary mesenchyme and has been shown to stimulate embryonic epithelial cell proliferation (Bellusci et al., 1997b; Park et al., 1998). In addition to its role in epithelial cell proliferation, there is evidence that FGF-1 also possesses chemotactic properties for pulmonary epithelium, albeit less strong than that seen with FGF-10 (Park et al., 1998).

The complexity of lung morphogenesis has been underscored recently by the identification of a negative regulator of FGF signaling. In *Drosophila* one of the downstream targets of *branchless* and *breathless* was found to be *sprouty* (Hacohen et al., 1998). Inactivation of *sprouty* results in increased tracheal branching, indicating that *sprouty* functions as an antagonist of the *branchless/breathless* pathway (Hacohen et al.,

1998). At present, four murine and three human sprouty homologues have been identified. Only murine sprouty-2 (pulmonary endoderm) and –4 (pulmonary mesenchyme) are expressed in the lung (de Maximy et al., 1999; Hacohen et al., 1998; Tefft et al., 1999). Inhibition of sprouty-2 function with antisense oligonucleotides in organ explant cultures leads to increased branching and surfactant protein gene expression (Tefft et al., 1999). Although the mechanism of sprouty function is still not completely understood, it is most likely that sprouty-2 inhibits FGF-10 expression through inhibition of signal transduction pathways (Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999).

1.7.2. Shh/patched receptor/smoothened/Gli pathway

Shh is the mammalian homologue of the *Drosophila* segment polarity gene hedgehog. It belongs to a family of hedgehog proteins consisting of Sonic, Indian, Desert and Tiggy-Winkle [for review see (Hammerschmidt et al., 1997)]. Shh is expressed throughout pulmonary development in the respiratory epithelium being highest in the endodermal tips during branching morphogenesis (Bellusci et al., 1997a). Genetic ablation of Shh function results in the disturbance of dorsal ventral axis patterning during early embryogenesis (Chiang et al., 1996). As stated before, in Shh null mice the trachea and esophagus remain fused and two rudimentary sacs are attached to this tube forming the lungs (Litingtung et al., 1998; Pepicelli et al., 1998). In the two rudimentary sacs, epithelial cell differentiation along the proximo-distal axis occurred normally (Pepicelli et al., 1998). Overexpression of Shh in distal epithelial cells suggests that Shh functions as a mitogen (Bellusci et al., 1997a).

The Shh signal is transmitted by patched, a multi-membrane-spanning protein identified as the receptor for hedgehog in *Drosophila* (Chen and Struhl, 1996; Marigo et al., 1996; Stone et al., 1996). Patched is predominantly expressed in the mesenchyme surrounding the endodermal tips expressing highest Shh levels (Bellusci et al., 1997a; Pepicelli et al., 1998). Shh induces patched expression (Bellusci et al., 1997a). Abrogation of patched function with antisense oligonucleotides results in diminished branching morphogenesis *in vitro*. When Shh binds to patched, the latter releases another segment polarity gene, smoothened. Smoothened then activates the zinc finger Gli proteins to function as a transcriptional activator (Murone et al., 1999). Gli1, Gli2 and Gli3 are the mammalian homologues of *Drosophila* cubitus interruptus (Hui et al., 1994). As described before, the Gli proteins have essential functions during normal embryonic foregut development and lung morphogenesis (Motoyama et al., 1998). The downstream

target genes of the Gli proteins are as yet unknown. Taken together, it is clear that the Shh/patched/smoothened/Gli pathway plays an essential role in morphogenesis of the foregut and the lung.

1.7.3. Epidermal growth factor family

Epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), and amphiregulin (AR) are members of the EGF family of growth factors that act through the common EGF receptor (EGFR). The mRNAs of all three are localized in the mesenchyme (Ruocco et al., 1996; Snead et al., 1989), and the proteins in the epithelium (Ruocco et al., 1996; Snead et al., 1989; Strandjord et al., 1995a; Strandjord et al., 1994), suggesting that they are produced in the mesenchyme, but act on the epithelium. The EGFR is expressed in the epithelium (Ruocco et al., 1996; Strandjord et al., 1995a; Strandjord et al., 1994; Warburton et al., 1992). All three proteins stimulate growth and branching morphogenesis of embryonic murine lung *in vitro* (Warburton et al., 1992). However, genetic ablation of EGFR in mice has led to conflicting interpretations regarding a role of EGF/TGF-α in branching morphogenesis. Mice homozygous for an EGFR null mutation have been reported to die postnatally of respiratory failure which was attributed to lung immaturity (Miettinen et al., 1995; Miettinen et al., 1997). In contrast, another null EGFR mutation with a different genetic background did not display any major lung abnormality during development (Threadgill et al., 1995). Also, expression of a dominant negative EGF receptor under control of the SP-C promoter does not alter lung morphogenesis in transgenic mice (Hardie et al., 1996). Moreover, mice having EGFR signaling deficiencies, *wa-2/wa-2* mice (Fowler et al., 1995), have normal lung morphology (Hardie et al., 1996). Thus, it appears that EGFR signaling is not essential for lung branching morphogenesis although it is possible that other receptors have taken over EGFR signaling in these loss-of-function experiments.

1.7.4. Insulin-like growth factor family

The insulin-like growth factor (IGF) family consists of two related peptides, IGF-I and IGF-II, six IGF binding proteins (IGFbps) and two specific IGF receptors. The type 1 receptor transmits most biological responses, such as proliferation and differentiation, while the type 2 IGF/mannose-6-phosphate receptor appears to function in internalizing and transporting IGF-II to the lysozomes (Jones and Clemmons, 1995). Ample evidence suggests that the IGF system has a role in pulmonary development (Stiles and D'Ercole,

1990). All components of the IGF system, IGF-I and –II, their receptors, and the IGFbps are expressed in both rodent and human lung. During pulmonary development, there is hardly any change in distribution or level of expression of IGF-I and –II or their receptors. IGF-I is expressed in the pulmonary mesenchyme (Han et al., 1987; Klempt et al., 1992; Retsch-Bogart et al., 1996), and IGF-II predominantly in the pulmonary epithelium (Retsch-Bogart et al., 1996). The type 1 receptor is expressed in almost all cells of the lung (Retsch-Bogart et al., 1996), whereas expression of the type 2 receptor is limited to pulmonary mesenchyme (Retsch-Bogart et al., 1996). In contrast, the IGFbps are differentially regulated and dynamically expressed during pulmonary development (Moats-Staats et al., 1995; Retsch-Bogart et al., 1996). The function of each IGFbp is not completely known yet, but their spatial and temporal expression patterns suggest a role in controlling IGF action at specific sites during pulmonary development. Studies with transgenic mice argue against a direct role for IGFs in branching morphogenesis. Mice carrying a disrupted IGF-I gene are growth retarded and die at birth from respiratory failure. However, no obvious histopathological defect of pulmonary development other than uninflated lungs, is observed (Liu et al., 1993). Mice with no functional type 1 IGF receptor display a similar phenotype (Liu et al., 1993). Transgenic mice with a disrupted IGF-II gene are small and have small lungs, but no abnormal lung morphology is noted (DeChiara et al., 1990). The type 2 IGF receptor is paternally imprinted and mice with a maternally inherited disrupted type 2 IGF receptor gene, which die shortly after birth from heart failure, have abnormal alveoli but no major branching defect (Lau et al., 1994; Wang et al., 1994). In conclusion, although the IGFs may play a role during pulmonary development, they appear not to be a major determinant in early lung morphogenesis.

1.7.5. Hepatocyte growth factor

A heparin-binding mitogen with morphogenetic and motogenic activities is hepatocyte growth factor/scatter factor (HGF/SF). During mouse development, HGF/SF and its receptor *c-met*, are expressed in different but adjacent tissues, suggesting that they transduce mesenchymal-epithelial signaling (Rosen et al., 1994; Sonnenberg et al., 1993). HGF/SF is produced by fetal lung fibroblasts and stimulates the proliferation of adult type II cells (Mason et al., 1994; Panos et al., 1993; Shiratori et al., 1995). *In vitro*, exogenous HGF/SF or a neutralizing antibody to HGF/SF has no effect on early branching morphogenesis of mouse lung (Shiratori et al., 1996). These data are consistent with genetic ablation experiments. Mouse bearing a null mutation in the HGF/SF gene do not

survive beyond 14.5 days of gestation *in utero*, but show normal branching morphogenesis until that gestational age (Schmidt et al., 1995; Uehara et al., 1995). Also, branching and growth appears normal when lungs of day 14.5 mutant embryos are cultured *in vitro* (Uehara et al., 1995).

1.7.6. Platelet-derived growth factor family

Platelet-derived growth factor (PDGF) has been implicated in embryonic development (Goustin et al., 1985; Mercola et al., 1990). It is a dimeric molecule composed of two distinct but related polypeptides (A and B). The two chains assemble as a heterodimer, PDGF-AB, or as homodimers, PDGF-AA or PDGF-BB (Raines et al., 1991). PDGF exerts its effect via specific cell surface receptors (Claesson-Welsh, 1994). Two related transmembrane tyrosine kinase receptors, PDGF-α and PDGF-β receptors have been characterized. The PDGF-α receptor binds all three isoforms of PDGF whereas the PDGF-β receptor binds only PDGF-BB with high affinity (Claesson-Welsh, 1994). Both PDGF homodimers, AA and BB, and both PDGF receptors are present in the early embryonic rat lung (Han et al., 1993; Han et al., 1992). At this early stage of pulmonary development, the mesenchyme is enriched in the expression of both receptors (Souza et al., 1995). In contrast, expression of both PDGF homodimers, AA and BB, is confined to the epithelium of the embryonic lung (Han et al., 1992; Souza et al., 1995; Souza et al., 1994). However, PDGF-A and PDGF-B transcripts and proteins are detected in both tissue layers with advancing gestation (Han et al., 1992; Souza et al., 1995; Souza et al., 1994). Expression in separate but adjacent tissue layers of ligands and receptors for PDGF has been noted in many tissues during development, including mouse lung (Orr-Urtreger and Lonai, 1992), submandibular salivary gland (Orr-Urtreger and Lonai, 1992) and placenta (Holmgren et al., 1991).

The appositional expression of PDGF and its receptors during early lung development is compatible with a role for PDGF in epithelial-to mesenchymal signaling during branching morphogenesis. The physiological function of PDGF in lung branching morphogenesis has been partly delineated. Translation arrest of endogenous PDGF-B with antisense oligonucleotides in embryonic rat lung results in smaller lungs but the degree of branching is not affected (Souza et al., 1994). Similarly, antisense PDGF-β receptor oligonucleotides reduce lung size without affecting branching (Souza et al., 1996). Mice deficient for PDGF-B (Leveen et al., 1994) and PDGF-β receptor (Soriano, 1994) show no abnormal lung branching phenotype, suggesting that PDGF-BB is not involved in the

regulation of lung branching. Using an antisense strategy, Souza *et al.* (Souza et al., 1995) reported that PDGF-AA influences early lung branching morphogenesis. The number of terminal buds of embryonic rat lung explants was significantly reduced in the presence of antisense PDGF-A oligonucleotides. Addition of PDGF-AA, but not PDGF-BB, to the culture system attenuated the inhibitory effect of antisense PDGF-A on early lung branching. Also, antisense PDGF-α receptor oligonucleotides inhibited early lung branching (Souza et al., 1996). It should be noted that blocking of function of PDGF-A or PDGF-α receptor does not completely block branching, indicating that other factors are involved in regulating this morphogenetic process.

A null mutant for PDGF-A is lethal at two restriction points: one prenatally prior to 10.5 days of gestation, implying a role in early embryonic development, and one postnatally (Bostrom et al., 1996). The lung phenotype of PDGF-A null embryos dying prior to 10.5 days of gestation was not further investigated and, thus, it is not completely clear whether PDGF-AA is involved in the early formation of the lung. The observation that PDGF-A-deficient mice surviving postnatally have normal fetal and early postnatal pulmonary development argues against PDGF-AA being a major determinant in lung branching morphogenesis. However, it is possible that the role of PDGF-A is replaced by other non-PDGF factors or that maternal leakage of PDGF rescues the early developmental events in the knockout mice. Interestingly, homozygous mice that survive the first bottleneck at 10.5 days of gestation develop postnatally lung emphysema secondary to the failure of alveologenesis attributed to a lack of alveolar myofibroblast differentiation (Bostrom et al., 1996). Homozygous mutant mice carrying a targeted disruption in the PDGF-α receptor gene die during embryonic development and exhibit severe developmental defects. Most interestingly, the phenotype of the PDGF-α receptor mutant mice differs from that of the PDGF-A mutant mice. However, early pulmonary development proceeds normally in the PDGF-α receptor null mutants. *Patch* mutant mice which are homozygous for a large deletion of the PDGF-α receptor gene (Bostrom et al., 1996), also die prenatally before 10.5 days of gestation (Orr-Urtreger et al., 1992). Heterozygotes, expressing only 50% of the PDGF-α receptor on their cells compared with their wild-type littermates, develop normal lungs. Recently, the role of the PDGF-α receptor was better specified. Using a human YAC transgene to overexpress the PDGF-α receptor in PDGF-α receptor knockout mice, researchers were able to overcome the early embryonic lethality in the knockout mice. However, the mice died after birth because of respiratory failure, since the transgene did not rescue the lung phenotype, suggesting an

important role in prenatal pulmonary development (Sun et al., 2000). Hence, although the genetic data support a role for the PDGF-AA/α receptor complex in embryonic development, further studies such as double mutant mice for ligands and receptors are needed to determine whether the PDGF/receptor complex has a regulatory role in lung branching morphogenesis.

1.7.7. Transforming growth factor beta family

Transforming growth factor beta (TGF–β) is a multifunctional peptide that elicits a wide variety of morphogenetic responses in many organs. Several lines of evidence suggest that TGF–β is also important for fetal pulmonary development. All three mammalian TGF–β subtypes (β1, β2, and β3) have been detected in the developing murine lung (Pelton et al., 1990; Schmid et al., 1991). TGF–β1 mRNA expression is prominent throughout the mesenchyme while TGF–β2 message is mainly localized to the epithelium of the developing distal airways. The TGF–β3 transcripts are initially found in the tracheal mesenchyme, but are also found in the epithelium of the growing bronchioles during the pseudoglandular stage of murine pulmonary development. Both TGF–β1 and TGF–β2 inhibit branching morphogenesis *in* vitro (Serra et al., 1994; Zhao et al., 1996b). Although numerous TGF–β binding proteins have been identified, type I and type II receptors are responsible for most, if not all, biological activities of TGF–βs (Attisano et al., 1994). Just recently, the group of Post has extensively investigated the role of the three mammalian TGF-β subtypes and their receptors in early pulmonary organogenesis *in vitro*. By using antisense oligonucleotides and neutralizing antibodies in lung explant culture, they demonstrated that TGF-β2, but not TGF-β1 and TGF-β3 are essential for regulating pattern formation in the early lung. The signals are thereby predominantly mediated via the TbetaR-I(ALK-5)/TbetaR-II heteromeric complex (Liu et al., 2000).

TGF–β can exert a variety of biological effects, including regulation of extracellular matrix (ECM) production and integrin expression (Roberts and Sporn, 1991). Thus TGF–β may increase cell-ECM interactions and, by doing so, influence branching morphogenesis (Minoo and King, 1994). A recent study has shown that TGF–β1 co-localizes with collagen I and II, fibronectin, and glycoaminoglycans at the epithelial-mesenchymal interface of clefts of branching lung (Heine et al., 1990). High dosages of exogenous TGF–β1 inhibit lung branching morphogenesis *in vitro* (Serra et al., 1994). The mechanism of inhibition of branching by TGF–β1 is unknown. TGF–β1

has been suggested to inhibit the action of N-*myc* protein in lung growth (Serra et al., 1994), which has been implicated in lung growth and branching (Moens et al., 1992; Sawai et al., 1993). Interestingly, mice bearing a partial mutation of the N-*myc* gene display severe pulmonary hypoplasia resulting in neonatal lethality (Moens et al., 1993). Recent studies have demonstrated that PDGF α receptor expression in lung fibroblasts is also negatively modulated by TGF–β1 (Bonner et al., 1995). As the PDGF-AA/α receptor complex may play a role in lung branching morphogenesis *in vitro* (Souza et al., 1995; Souza et al., 1996), another possibility is that high dosages of TGF–β1 disrupt this complex by adversely influencing PDGF α receptor expression in embryonic mesenchymal cells. Whether TGF–β2 and TGF–β3 have similar effects on N-*myc* or PDGF α receptor expression as TGF–β1 remains to be elucidated. Studies with transgenic mice have implicated TGF–β in regulating late but not early lung organogenesis. Overexpression of TGF–β1 in lung epithelium of transgenic mice causes a pseudoglandular arrest of lung development (Zhou et al., 1996a). However, caution is warranted in extrapolating these data to normal development as TGF–β1 is mainly expressed in the lung mesenchyme of developing lung (Pelton et al., 1990; Schmid et al., 1991). Surprisingly, inactivation of the mouse TGF–β1 gene does not affect lung branching (Kulkarni et al., 1993; Shull et al., 1992). Mice lacking TGF–β3 die within several hours after birth and exhibit an arrest in pulmonary development at the late pseudoglandular stage (Kaartinen et al., 1995). The TGF–β2 null mice exhibit perinatal mortality and a wide range of developmental defects (Sanford et al., 1997). Prenatal lung morphology of the mutant mice appeared to be normal while newborn mice had collapsed conducting airways. Together, these genetic analyses suggest that none of the TGF–β isoforms play a major biological role in early lung branching morphogenesis. Alternatively, it is possible that redundancy or maternal leakage of TGF–βs rescues the early developmental events in these knockout mice. Analysis of double or triple mutants is now feasible, which may help to further understand the requirements of TGF–βs during pulmonary development.

BMPs are members of the TGF–β family that are important for pulmonary development. BMP-3, 4 and 7 are expressed in the lung during embryonic development (Bellusci et al., 1996; Takahashi and Ikeda, 1996). BMP-4 transcripts are expressed in the epithelium at the tips of terminal lung buds (Bellusci et al., 1996). Transgenic studies with mice overexpressing BMP-4 in distal epithelium results in smaller lungs with dilated

terminal sacs, with a reduced number of differentiated type II cells (Bellusci et al., 1996). Recently, BMP-4 signaling was blocked using a dominant negative approach and a BMP-4 inhibitor, *Xenopus Noggin* in transgenic mice. This resulted in a disruption of distal differentiation and thus proximalization of the lung (Weaver et al., 1999). These data indicate that BMP-4 is involved in specifying cell fates along the proximo-distal axis. Furthermore, the interaction between FGF-10 and BMP-4 signaling has been investigated intensively (Lebeche et al., 1999; Weaver et al., 2000). BMP-4 expression is induced by FGF-10 *in vitro* (Lebeche et al., 1999). In addition, BMP-4 has been demonstrated to serve as an inhibitor for FGF-10 induced lung bud outgrowth (Weaver et al., 2000). There are two other factors involved in regulation of BMP-4, namely TTF-1 (Minoo et al., 1999) and Pod-1 (Quaggin et al., 1999). Pod-1 belongs to the basic helix-loop-helix transcription factors. Its expression is confined to the mesenchyme of organs where epithelial-mesenchymal interactions occur, such as the kidney, lung, intestine and pancreas (Quaggin et al., 1998). Blocking Pod-1 function with antisense oligonucleotides results in diminished mesenchymal cell condensation in embryonic kidney culture (Quaggin et al., 1998). Genetic ablation of Pod-1 results in pulmonary hypoplasia and disruption of epithelial cell differentiation (Quaggin et al., 1999). In the null mutants, BMP-4 expression is downregulated, whereas FGF-10 expression is not altered (Quaggin et al., 1999).

1.8. Extracellular matrix and lung branching

Extracellular matrix (ECM) plays a complex role in regulating the behavior of cells that contact it, influencing their development, migration, proliferation, and morphology, as well as metabolic functions. In particular, the basement membrane (BM), which separates the epithelium from the surrounding mesenchyme, has been implicated in mediating morphogenetically significant tissue interactions. The epithelial-mesenchymal interface contains laminin, fibronectin, proteoglycans, and collagen as major matrix components.

1.8.1. Collagens

In addition to their structural roles, collagens may have numerous developmental and physiological functions (Bienskowski, 1991; Hay, 1991). In the adult lung, collagen accounts for 60% of the lung connective tissue protein mass. Collagen types I and III are predominantly associated with mesenchymal connective tissue in the lung. Lung collagen types IV and V are mainly found in BMs, although the latter may also be associated with

the interstitial matrix (Clarke et al., 1983). The amount of collagen has been shown to increase with development (Bradley et al., 1974). Several *in vitro* studies with inhibitors of collagen have suggested that it is important for branching morphogenesis of the lung (Spooner and Faubion, 1980). However, these results have not yet been confirmed in studies with mice that do not produce collagen (Kratochwil et al., 1986), and therefore, the precise role of collagen in pulmonary development remains obscure.

1.8.2. Laminins

Laminins are structurally related glycoproteins found mainly in BMs. Laminins are large multidomainglycoproteins composed of three polypeptide subunits: α, β, and γ (Burgeson et al., 1994). Structural isoforms exist for each of these subunits and the association of these isoforms in heterotrimers gives rise to different laminins. Laminins stimulate cell adhesion and migration, growth, and morphology, as well as gene expression in a variety of cells (Timpl and Brown, 1994). At least 10 laminin variants are involved in pulmonary development. The precise role of the different laminins during pulmonary development is not yet completely understood. Laminin-1 (α1, β1, γ1), laminin-2 (α2, β1, γ1) and laminin-3 (α1, β2, γ1) have been implicated in branching probably through their involvement in the organization of the basement membrane (Schuger et al., 1990a; Schuger et al., 1990c). The laminin domains involved in cell attachment (cross-intersection of laminin structure and carboxy terminus of α chain) and heparin binding activity (inner globular region of β1 chain) may facilitate lung branching morphogenesis (Schuger et al., 1991). Recent studies suggest that an interaction between laminin and heparan sulfate proteoglycan (HSPG) is critical for the development of epithelial polarization and lumen formation (Schuger et al., 1990b; Schuger et al., 1996). Taken together, the exact mechanism of laminin function in pulmonary development has to be determined, however, preliminary evidence suggests that it is a key regulatory matrix component in branching morphogenesis.

1.8.3. Fibronectin

Fibronectin is an adhesive glycoprotein that plays a major role during morphogenesis by promoting cell attachment (Ruoslahti, 1988a). In the developing mouse lung, fibronectin is localized to areas of airway bifurcations (Roman and McDonald, 1992). Inhibition of ligand binding to the fibronectin receptor with synthetic peptides diminishes but does not abolish lung branching (Roman et al., 1991a; Roman et al., 1991b).

1.8.4. Anti-adhesive ECM proteins

Since adhesion of cells to the extracellular matrix appears to be critical for lung branching, molecules that disrupt cellular adhesion may also modulate this morphogenetic process. Anti-adhesive properties have been ascribed to several ECM proteins, such as SPARC (secreted protein, acidic and rich in cysteine), and tenascin (Sage and Bornstein, 1991). SPARC is expressed in the pulmonary epithelium during early rat pulmonary development (Strandjord et al., 1995b). Inhibition of SPARC function results in inhibition of branching morphogenesis *in vitro* (Strandjord et al., 1995b). The tenascins are a family of extracellular matrix glycoproteins of typical multidomain structure (Chiquet-Ehrismann, 1995). Only tenascin-C has been implicated in lung organogenesis (Young et al., 1994). Antibodies against tenascin-C inhibit branching *in vitro* (Young et al., 1994). In contrast, disruption of the tenascin-C gene in mice does not affect normal pulmonary development (Saga et al., 1992). The latter finding makes it questionable whether tenascin plays an essential role during lung branching morphogenesis. However, it is possible that the other members of the tenascin family compensate for the loss of tenascin-C.

1.8.5. Proteoglycans

Proteoglycans (PGs) are major components of the basal laminae. The PGs constitute a family of multidomain core proteins to which glycosaminoglycans (GAGs) are attached. GAGs bind to other matrix components, cell adhesion molecules, and growth factors (Juul et al., 1991; Ruoslahti, 1988b; Ruoslahti, 1989). These properties suggest that GAGs may mediate, at least in part, cell adhesion, structural organization, and cell proliferation during fetal pulmonary development. Inhibitors of GAG synthesis such as 6-diazo-5-oxo-L-norleucine and azaserine inhibit branching morphogenesis of cultured embryonic lung rudiments (Spooner et al., 1986). The exact mechanism by which PG/GAGs direct branching morphogenesis is unknown. Increasing evidence suggests that PG/GAGs play an important role as modulators of growth factor activities. Several isoforms of FGF bind to high-affinity receptors and are active when bound to specific cell-surface heparan sulfate proteoglycans (Klagsbrun and Baird, 1991). Similarly, the membrane proteoglycan betaglycan increases the binding of TGF-β to the signaling TGF-β receptor (Lopez-Casillas et al., 1993). These properties suggest that PG/GAG may mediate morphogenetic actions of growth factors, such as FGF and TGF-β, on cell adhesion and cell proliferation during early pulmonary development.

1.8.6. Integrins

Integrins are cell surface proteins that have been identified to serve as receptors for ECM molecules (Albelda and Buck, 1990; Hynes, 1992). Integrins are transmembrane glycoproteins that consist of αβ heterodimers. The response of a cell to a particular form of an ECM molecule will depend on the number and subunit composition of integrins expressed on its surface and their binding properties. The integrins α1β1 and α2β1 function as collagen-binding receptors in fetal rat lung fibroblasts (Caniggia et al., 1995). The α1 integrin subunit is not crucial for lung branching as a null mutation in the α1 integrin gene does not result in any loss of viability or obvious phenotype (Gardner et al., 1996). The expression of the fibronectin receptor α5β1 in the developing lung has been reported (Roman and McDonald, 1992) and inhibition of ligand binding to fibronectin receptor affects lung branching *in vitro* (Roman et al., 1991b; Roman and McDonald, 1992). A null α5 mutation leads to embryonic death prior to lung formation (Yang et al., 1993). As discussed earlier, laminin appears to be a key determinant for regulating lung branching morphogenesis. Several laminin integrins have been identified (Mercurio and Shaw, 1991), of which α2β1, α3β1, and α6β1 are expressed in the developing human lung (Virtanen et al., 1996). Recently, genetic ablation of the α3 integrin gene has been shown to result in reduced bronchial branching (Kreidberg et al., 1996). This suggests that the laminin/α3β1 interaction is crucial for lung organogenesis.

1.9 Differentiation and maturation of pulmonary tissues

At the end of the pseudoglandular stage, when branching morphogenesis has almost come to an end, the airways are lined by undifferentiated columnar epithelium (Ten Have-Opbroek, 1981). Subsequently, this primordial epithelium gives rise to the prospective bronchial columnar epithelium and the prospective respiratory low columnar/cuboidal epithelium (Ten Have-Opbroek, 1981). This event establishes a proximal conducting region and a distal gas exchange region. The process of differentiation and maturation then continues in the canalicular, terminal sac and alveolar periods. The cuboidal cells lining the pulmonary acinus are type II (precursor) cells. These cells will eventually mature into surfactant-producing type II cells or differentiate into flattened type I cells (Otto-Verberne et al., 1988).

Surfactant is a compound that lowers the surface tension of the alveoli, so that collapse of the alveoli is prevented on expiration (Hawgood, 1991). As mentioned, type II cells produce surfactant. These type II cells have matured from precursor type II cells

during the canalicular phase of pulmonary development. The main characteristic that distinguishes precursor type II cells from mature type II cells is the decrease in glycogen content which in turn gives rise to an increase in number and size of lamellar bodies, which are the storage pools of pulmonary surfactant (Kresch and Gross, 1987). Surfactant consists of approximately 90% phospholipids, 5-10% proteins, and a small amount of carbohydrate (Kresch and Gross, 1987; Post and van Golde, 1988). The phospholipid fraction is composed of 75% phosphatidylcholine (PC) and 70% of this is saturated (Kresch and Gross, 1987; Post and van Golde, 1988; Rooney et al., 1994). The main enzyme involved in PC synthesis is cholinephosphate cytidylyltransferase (CT), which forms also the rate-limiting step of PC synthesis (Kresch and Gross, 1987; Post and van Golde, 1988; Viscardi and McKenna, 1994; Zimmermann et al., 1993).

Four different surfactant-associated proteins can be distinguished: Surfactant protein-A (SP-A), -B (SP-B), -C (SP-C), and –D (SP-D) [for review see (Johansson et al., 1994)]. SP-A is the most abundant surfactant protein. It is synthesized by type II cells, but also by the Clara cells of the respiratory bronchioles. Interestingly, SP-A has no surface tension-reducing properties. It is involved in pulmonary host defense processes, and functions as a cytokine and opsonin (Ikegami et al., 1997; Ikegami et al., 1998; LeVine et al., 1999; LeVine et al., 1998). SP-D has also no function in reducing the surface tension. It functions as a cytokine, and is involved in regulation of the function of pulmonary macrophages (Botas et al., 1998; Ikegami et al., 2000; Wert et al., 2000). The other two surfactant proteins, SP-B and SP-C are involved in reducing the surface tension of the alveoli. SP-B is mainly involved in processing of the surfactant pools and regulates surfactant homeostasis (Akinbi et al., 1997; Stahlman et al., 2000; Tokieda et al., 1999; Tokieda et al., 1997). SP-C promotes the rapid adsorption of phospholipid films, thereby reducing the alveolar surface tension at the air-liquid interface.

1.10. Hormonal modulation of pulmonary growth

Several growth factors and hormones have been demonstrated to be involved in regulating fetal pulmonary maturation. Glucocorticoids, thyroid hormones, epidermal growth factor, adenosine 3', 5'-cyclic monophosphate (cAMP), estrogens, and prolactin all positively regulate lung maturation (Gross, 1990). On the other hand, insulin, androgens and transforming growth factor-β have all been implicated in negatively influencing pulmonary maturation (Gross, 1990). Herein, we will only focus on the influence of

glucocorticoids and thyroid hormones, since they have actually made it to clinical practice, whereas the other mentioned factors have been investigated only experimentally.

1.10.1. Glucocorticoids

Since the early seventies, the influence of hormones on pulmonary maturation has been well recognized. At that time, Liggins (Liggins, 1969) demonstrated that administration of glucocorticoids to premature fetal sheep results in less respiratory distress and a better survival. Since then, the application of hormonal modulation of fetal pulmonary growth has been studied extensively. It is now well established that steroids, i.e. glucocorticosteroids, as well as thyroid hormones play a role in fetal lung development and in pulmonary surfactant production. Glucocorticoids play a critical role in regulating numerous physiological and developmental processes (Ballard and Ballard, 1995). During fetal and postnatal development, glucocorticoids function as signaling molecules to modulate the orderly sequence of differentiation in most tissues. From mid-gestation onwards, the fetus is exposed to increasing levels of cortisol of primarily fetal origin. Both in humans and in other mammals there is strong evidence that the administration of glucocorticoids to the immature fetus results in an accelerated maturation of the fetal lungs. Also, "physiological stressors" such as infection and preterm prelabor rupture of the membranes have been shown to accelerate fetal lung maturation (Bauer et al., 1974; Kotas, 1973). However, there is also strong evidence that endogenous glucocorticoids do not initiate alveolar epithelial maturation, but that they are involved in the modulation of genes responsible for surfactant production [for review see (Ballard, 1987)].

The positive effects of glucocorticoids on pulmonary maturation are attributed to an increase in surfactant protein synthesis, but also to an increased collagen and elastin content, both resulting in an improved lung compliance (Ballard and Ballard, 1995; Kresch and Gross, 1987). Together with these positive effects on surfactant production, glucocorticoids stimulate antioxidant enzyme activity, resulting in higher levels of antioxidant enzymes (Frank et al., 1985; Walther et al., 1991). The exact mechanisms by which glucocorticoids exert these positive effects are not completely understood. The effects have been suggested to be mediated via a Fibroblast Pneumocyte Factor (FPF), that is produced by pulmonary fibroblasts and stimulates surfactant synthesis by type II cells (Smith and Post, 1989). All these effects are mediated through specific glucocorticoid receptors that, upon binding of glucocorticoids, regulate transcription of the different involved genes, such as the surfactant protein genes (Ballard, 1983).

Concomitant with a rise in glucocorticoid plasma levels near term, a developmentally timed enhancement of glucocorticoid receptor gene expression has been shown by Sweezy and coworkers in the fetal rat (Sweezey et al., 1995; Sweezey et al., 1998). Autoradiographic localization studies demonstrate that glucocorticoid receptor gene expression is increased in the mesenchyme, and more specifically in the mesenchymal cells that are adjacent to the terminal saccular epithelium, the cell population responsible for fibroblast-pneumocyte factor production (Beer et al., 1984; Caniggia et al., 1991).

Already in 1972, Liggins and Howie reported the first trial of antenatal glucocorticoid treatment in humans. Antenatal glucocorticoids significantly decreased the incidence of respiratory distress syndrome (RDS) in preterm born infants (Liggins and Howie, 1972). Meta-analysis of published studies on antenatal glucocorticoids demonstrated statistically significant beneficial effects on neonatal outcome for infants born at less than 34 up until 24 weeks of gestation (Crowley, 2000; Crowley, 1995). Hence, the NIH consensus recommends antenatal administration of corticosteroids for 24 hours, but if possible, for 48 hours, to all fetuses between 24 and 34 weeks of gestation at risk of preterm delivery (1995b).

1.10.2. Thyroid hormones

The beneficial effects of the thyroid hormones tri-iodothyronine (T3) and thyroxine (T4) on pulmonary maturation are more controversial. On the one hand thyroid hormones have been demonstrated to stimulate PC synthesis and CT activity, and hence stimulate surfactant synthesis (Kresch and Gross, 1987; Post and van Golde, 1988). On the other hand, there is no regulatory effect on surfactant protein synthesis and moreover, a negative effect on fatty acid synthase (Gross, 1990; Kresch and Gross, 1987). Since thyroid hormones do not normally cross the blood-placenta barrier, thyrotropin-releasing hormone (TRH) is administered to mothers in order to increase T3 levels in the fetus (Thorpe-Beeston and Nicolaides, 1993). However, TRH only stimulates surfactant excretion, and not synthesis (Post and van Golde, 1988). Moreover, TRH has been demonstrated to negatively influence the activity of antioxidant enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase (Chen et al., 1993). These enzymes are required for the detoxification of reactive O_2 metabolites such as O_2 free radicals (Frank and Sosenko, 1987a; Frank and Sosenko, 1987b). These O_2 metabolites are likely to be produced when premature born neonates are exposed to high oxygen concentrations during artificial ventilation (Fardy and Silverman, 1995; Frank and Sosenko, 1987a).

In the early nineties, the administration of glucocorticoids combined with thyroid hormones was advocated for their supposed synergistic effects. However, the extra effect of adding thyroid hormones to glucocorticoids is becoming more controversial. The combined administration of glucocorticoids and TRH was presumed to have effects on surfactant synthesis. However, these synergistic effects were not confirmed in multicenter clinical trials. The negative effects of TRH on antioxidant enzyme expression and the unclear synergistic effects do not justify the addition of TRH to glucocorticoids in clinical practice these days [for review see (Keijzer et al., 2000)].

The rationale for clinical studies with TRH was based on numerous animal and *in vitro* studies, but the clinic itself also gave some suggestions for the involvement of thyroid hormones in lung development. Lower circulating T3 levels were observed in human preterm infants who develop RDS as compared to healthy neonates of similar gestation (Abbassi et al., 1977). More evidence that thyroid hormones are directly involved in lung development came from the observations that, in the human fetus, the lungs have been shown to contain high levels of T3 receptors (Bernal and Pekonen, 1984), and T3 receptors have been demonstrated in adult type II cells (Smith and Hitchcock, 1983). deMello et al. found that fetal and neonatal lung maturation is delayed in mice with primary hypothyroidism (deMello et al., 1994). Using a different experimental mouse model of fetal hypothyroidism, van Tuyl et al. could not demonstrate an effect on fetal surfactant proteins; yet, based on morphometric analysis, we found a striking delay in differentiation from day 7 to 28 postnatally (unpublished results, van Tuyl personal communication).

In 1992, Ballard et al. published the results of a multicenter, blinded, randomized trial (Ballard et al., 1992). TRH plus betamethasone treatment significantly reduced the development of chronic lung disease in infants weighing less than 1500 g, but TRH treatment alone did not affect the incidence of RDS. In contrast, the ACTOBAT study, the Australian multicenter randomized trial, showed that the combined use of glucocorticoids and TRH in preterm labor resulted in an increased frequency of RDS and increased need of ventilation in the newborn. Moreover, this study reported a significant increase in maternal side effects, such as nausea, vomiting, lightheadedness, and a rise in blood pressure to 140/90 mm Hg or higher (1995a). The collaborative Santiago Surfactant Group was also not able to find any additional benefit of a combined antenatal use of TRH and corticosteroids over corticosteroids alone in the prevention of RDS (1998). In 1998, Ballard et al., now including all treated infants, could not show any beneficial effect

of antenatal TRH and glucocorticoids treatment over glucocorticoid treatment alone (Ballard et al., 1998). Taken together, these data indicate that the additional effects of TRH observed in experimental models have not been substantiated in clinical trials and the antenatal use of TRH should therefore be abandoned until further data become available.

1.11 Vascularization

In contrast to the large amount of information on the development of the pulmonary tissues and their interaction, much less is known about vascularization of the lung. As in differentiation of pulmonary tissues, pulmonary vascularization is guided by a well-orchestrated interplay between extracellular matrix proteins, cell adhesion receptors, growth factors and their receptors (Baldwin, 1996). In a classical study, deMello *et al.* investigated neovascularization of the lung using Electron Microscopy. They postulate that pulmonary vascularization is dependent on two processes: vasculogenesis and angiogenesis. In the first process, vasculogenesis, blood vessels develop *de novo* from blood lakes present in the mesenchyme of the E9 embryonic mouse lung. Subsequently, the blood lakes coalesce and form irregular sinusoidal structures. The second process, angiogenesis, starts a few days later around E12, when arteries and veins start to sprout from the central pulmonary vessels. At a certain point during development (around E14), the peripheral sinusoids and central vessel sprouts will connect and establish a vascular network in the lung (deMello et al., 1997). This connecting of peripheral and central vascular structures is accompanied by extensive branching of the vessels which follow the branching pattern of the airways (deMello et al., 1997). However, it is not clear whether branching morphogenesis of the airways dictates branching of the vessels, or *vice versa*.

Just recently, the first factors involved in pulmonary endothelial cell differentiation have been identified. In addition to growth factors that were already known for their role in pulmonary tissue differentiation, such as members of the FGF-, TGF-β- and PDGF-family, new growth factors such as Vascular Endothelial Growth Factor (VEGF) and its receptors flk-1 and flt-1, have been implicated in pulmonary vessel formation (Drake and Little, 1995; Flamme et al., 1995a; Flamme et al., 1995b; Gebb and Shannon, 2000; Yamaguchi et al., 1993). Overexpression of VEGF in pulmonary epithelium using the SP-C promoter in transgenic mice, results in abnormal pulmonary morphogenesis combined with an increase in vascularization surrounding the airways (Zeng et al., 1998). This indicates that neovascularization and lung morphogenesis are closely related.

Very recently, Endothelial Monocyte Activating Polypeptide (EMAP) II was identified as an important anti-angiogenic factor preventing unorganized vessel growth in the fetal lung (Schwarz et al., 1999). *In vitro*, EMAP II inhibits fetal lung neovascularization and alters lung epithelial morphology. In conclusion, pulmonary neovascularization is dependent on a balanced interplay between positive and negative modulators of angiogenesis. In addition, pulmonary vascularization and pulmonary morphogenesis are closely related during fetal development, and disturbing normal vessel formation results in abnormal pulmonary morphogenesis.

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Chapter 2

Abnormal pulmonary development

Based on:
Keijzer, R., van Tuyl, M., and Tibboel, D. (2000) Hormonal modulation of fetal pulmonary development: relevance for the fetus with diaphragmatic hernia. *Eur J Obstet Gynecol Reprod Biol*, 92(1): p. 127-33.

2.1 Introduction

Disturbance of the normal order of developmental events during pulmonary organogenesis results in abnormal pulmonary development, or most frequently to underdevelopment of the lungs, which is generally referred to as pulmonary hypoplasia. Pulmonary hypoplasia can occur as an isolated entity, but is usually part of a congenital anomaly. The most important ones discussed herein are, oligohydramnios sequence, Potter syndrome, obstructive uropathy, anencephaly, and congenital diaphragmatic hernia (CDH). When children suffering from these anomalies are born, pulmonary hypoplasia is in many cases the most important prognostic factor, due to the resulting respiratory insufficiency which may eventually lead to therapy resistant respiratory failure and death. During the past decades, tremendous progress has been made in treating congenital anomalies due to the increase in sophisticated technological treatment modalities which are nowadays at the disposal of neonatologists and pediatric surgeons. Amongst the most recent treatment modalities are for instance extracorporeal membrane oxygenation (ECMO), high frequency oscillation (HFO) and fetal surgery. However, this increased assortment of treatments did not really influence survival in the children suffering from severe pulmonary hypoplasia. Therefore, instead of finding alternative aids for treating pulmonary hypoplasia, the emphasis of research is (and should be) shifted towards understanding pulmonary hypoplasia better. In this way, future treatment modalities can be designed at preventing or reversing pulmonary hypoplasia, which will hopefully prove to be better than "just" treating respiratory insufficiency postnatally. Herein, the characteristics of pulmonary hypoplasia and the most important congenital anomalies that are associated with pulmonary hypoplasia will be described. In addition, one of the anomalies, CDH, will be discussed in more detail, because an important part of the work described in this thesis was motivated by the remaining questions in the pathogenesis of pulmonary hypoplasia in CDH and consequently performed in an animal model for CDH.

2.2 Pulmonary hypoplasia

Hypoplasia is derived from the Greek word *hypoplasis*, which means "undermolding" or underdevelopment. Usually this is due to a decrease in the number of cells. Hence, pulmonary hypoplasia can be described as underdevelopment of the lungs. The criteria for pulmonary hypoplasia have first been described by Askenazi and Perlman (Askenazi and Perlman, 1979).

They are:

- decreased lung-body weight ratios
- decreased radial alveolar counts (RAC), which is a measure of the complexity of the pulmonary acinus (Emery and Mithal, 1960; George et al., 1987; Nakamura et al., 1991; Reale and Esterly, 1973)
- lower number of airway generations (Areechon and Reid, 1963; Kitagawa et al., 1971)
- lower number of vascular generations (Kitagawa et al., 1971)
- greater muscularity of the peripheral arteries (Naeye et al., 1976).

The first three criteria are observed as part of different congenital anomalies, such as oligohydramnios, Potter syndrome, anencephaly, and CDH. However, the latter two criteria are only observed in pulmonary hypoplasia associated with CDH, and not with the other congenital anomalies (Hislop et al., 1979).

In case of oligohydramnios, there is less than normal or virtually no amniotic fluid, due to a leakage of amniotic fluid or insufficient production of urine. This clinical condition is often caused by congenital malformations of the urinary tract especially obstructive uropathy (Hislop et al., 1979; Perlman and Levin, 1974; Potter, 1965). In the classical description of the Potter syndrome, oligohydramnios is the result of bilateral agenesis of the kidneys (Potter, 1965). Because of the absence of kidneys, no urine is produced. The prevalence of Potter syndrome is approximately 1 in every 3000 births. Next to oligohydramnios, characteristic facial changes and limb deformities, together with pulmonary hypoplasia are associated with this syndrome (Potter, 1965). The facial changes and limb deformities are thought to be caused by compression of the face and limb *in utero*. The absence of liquid in the lungs during development and interference with fetal breathing movements are held responsible for resulting in pulmonary hypoplasia, which is the cause of death in these children. For normal pulmonary development to occur, the presence of liquid in the lungs as well as fetal breathing movements are absolutely required (Harding, 1997; Hooper and Harding, 1995; Lawrence and Rosenfeld, 1986). Hence, the absence of both probably results in pulmonary hypoplasia in case of anencephaly also. Because these children cannot swallow amniotic fluid nor perform normal fetal breathing movements, they are born with hypoplastic lungs (Reale and Esterly, 1973).

2.3 Congenital diaphragmatic hernia

2.3.1. Introduction and embryology

The most common congenital anomaly associated with pulmonary hypoplasia is CDH. The first literature on CDH dates back to 1679, when Lazarus Riverius described the postmortem findings of a 24-year old man (Riverius, 1679). Subsequently, Sir Charles Holt described the first pediatric case of CDH in 1701 (Holt, 1701). In 1761, Giovanni Battista Morgagni was the first to describe the association of pulmonary hypoplasia with CDH (Morgagni, 1769). In 95% of the cases the diaphragmatic defect is localized posterolateral. This form of CDH is called a hernia of Bochdalek after the Hungarian anatomist who first described this form of CDH and the localization of the remaining foramen in 1848 (Bochdalek, 1848). Approximately 60 years after the first speculation on surgical repair of CDH by Laennec (Laennec, 1827), the first (unsuccessful) attempt was undertaken (Naumann, 1888). The first series of survivors is attributed to Robert Gross based on his experience in the Boston Children's Hospital (Gross, 1946). Since then, numerous reports have been published on the surgical repair of CDH, and with the increased peri-operative care in the twentieth century, the operation itself is not longer considered the limiting factor for survival. However, overall survival remained troublesome, due to the inability to treat pulmonary hypoplasia, persistent pulmonary hypertension and the negative effects of artificial ventilation.

The diaphragmatic defect occurs eight times more often on the left side than on the right (Benjamin et al., 1988; Tibboel and Gaag, 1996). This is due to the fact that closure of the diaphragm occurs earlier on the right side than on the left. The foramen of Bochdalek normally closes during development, however, a disturbed development of the pleuroperitoneal fold results in defective closure (Greer et al., 2000). Besides this pleuroperitoneal fold, the diaphragm originates from the septum transversum, the body wall, and the dorsal mesentery of the esophagus (Allan and Greer, 1997). As a consequence of defective closure of the foramen, herniation of abdominal viscera into the thorax can occur. Classically the herniation of abdominal organs is held responsible for the occurrence of pulmonary hypoplasia, because of a competition for intrathoracic space between the developing lungs and the herniated abdominal organs (Molenaar et al., 1991) leaving the original theory of compression of the developing lungs behind. However, since the introduction and extensive study of an animal model for CDH, an alternative

hypothesis has been proposed (Iritani, 1984). This hypothesis will be discussed in detail below when the animal models for CDH are described.

2.3.2. Clinical background

The prevalence of CDH is 1 in 3000 total births (Torfs et al., 1992). Even recently developed management techniques, such as fetal surgery, ECMO, HFO, Nitric oxide inhalation and the concept of gentle handling, have not significantly influenced the mortality rate of CDH in high-risk patients and consequently, CDH remains a major problem in pediatric surgery and neonatology. A recent review shows that the mortality remains high at 50 to 60% in high-risk patients (Katz et al., 1998). However, very recent reports suggest a modest reduction in mortality to approximately 30% in comparable series of patients (Azarow et al., 1997; Kays et al., 1999; Reickert et al., 1998; Wung et al., 1995). In particular, the degree of pulmonary hypoplasia is almost impossible to evaluate pre- and immediately postnatal, and in a number of cases the degree of pulmonary hypoplasia is an important determinant of the outcome (Thebaud et al., 1998). Today, even severe cases of pulmonary hypoplasia can sustain life for a limited period of time, but will die due to the high vulnerability for ventilation induced lung damage and the inability to dilate the abnormal pulmonary vasculature.

The pathogenesis and etiology of CDH are incompletely understood (Tibboel and Gaag, 1996). Familial cases of CDH have been described (Norio et al., 1984), however, the recurrence rate in a family is only 3% (Crane, 1979). Besides the description of isolated cases of CDH as the result of prenatal use of thalidomide, anticonvulsants and quinine in mothers (Hobolth, 1962; Kup, 1967; Powell and Johnstone, 1962), no environmental factors have been identified to be involved in the etiology of CDH in humans. In 50% of the cases, CDH is associated with other malformations such as neural tube defects and cardiac abnormalities (Benjamin et al., 1988; Fauza and Wilson, 1994) and (genetic) abnormalities, albeit not uniform (Sweed and Puri, 1993; Tibboel and Gaag, 1996). These associated major malformations negatively influence survival of the children. The most common chromosomal abnormalities found in CDH patients are: trisomy 18, 13, 21, or monosomy 9p and 4p (Cunniff et al., 1990). A special syndrome associated with CDH is the so-called Fryns syndrome (Lubinsky et al., 1983).

As stated above, the main limiting prognostic factors are determined by the degree of pulmonary hypoplasia and persistent pulmonary hypertension. Although the exact underlying pathogenetic events are not clear, pulmonary hypoplasia was found to be

based on a decrease in the number of terminal bronchioli and volume of alveoli in CDH (Areechon and Reid, 1963). On the other hand, persistent pulmonary hypertension is caused by abnormal muscularity of the pulmonary arteries (Kitagawa et al., 1971; Naeye et al., 1976), and in addition, increased sensitivity of the vascular tissue to regulators of vascular tone (Stolar et al., 1985).

2.3.3. Pathogenesis and animal models for CDH

The pathogenesis of pulmonary hypoplasia and CDH remains unclear. At present, there are two hypotheses (see Figure 1). The first is classical and considers pulmonary hypoplasia as a result of the diaphragmatic defect. Subsequent to the appearance of the diaphragmatic defect, the abdominal organs herniate into the thorax, thereby hindering normal pulmonary development due to a competition for intrathoracic space (Molenaar et al., 1991). The second (alternative) hypothesis suggests that primary disturbance of pulmonary growth into the pleuroperitoneal canal results in abnormal diaphragm development, leading to the diaphragmatic defect. This alternative hypothesis was first postulated by Irritani based on his observations made in a mouse model of CDH using a teratogenic agent (Iritani, 1984).

There are various animal models to investigate either one of the hypotheses. One model is based on a strain of pigs in which CDH occurs naturally (Ohkawa et al., 1993). The involved gene is not cloned yet, but the authors postulate that it has an autosomal recessive mode of inheritance. Naturally, this is the most promising one, since it offers the possibility to clone the responsible genes and generate transgenic animals. Recently, compound null mutations of retinoic acid receptor (RAR) genes, the receptor through which vitamin A exerts its effects, were described with variable foregut anomalies. Some mice displayed a CDH-like phenotype, whereas others suffered from an absent tracheoesophageal septum and left lung agenesis with hypoplasia of the left and/or right lungs (Mendelsohn et al., 1994). Besides these "real" genetic models, all other animal models for CDH are either based on mechanical (surgical) or teratogenic interference with diaphragm development.

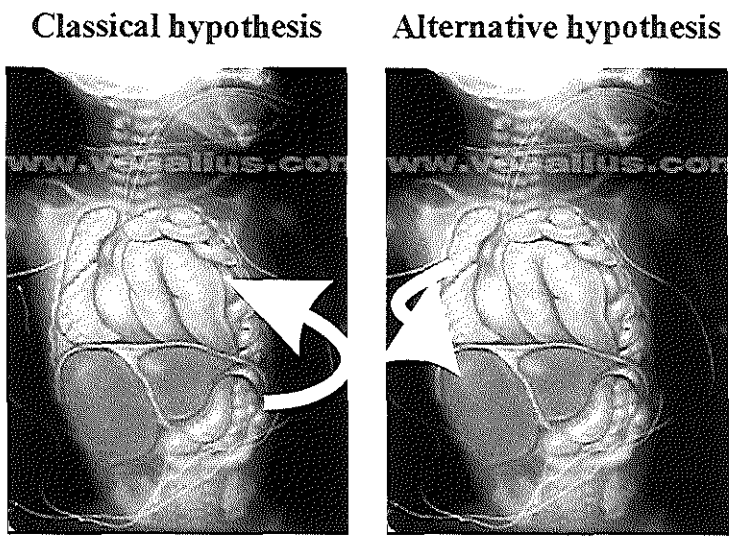

Figure 1 Schematic representation of the two hypotheses regarding the pathogenesis of pulmonary hypoplasia and CDH. On the left the classical hypothesis, which states that the diaphragmatic defect is the primary defect, and pulmonary hypoplasia is the result of herniation of the abdominal organs into the thorax. On the right the alternative hypothesis stating that pulmonary hypoplasia is the primary defect, and abnormal diaphragm development occurs separately or as a result of abnormal pulmonary development.

The surgical model has been extensively studied in sheep and to a lesser extent in rabbits. De Lorimer et al. were the first to describe the surgical model for CDH in sheep. In fetal lambs, a hole in the diaphragm is surgically created during the late pseudoglandular or canalicular stage of lung development and the bowels are manipulated into the thoracic cavity, in order to mimic the herniation of abdominal viscera (De Lorimier et al., 1967). Most aspects of pulmonary hypoplasia seen in the human infant with CDH were also seen in the lambs with surgically created CDH (Harrison et al., 1980). However, this does not hold true for the pulmonary vascular morphologic changes (Adzick et al., 1985). The group of Harrison slightly modified the model, since they positioned an inflated balloon in the thorax in order to simulate intrathoracic herniation of the abdominal organs (Harrison et al., 1980). The sheep model has proven extremely valuable in investigating several aspects of the late-gestational and perinatal pathophysiology, as well as *in utero* repair of the diaphragmatic defect. With the increased use of antenatal ultrasound, more fetuses with CDH are being diagnosed before

birth. The high mortality from CDH has prompted approaches to correct the malformation *in utero* (Mychaliska et al., 1996). Whereas anatomical repair of the defect is possible *in utero* with open fetal surgery, such an approach proved possible only when the liver was not herniated into the thorax. *In utero* surgical correction of cases with "liver up" did not result in a reduced mortality rate when compared to postnatal treatment results (Harrison et al., 1993). Another antenatal therapeutic strategy that has been introduced recently is known as 'Plug the Lung Until it Grows' (PLUG) (Hedrick et al., 1994) and is materialized also in the application of a tracheal ligation technique as described by Wilson and colleagues (DiFiore et al., 1994). There have been reported survivors using this approach, but there are concerns that tracheal occlusion may delay the maturation of the lungs and the risks of preterm birth associated with *in utero* surgical interventions continue to limit fetal interventions (Benachi et al., 1998). Harrison *et al.* recently suggested that high-risk fetuses with CDH, those with liver in the chest or low lung to head ratios, can be successfully treated with fetoscopic temporary tracheal occlusion by a detachable balloon (Harrison et al., 1998). However, the results of this NIH sponsored FETENDO technique have to be awaited.

Despite these positive characteristics of the sheep model, the most important shortcoming of this model is that the defect is created relatively late in gestation, and as a consequence it is not really possible to investigate the pathogenesis of CDH. In addition, because of the limited number of fetuses in a litter in sheep it is difficult to design adequate controlled studies leading to statistically significant results. Moreover, there are considerable amounts of money associated with this animal model, which makes it less attractive. The latter might be overcome by the use of rabbits instead of sheep for the surgical model. In rabbits (with an average litter size of approximately 6), it is also possible to create a left diaphragmatic defect during the pseudoglandular stage of lung development (Fauza et al., 1994; Wu et al., 2000). The group of Deprest has even demonstrated that it is possible to repair this defect *in utero* or perform tracheal ligation later on during gestation (personal communication).

The other widely used animal model for CDH is based on the teratogenic effects of the herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (Nitrofen). When administered to pregnant rodents, disturbed organogenesis of the diaphragm, lungs, heart and kidneys will occur (Ambrose et al., 1971). In 1984 Iritani published the first data on using Nitrofen to induce CDH and pulmonary hypoplasia in fetal mice through administration of Nitrofen to the mother during pregnancy (Iritani, 1984). Iritani was also the first one to propose

pulmonary hypoplasia to be the primary defect in CDH, and he even suggested that pulmonary hypoplasia might be causing the diaphragmatic defect in CDH (Iritani, 1984). This model was later adjusted by Kluth (Kluth et al., 1990) and by Tenbrinck (Tenbrinck et al., 1990), who demonstrated that Nitrofen has similar effects in rats. Subsequently, the rat model has been extensively studied in order to gain more insight into the pathogenetic events in CDH. Interestingly, whereas all fetuses exposed *in utero* display pulmonary hypoplasia, only in 80-90% of the fetuses a diaphragmatic defect is obtained. The fact that pulmonary hypoplasia is more severe when a diaphragmatic defect is present and also more severe on the ipsilateral side of the diaphragmatic defect suggests that there might indeed be a causative relationship between pulmonary hypoplasia and the diaphragmatic defect (Brandsma et al., 1994a; Nakao and Ueki, 1990; Tenbrinck et al., 1990). Just recently, studies with a Nitrofen mouse model were published (Cilley et al., 1997; Coleman et al., 1998). This will make it possible to use Nitrofen in transgenic mice and thus, making a whole new set of research tools available for investigating Nitrofen and its effects on lung and diaphragm development.

The exact mechanism of action of Nitrofen is not completely understood. There have been speculations about the finding that Nitrofen might function through alteration of the thyroid hormone status (Manson, 1986). The significance of thyroid hormones on lung development in this CDH model, however, is based on the following observations. Nitrofen has a high stereochemical similarity to thyroid hormones and Manson *et al.* demonstrated that following concomitant administration of thyroxine and Nitrofen to pregnant rats, a drastic reduction (70%) in the number of malformations in the offspring was observed (Manson et al., 1984). Brandsma *et al.* showed that Nitrofen *in vitro* decreases the binding of triiodothyronine to the alpha 1 and beta 1 form of the thyroid hormone receptor in a non-competitive manner (Brandsma et al., 1994b). They concluded that rat lung hypoplasia might result from the decreased binding of triiodothyronine to its receptor via exposure to Nitrofen during fetal development. Despite this, no evidence has been published that demonstrates the direct interaction of Nitrofen with thyroid hormone metabolism. Of interest is the recent finding that antenatal treatment with vitamin A of Nitrofen-induced CDH rats, reduces the incidence and severity of CDH, and improves pulmonary growth and maturation (Thebaud et al., 1999).

The main advantage of the Nitrofen model is that CDH in this case is induced very early in gestation, enabling the investigation of early pathogenetic events. Other advantages of this model are the large litter sizes, the short duration of gestation and the

relative low costs of rodents. A disadvantage of the model is that the small size of the fetuses limits the possibilities for functional studies such as artificial ventilation and surgical interventions. Despite the proven usefulness of the Nitrofen model over the years in particular for characterizing abnormal lung growth and pulmonary vascular abnormalities, no correlation between either Nitrofen or thyroid hormone status has been proven to play a pivotal role in the etiology of CDH in humans (Bos et al., 1994; Tibboel and Gaag, 1996). Hence, the results obtained with the Nitrofen model should be extrapolated to the human situation with a certain amount of reserve.

2.3.4. Main characteristics of pulmonary hypoplasia in the animal models for CDH

As stated above, the sheep model of surgically-induced CDH has been mainly used for functional and *in utero* repair studies, and to a lesser extent for structural and morphological studies. The degree of pulmonary hypoplasia is dependent on the time of gestation when the diaphragmatic defect is produced. The earlier the defect is produced, the more severe is the hypoplasia. However, one should bear in mind that the defect in the diaphragm is produced after normal closure, and pulmonary development was up till that moment normal.

Pulmonary hypoplasia associated with surgically-induced CDH is characterized by the same morphological abnormalities as seen in the human situation (Harrison et al., 1980). In addition, disturbed alveolar cell differentiation results in diminished pulmonary maturation, which is characterized by surfactant deficiency (Glick et al., 1992; Wilcox et al., 1995). Surprisingly, normal lecithin/sphingomyelin (l/s) ratios and phophatidylglycerol concentrations, both markers of lung maturity, have been found in the amniotic fluid of CDH lambs (Wilcox et al., 1995). In humans, these markers have been reported to be decreased (Hisanaga et al., 1984; Sullivan et al., 1994). In contrast, Pringle et al. found an increase in number and size of type II cells when they investigated the hypoplastic lungs of CDH lambs using electronmicroscopy (Pringle et al., 1984). Furthermore, the muscle mass is increased in the pulmonary vessels of CDH lambs, mimicking the findings in persistent pulmonary hypertension (Harrison et al., 1986).

Since the introduction of the Nitrofen model, speculations have been made about pulmonary hypoplasia being the primary defect in CDH. Iritani has even postulated the idea that Nitrofen interferes with normal pulmonary development, and hence, the lung buds cannot grow normally into the pleuroperitoneal canal, thereby disturbing normal development of the posthepatic mesenchymal plate. It is this posthepatic mesenchymal

plate that forms the main tissue of origin for the diaphragm, and the disturbance of its normal development will result in a diaphragmatic defect (Iritani, 1984). However, Iritani did not provide any direct evidence to show an interaction between lung and diaphragm development. Moreover, Nitrofen was given on a daily basis from day 9 of gestation till term.

Only a limited amount of information is available on the contribution of diminished expression of growth factors, transcription factors and neuropeptides to pulmonary hypoplasia and CDH. Most of this data has been obtained in the Nitrofen model. Just recently, the expression of some of the key lung morphogenetic genes (FGF-10 and Bmp-4) was demonstrated to be disturbed in the Nitrofen CDH model, whereas the expression of others (Shh and Sprouty2) was not (Thebaud, personal communication). Different studies focused more on the delayed maturation and cell differentiation of hypoplastic lungs of Nitrofen treated rats. These lungs display altered SP-A protein expression indicating that type II cell differentiation into type I cells is altered (Brandsma et al., 1994a). Recently it was shown that Nitrofen induced pulmonary hypoplasia results in diminished expression of SP-B and SP-C mRNA (Guilbert et al., 2000). However, others have shown that this surfactant deficiency is no longer present at term (Batenburg et al., 1996). Interestingly, there was no difference in the amount of lamellar bodies in Nitrofen lungs, however, the appearance of these lamellar bodies was abnormal (Brandsma et al., 1993). In addition, conflicting results have been obtained when markers for lung maturation were investigated (Brandsma et al., 1993; Hisanaga et al., 1984; IJsselstijn et al., 1998b; Moya et al., 1995; Nakamura et al., 1991; Sullivan et al., 1994). Preliminary data of Brandsma *et al.* demonstrated that the expression of several extracellular matrix proteins is not altered in Nitrofen–exposed lungs (personal communication). Recently, the group of Chinoy has demonstrated in their Nitrofen mice model that laminin protein is reduced in late gestation hypoplastic lungs when compared to control lungs (personal communication).

Antenatal hormonal modulation of pulmonary growth and maturation has been investigated in both the surgical and Nitrofen CDH model. The administration of glucocorticoids and thyrotropin releasing hormone (TRH) to Nitrofen-treated pregnant rats resulted in an increase in di-saturated PC in the fetal rats, but had no effect on the glycogen concentration, lung compliance or lung morphology (Losty et al., 1996; Losty et al., 1995; Suen et al., 1994a; Suen et al., 1994b). In addition, the combination of dexamethasone and TRH treatment in the Nitrofen CDH model did not change survival of

the affected pups when compared to sham-treated rats. (Ijsselstijn et al., 1997b). As corticosteroids are known to exert their effect primarily on mesenchymal cells, the group of Martin Post performed a number of experiments using isolated type II cells (unpublished results, personal communication). The rate-limiting enzymes for surfactant synthesis together with the effect of corticosteroids on mesenchymal cells in Nitrofen-induced CDH pulmonary cells and age-matched controls were evaluated. They observed that mesenchymal cells in CDH could not be stimulated by corticosteroids in the same manner as controls. This raises intriguing questions on the effects of corticosteroids at the cellular level.

After a period of uncertainty about the optimal way to modulate lung growth and/or differentiation by hormones, nowadays consensus exists on the role of corticosteroids in threatened preterm births. Based on this consensus, some clinicians have started to use corticosteroids in antenatally diagnosed cases of CDH. So far, the few reports that are available consist of personal communications, individual case reports or small series, but no properly designed randomized trial. The rationale for using corticosteroids in CDH is based on observations in the surgical CDH sheep model, or the Nitrofen-based rat model. In these models, changes in morphometric measurements, mRNA levels of surfactant proteins and functional changes following artificial ventilation have been investigated. Researchers have even suggested that, in the Nitrofen model, antenatal use of corticosteroids changes the pulmonary vascular architecture. However, no reports on the expression of the glucocorticoid receptor in the hypoplastic lung are available thus far.

In the clinical setting, a randomized clinical trial evaluating the use of corticosteroids in antenatally diagnosed CDH has recently been started. Under the guidance of Dr. Kevin Lally, Texas, over 30 centers both in Europe and the U.S. plan to include 150 cases in both arms of the study to obtain an answer to the question whether antenatal corticosteroids are of benefit in human CDH. Mothers with fetuses diagnosed with CDH will be given multiple courses of betamethasone after 34 weeks of gestation.

The antioxidant enzyme system appears to be unaltered in the Nitrofen CDH model during development. However, when CDH rats were exposed to high oxygen artificial ventilation, their lungs displayed more pronounced oxygen-induced lung damage (Ijsselstijn et al., 1997b; Sluiter et al., 1992). Of interest is the finding that infants with CDH have decreased vitamin A (a major antioxidant) blood levels (Major et al., 1998). This corroborates with a recently published study that demonstrated a better survival and

decreased congenital anomalies in litters which had received vitamin A together with the Nitrofen treatment (Thebaud et al., 1999). Neuropeptide expression was shown to be increased and delayed in the rat model of CDH (IJsselstijn et al., 1998a; IJsselstijn et al., 1995). This corresponds to the increased bombesin immunoreactivity in infants with CDH (Ijsselstijn et al., 1997a), but is in contrast to the decreased expression of gastrin-releasing peptide (Durbin et al., 1996).

The importance of abnormal vascular development as a determinant of survival in CDH has just recently been appreciated. The results of studies on this subject are just beginning to emerge. In the rat model similar abnormalities have been observed in the pulmonary vessels as in infants: increased muscle mass and muscle found in arteries of smaller diameter as normal (Kitagawa et al., 1971; Naeye et al., 1976). One of the important determinants of vascular development which has been shown to be absent in lungs of CDH rats is VEGF (Okazaki et al., 1997). However, contrasting results have been obtained for VEGF and its receptors flk-1 and flt-1 by others using mRNA *in situ* hybridization (Guilbert et al., 2000). To make the situation even more confusing, VEGF expression was shown to be enhanced in lungs of humans with CDH when compared to age-matched controls (Shehata et al., 1999)

Besides studies investigating the structural morphological factors in Nitrofen-induced abnormal pulmonary vascular development, many other studies have focused more on the presumed altered pulmonary vascular reactivity as the underlying mechanism of persistent pulmonary hypertension in CDH. It was shown that one of these regulators, endothelin-1 and its receptor A were upregulated in lungs of CDH rats (Okazaki et al., 1998). In a different study, both decreased Nitric oxide synthase (NOS) expression and NOS activity were observed in CDH rat lungs (Karamanoukian et al., 1996). As in the human situation (Nakayama et al., 1992), several lung eicosanoids are increased after birth in CDH rats when compared to controls (Ijsselstijn et al., 1997c).

2.4 Concluding remarks and aims of the studies

A non-conclusive overview of the (in my opinion) most important and interesting aspects of normal and abnormal pulmonary development which have been already investigated was given herein. In most clinical studies on pulmonary hypoplasia associated with CDH, decreasing the high mortality and morbidity was the primary goal mainly by searching for new treatment modalities. However, even sophisticated new techniques such as ECMO and fetal surgery did not bring the long-awaited significant improvement in survival of

high-risk CDH patients. Over the past years, research in the field has shifted into the direction of finding ways to better understand the abnormal lung from a developmental perspective. In order to better understand abnormal development it is most important to have a good understanding of the processes involved in normal development.

This thesis describes studies which are aimed at unraveling certain aspects of normal and abnormal pulmonary development. Therefore, the studies in this thesis were designed in order to answer the following questions:

1. What role does the transcription factor GATA6 play during fetal pulmonary development?
2. What is the role of proliferation and apoptosis in normal lung morphogenesis and does the balanced interplay between the two influences this process?
3. How are the thyroid hormone receptors involved in fetal (pulmonary) organogenesis?
4. What are the direct effects of Nitrofen on pulmonary development, and how is this related to the diaphragmatic defect?
5. Are members of the steroid hormone receptor superfamily differently expressed in Nitrofen-induced hypoplastic lungs?

The first three questions are discussed in the studies described in Part II of this thesis, and the last two questions in the studies described in Part III.

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Part II

NORMAL MECHANISMS OF PULMONARY DEVELOPMENT

Chapter 3

The transcription factor GATA6 is essential for branching morphogenesis and epithelial cell differentiation during fetal pulmonary development

Based on:
Keijzer, R., Van Tuyl, M., Meijers, C., Post, M., Tibboel, D., Grosveld, F., and Koutsourakis, M. (2001) The transcription factor GATA6 is essential for branching morphogenesis and epithelial cell differentiation during fetal pulmonary development. *Development*, 128: p. 503-11.

3.1 Abstract

Recent loss-of-function studies in mice show that the transcription factor GATA6 is important for visceral endoderm differentiation. It is also expressed in early bronchial epithelium and the observation that this tissue does not receive any contribution from *GATA6* double mutant embryonic stem (ES) cells in chimeric mice suggests that GATA6 may play a crucial role in lung development. The aim of this study was to determine the role of GATA6 in fetal pulmonary development. We show that *GATA6* mRNA is expressed predominantly in the developing pulmonary endoderm and epithelium, but at E15.5 also in the pulmonary mesenchyme. Blocking or depleting GATA6 function results in diminished branching morphogenesis both in vitro and in vivo. TTF1 expression is unaltered in chimeric lungs whereas SPC and CC10 expression are attenuated in abnormally branched areas of chimeric lungs. Chimeras generated in a ROSA26 background show that endodermal cells in these abnormally branched areas are derived from *GATA6* mutant ES cells, implicating that the defect is intrinsic to the endoderm. Taken together, these data demonstrate that GATA6 is not essential for endoderm specification, but is required for normal branching morphogenesis and late epithelial cell differentiation.

3.2 Introduction

The formation of a lung requires a well organized program to coordinate the balanced interplay between activation and repression of gene transcription as it develops from a cluster of cells in the floor of the primitive foregut to the highly vascularized gas exchange organ (Hogan, 1999; Warburton et al., 2000). Several evolutionarily conserved signaling pathways have been implicated in the different stages of fetal pulmonary development. In particular members of the fibroblast growth factor, bone morphogenetic protein, hedgehog/Gli, wingless and epidermal growth factor families have been demonstrated to be key regulatory factors for lung morphogenesis and epithelial differentiation, but lung endoderm specification is less well understood (for review see Hogan, 1999; Warburton et al., 2000). Recently, a member of the GATA family of transcription factors, GATA6, has been implicated in endoderm specification (Morrisey et al., 1998). In vertebrates, the GATA family of transcription factors contains a conserved zinc-finger motif that binds to the consensus sequence A/TGATA/G resulting in

transcriptional regulation of genes in different cell lineages (for review see Charron and Nemer, 1999; Evans, 1997; Jordan and Van Zant, 1998; Orkin, 2000). The GATA transcription factors can be subdivided into two main groups. First, GATA1, GATA2 and GATA3, which all have unique functions in the hematopoietic system (Leonard et al., 1993; Pandolfi et al., 1995; Pevny et al., 1991; Simon et al., 1992; Ting et al., 1996; Tsai et al., 1994; Weiss et al., 1994; Whyatt et al., 2000) and, in case of GATA2 and GATA3, in many other tissues, including the nervous system and the kidneys. Second, GATA4, GATA5 and GATA6, which are mainly expressed and involved in the formation of the extra-embryonic and embryonic endoderm, as well as in the cardiogenic mesoderm (Arceci et al., 1993; Koutsourakis et al., 1999; Laverriere et al., 1994; Morrisey et al., 1996; Morrisey et al., 1997a; Morrisey et al., 1997b; Morrisey et al., 1998).

Radioactive in situ hybridization experiments have demonstrated that GATA6 mRNA is expressed in the developing embryonic bronchial epithelium and in chimeric experiments this endodermally derived tissue did not receive any contribution from *GATA6*-/- ES cells (Morrisey et al., 1996; Morrisey et al., 1998). The conclusion of these studies was that GATA6 is required for specification of lung endoderm. In addition, the promoters of several lung specific genes, such as the genes for human thyroid transcription factor 1, human surfactant protein C, mouse surfactant protein A and mouse Clara cell marker 10, contain GATA motifs (Ikeda et al., 1995; Korfhagen et al., 1992; Ray et al., 1993; Wert et al., 1993) and GATA6 has also been shown to activate both surfactant protein A and thyroid transcription factor 1 reporter gene transcription in vitro (Bruno et al., 2000; Shaw-White et al., 1999). Taken together, these data strongly suggest that GATA6 plays an important role during lung development.

We therefore searched to identify the specific role(s) of GATA6 in fetal pulmonary development. In order to obtain a more detailed insight into the spatial-temporal distribution of GATA6 mRNA, non-radioactive in situ hybridization during fetal lung development in vivo was performed. The observed expression patterns in early embryonic lungs suggest a role for GATA6 in branching morphogenesis, that we investigated using organotypic explant cultures with antisense oligonucleotides. To confirm and further investigate the role of GATA6 in lung development in vivo, we also generated highly chimeric embryos by injecting wild-type blastocysts with *GATA6*-/- embryonic stem (ES) cells. The latter experiments enabled us to overcome the early embryonic lethality in *GATA6*-/- mice, which is due to extra-embryonic defects (Koutsourakis et al., 1999; Morrisey et al., 1998), because in such chimeras, the extra-embryonic tissues are provided

by the wild-type host blastocyst. Highly chimeric lungs were histologically analyzed, and the expression of molecular markers for endoderm specification and epithelial cell differentiation (thyroid transcription factor 1, surfactant protein C and Clara cell marker 10) was investigated using immunohistochemistry and in situ hybridization.

Branching morphogenesis was dramatically affected in vitro when antisense oligonucleotides for *GATA6* were added to lung cultures. In chimeric embryos, pulmonary endoderm was formed but it branched abnormally and failed to undergo late epithelial cell differentiation, based on diminished surfactant protein C (SP C; Sftpc-Mouse Genome Informatics) and Clara cell marker 10 (CC 10; Utg-Mouse Genome Informatics) expression. Using ROSA26 recipient blastocysts, we demonstrate that this abnormal epithelium is derived from *GATA6-/-* ES cells. We conclude that GATA6 is essential for the later stages of branching morphogenesis and late epithelial cell differentiation, but not for lung endoderm specification.

3.3 Materials and methods

Whole-mount in situ hybridization

Wild-type embryonic day (E) 10.5 and E12.5 embryos were isolated from pregnant FVB female mice according to standard methods (Hogan et al., 1994) and the lungs were dissected from these embryos using microsurgical techniques. The lungs were fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 4 to 6 hours, dehydrated in a graded series of ethanol and kept in 100% methanol at -20°C until used. The whole-mount in situ hybridization protocol was adapted from the protocol described by Wilkinson (Wilkinson, 1993). In short, following hydration, the lungs were bleached for 1 hour in 6% (v/v) hydrogen peroxide in PBS containing 0.1% (v/v) Tween-20 (PBT) and permeabilized with proteinase K (10 $\mu g.ml^{-1}$ in PBT) for 15 minutes at room temperature. Subsequently, the lungs were post-fixed in 4% (w/v) paraformaldehyde in PBS and prehybridized in hybridization mixture, containing 50% formamide, 5x SSC (NaCl 0.75 mol/L, sodium citrate 0.075 mol/L, pH 4.5), 1% sodium dodecyl sulphate, 5 $\mu g.ml^{-1}$ yeast tRNA, 50 $\mu g.ml^{-1}$ heparin in water for 1 hour at 70°C. Hybridization was carried out for 16 to 18 hours at 70°C using the same hybridization mixture, but now containing a digoxigenin (DIG)-labeled sense or antisense GATA6 RNA probe. A 1.5 kb fragment of the 5' end of the mouse *GATA6* gene that was described previously (Brewer et al., 1999) was used to generate a probe for in situ hybridization on sections. A PstI-NotI

subclone from the 5' part of this 1.5 kb fragment was used to generate a probe for whole-mount in situ hybridization. The cDNA fragments were DIG labeled according to a protocol provided by the manufacturer (Roche Diagnostics, Almere, The Netherlands). The next day, the lungs were stringently washed and treated with RNAse to avoid aspecific background staining. Following a blocking step using 10% (v/v) sheep serum in 0.14 mol/L NaCl, 2.7 mmol/L KCl, 25 mmol/L Tris/HCl pH7.5 and 0.1% (v/v) Tween-20 in water (TBST) including 2 mmol/L levamisole, the lungs were incubated with an alkaline phosphatase-coupled antibody, 1:2000, against DIG for 16 to 18 hours at 4°C. For at least 3 days the lungs were then washed in TBST and 2 mmol/L levamisole. The lungs were changed to 0.1 mol/L NaCl, 0.1 mol/L Tris/HCl, pH 9.5, 0.05 mol/L $MgCl_2$ and 0.1% (v/v) Tween-20 (NTMT) and the hybridized probe was visualized using 337.5 $\mu g.ml^{-1}$ NBT and 175 $\mu g.ml^{-1}$ BCIP mixture as a substrate. Nonspecific labeling was removed in 95% ethanol, and the lungs were kept at 4°C in PBT containing 1 mmol/L EDTA.

Branching morphogenesis in vitro with antisense oligonucleotides

E11.5 and E12.5 lungs were harvested from wild-type embryos under a dissection microscope. The lungs were transferred to porous membranes (8 μm pore size) from Nucleopore (Whatman, NY), and incubated in 12-well culture plates from Costar (Corning, NY). The membranes were pre-soaked in MEM (Gibco) for 1 hour before the explants were placed on them. The explants were incubated as floating cultures in 500 μl Dulbecco's modified Eagle's medium, nutrient mixture F-12 (Gibco) supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin and 0.25 mg/ml ascorbic acid. The explants were cultured at 37°C in 95% air and 5% CO_2. Ten cultured lungs were treated with 40 μM phosphorothioated oligonucleotides, targeted against the translation initiation site of the murine GATA6 mRNA in the antisense direction with the following sequence: GTCAGTCAAGGCCAT. Ten cultured lungs were treated with the same concentration sense-orientated oligonucleotides with the following sequence: ATGGCCTTGACTGAC. Ten untreated cultured lungs served as controls. The lungs were cultured for up to 72 hours and branching morphogenesis was monitored daily and images captured using a dissecting microscope (Leica MZ12) and the Leica Digital Imaging Systems.

Generation of chimeric embryos

In contrast to standard procedures, we injected more than 20 *GATA6*-/-, or *GATA*+/- for the control experiment, ES cells into each blastocyst in order to obtain highly chimeric embryos. These injections were performed either in C57BL/6 or ROSA26 (Friedrich and Soriano, 1991) blastocysts and the ES cell lines used have been previously described (Koutsourakis et al., 1999). Chimeric lungs were dissected at E12.5, E13.5, E15.5 or E18.5 and either processed for explant cultures (E13.5), as described above, or fixed and used for histological analysis and in situ hybridization, as described above (E12.5, E15.5 and E18.5). Head or tail tissue of the embryos was used to determine the percentage of chimerism using Glucose Phosphate Isomerase (GPI) electrophoresis (Hogan et al., 1994).

In situ hybridization and immunohistochemistry

Non-radioactive in situ hybridization on 6 μm thick sections with SP C and CC 10 RNA probes was carried out essentially as described before by Motoyama et al. (Motoyama et al., 1998). In short, tissue sections were rehydrated and washed in PBS. Pretreatment included postfixation in 4% paraformaldehyde for 15 minutes, followed by proteinase K digestion (20 mg/ml) for 15 minutes at room temperature and acetylation (0.1 mol/L triethanolamine and 0.25% acetic anhydride) for 10 minutes at room temperature. Sections were then dehydrated and air-dried before addition of the hybridization solution. Digoxigenin-labeled probes were added to freshly prepared hybridization solution (50% deionized formamide, 10% dextran sulfate, 1.5× Denhardt's reagent, 0.5 mg/ml of yeast tRNA, 0.3 mol/L NaCl, 5 mmol/L EDTA and 25 mmol/L Tris, pH 7.5) at a concentration of 1 ng/μl. Following denaturation at 80°C, the probe was added to the tissue section and incubated for 16 to 18 hours at 55°C. After brief washes with 5× SSC and 50% formamide at 55°C, the tissue was treated with RNase A (10 μg/ml) for 30 minutes at 37°C. The digoxigenin nucleic acid detection kit (Roche Diagnostics, Almere, The Netherlands) was used for immunological detection of the hybridized probe. Tissue was then counterstained with Methyl Green and prepared for viewing.

Mouse specific SP C and CC 10 cDNA fragments (330 and 315 bases, respectively) were DIG labeled according to a protocol provided by the manufacturer (Roche Diagnostics, Almere, The Netherlands). Immunohistochemistry with a monoclonal antibody against TTF 1 (Neomarkers, CA, USA) in a concentration of 1 in 100 was carried out as described before (Keijzer et al., 2000).

3.4 Results

Spatial-temporal expression of GATA6 mRNA during fetal lung development

Starting from E10.5, when five primary lung buds have just evaginated from the foregut, GATA6 mRNA was detected in developing lung endoderm (Fig. 1A). At this stage the GATA6 mRNA was predominantly expressed at the tips of the growing lung buds. Two days later at E12.5, when branching morphogenesis of the lung buds is proceeding rapidly in order to establish the future bronchial tree, GATA6 mRNA was still observed in the branching endoderm. However, at this stage the signal was observed in the entire endoderm lining the developing lung buds, instead of being limited only to the tips of the growing buds (Fig. 1B). On sections of wild-type E15.5 lungs, in which branching morphogenesis is almost completed, and differentiation and vascularization are beginning, GATA6 mRNA was expressed in the endoderm lining the growing airways, as well as in parts of the mesenchyme surrounding these airways, albeit at a lower level (Fig. 1C). At E18.5, when the bronchial tree is complete, and the lung tissues are differentiating into the different cell types that will constitute the lung after birth, GATA6 mRNA was observed mainly in epithelial cells lining the bronchioli, but also to a lesser extent in the epithelial cells lining the sacculi (Fig. 1D). At this stage, the signal in the mesenchyme was difficult to distinguish, possibly because of the thinning of this tissue layer. Hybridization with sense RNA GATA6 probes generated no specific signal in either whole-mount or section in situ hybridization experiments at all stages (results not shown).

Antisense oligonucleotides against GATA6 reduce branching morphogenesis in vitro

Based on the mRNA expression pattern of GATA6, in particular expression in the endoderm lining the early lung buds, an important role for GATA6 in branching morphogenesis was anticipated. Antisense oligonucleotides targeted against the translation initiation site of *GATA6* were used in explant cultures of isolated fetal lungs to investigate this role. When E11.5 lungs were cultured for 72 hours in the presence of antisense GATA6 oligonucleotides (Fig. 2B), branching morphogenesis of these lungs was clearly reduced when compared with the patterns of branching morphogenesis of lungs exposed to sense GATA6 oligonucleotides (Fig. 2A) or to lungs cultured in medium alone without oligonucleotides (not shown). A similar reduction in branching morphogenesis was observed when E12.5 lungs were cultured for 72 hours with antisense (Fig. 2D) oligonucleotides. Again, lungs exposed to sense oligonucleotides (Fig. 2C) and

control lungs (not shown), which were cultured without oligonucleotides, demonstrated similar patterns of normal branching morphogenesis.

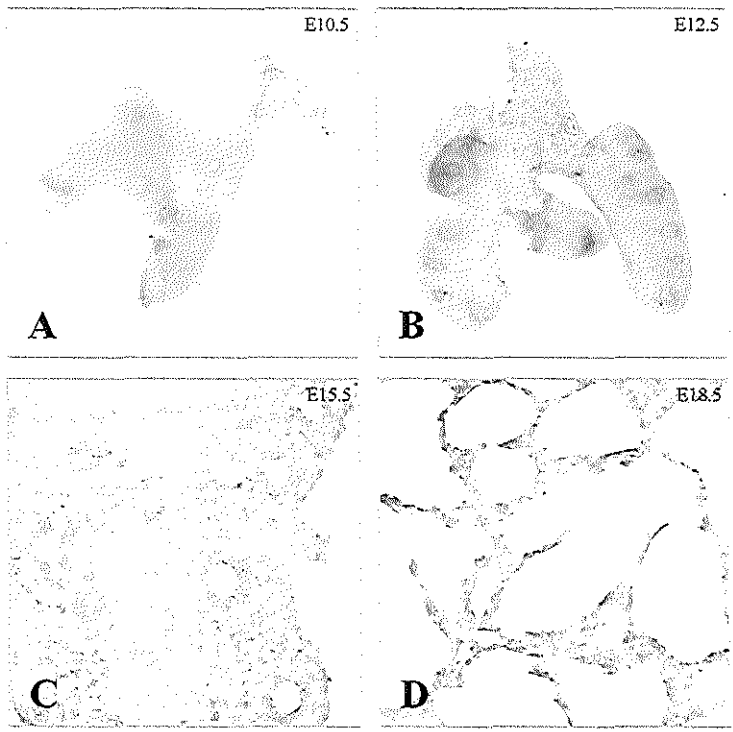

Figure 1
Spatial-temporal distribution of GATA6 mRNA during embryonic and fetal pulmonary development. At E10.5 GATA6 mRNA is predominantly expressed at the tips of the primary lung buds (A). Two days later at E12.5 GATA6 mRNA expression has expanded and is now expressed in the entire endoderm lining the actively branching lung buds (B). When branching morphogenesis is almost completed at E15.5, GATA6 mRNA is observed in the endoderm lining the growing airways, and in particular in the distal endoderm (C). In the mesenchyme GATA6 mRNA is also observed, but at a lower level. Finally during maturation and differentiation at E18.5, GATA6 mRNA is expressed in epithelial cells lining the sacculi (D).

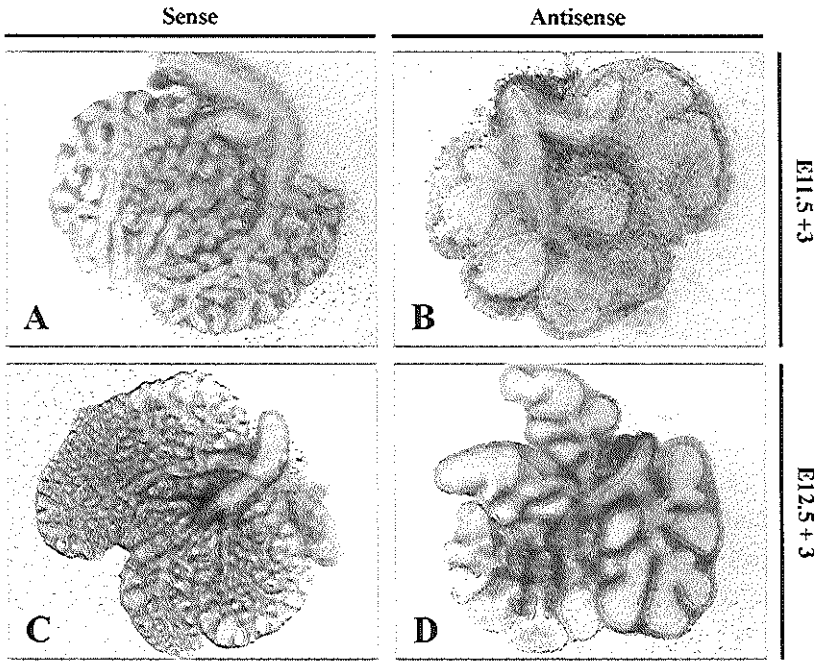

Figure 2
GATA6 antisense oligonucleotides inhibit branching morphogenesis in vitro. Representative pictures of experiments with E11.5 (A,B) or E12.5 (C,D) wild-type lungs, cultured as organotypic explants in the presence of sense (A,C) or antisense (B,D) oligonucleotides targeted against the translation initiation site of *GATA6*. All pictures were taken at the same magnification.

GATA6-/- chimeric lungs display diminished branching morphogenesis in vitro

In all experiments highly chimeric (more than 50%) embryos and lungs were selected based on GPI analysis (results not shown). Isolated E12.5 *GATA6-/-* chimeric lungs were smaller in size and had less lung buds (Fig. 3B) when compared with non-chimeric littermates (Fig. 3A). From a different litter, E13.5 *GATA6-/-* chimeric and non-chimeric lungs were cultured for 4 days as organotypic explants. At the time of isolation, both lungs were comparable in size, but the *GATA6-/-* chimeric lungs had fewer branches (Fig. 3D) than the non-chimeric lungs (Fig. 3C). After 4 days of culture, the *GATA6-/-* chimeric

lungs demonstrated diminished branching morphogenesis in certain areas of the lung (Fig. 3F, arrow), whereas in other areas, branching morphogenesis patterns were the same as wild-type (Fig. 3E).

GATA6-/- chimeric lungs display diminished branching morphogenesis in vivo resulting in respiratory insufficiency

When *GATA6*-/- chimeric lungs were isolated at E15.5 they appeared similar to lungs isolated from non-chimeric littermates. The size was comparable, the lungs had four lobes on the right, one lobe on the left side and no defect in branching morphogenesis was observed macroscopically (Fig. 4A,B). However, when these lungs were analyzed microscopically, a clear defect in branching morphogenesis indicated by the large airspaces was observed in the *GATA6*-/- chimeric lungs (Fig. 4D, arrow), when compared with non-chimeric lungs (Fig. 4C). When chimeras were generated with the parental heterozygous ES cells, which were used to generate the double mutant lines (Koutsourakis et al., 1999), no abnormalities were observed in highly chimeric E15.5 lungs (results not shown). Chimeric E18.5 lungs, generated with *GATA6*-/- ES cells (Fig. 4F) were again the same size as the non-chimeric lungs (Fig. 4E) from their littermates. In addition, chimeric lungs had four lobes on the right and one lobe on the left. However, at this stage a clear defect in branching morphogenesis was observed macroscopically in some *GATA6*-/- chimeric lungs (Fig. 4F, arrows). This defect in branching morphogenesis was confirmed at the microscopic level (Fig. 4H).

Eight pups were delivered by Caesarian section at E18.5 following blastocyst injection. Without knowing their chimerism, the pups were stimulated to breathe. Out of the eight pups, two were mummified and three were born dead. Out of the three pups that were born alive, two appeared purplish-blue, and one was pink. All three breathed normally. GPI electrophoresis demonstrated that only the pink pup was non-chimeric, and all the other pups were highly chimeric (results not shown).

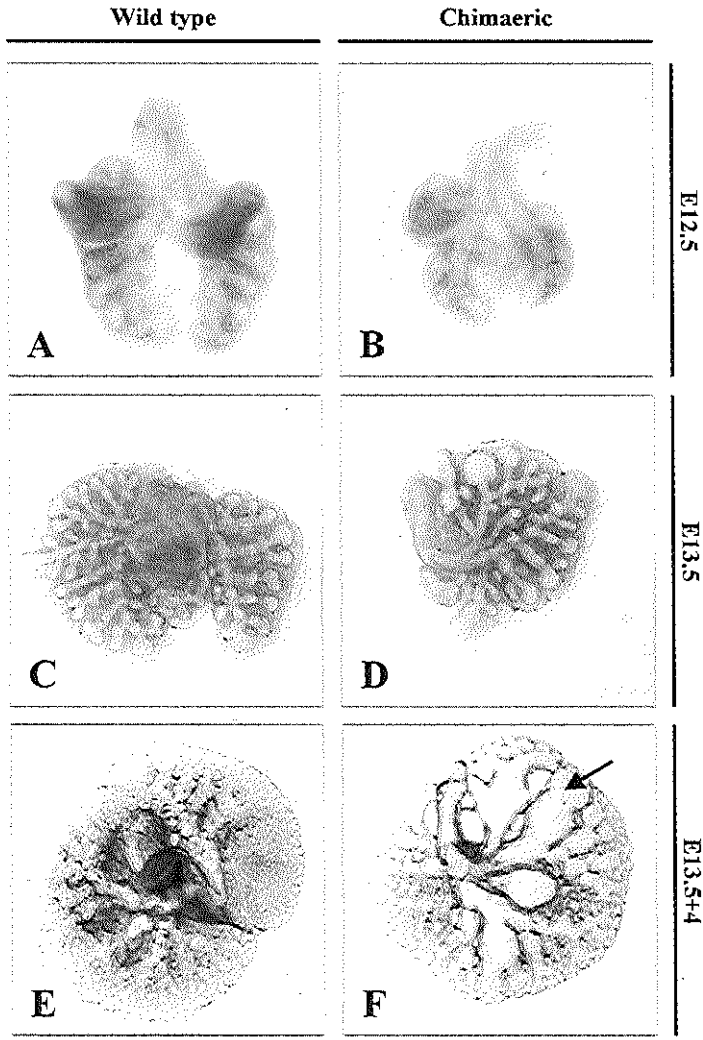

Figure 3

GATA6-/- chimeric lungs display diminished branching morphogenesis in vitro. When isolated at E12.5, *GATA6-/-* chimeric lungs (B) are smaller and have less branches than wild-type lungs isolated from a littermate (A). E13.5 wild-type (C) and *GATA6-/-* chimeric (D) lungs at day 0 of culture as organotypic explants. After 4 days of culture (E,F), the *GATA6-/-* chimeric lungs had areas of normal and areas of diminished branching morphogenesis (F), whereas in the wild-type lung branching morphogenesis had occurred normal (E). All pictures are representative of a series of experiments, and are at the same magnification. (Arrow indicates area with diminished branching morphogenesis)

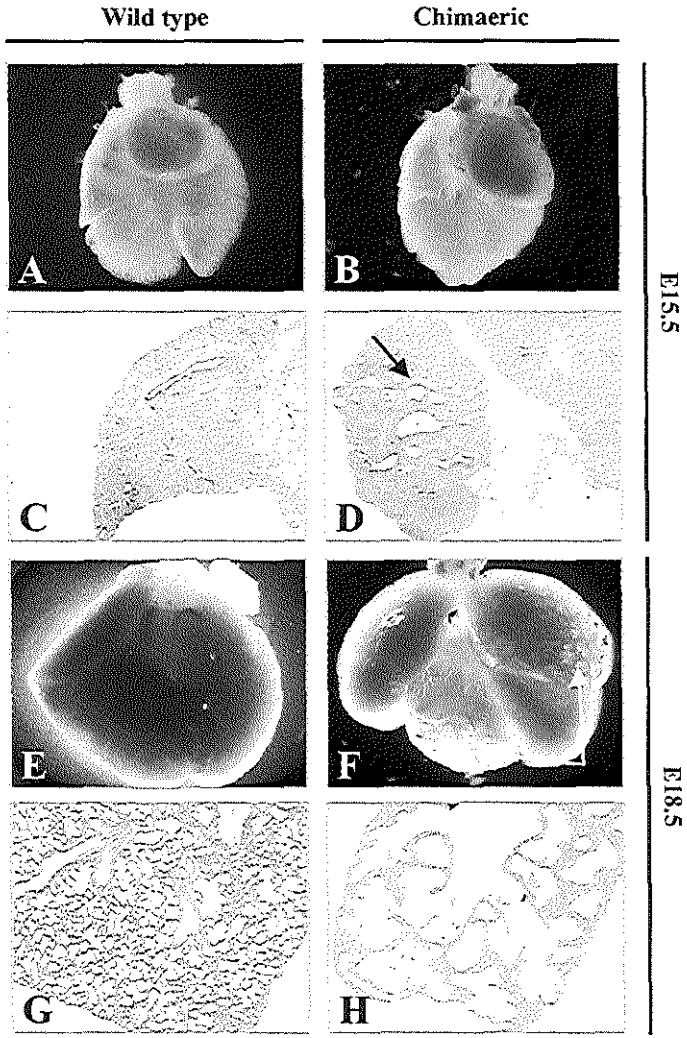

Figure 4 Branching morphogenesis in vivo is diminished in *GATA6-/-* chimeric lungs when compared to wild-type lungs. At E15.5 (A-D), lungs appeared macroscopically indistinguishable when isolated from wild-type (A) or *GATA6-/-* chimeric embryos (B). However, when microscopic sections were analyzed, *GATA6-/-* chimeric lungs had bigger airspaces (D) while lungs of wild-type embryos did not (C). At E18.5, *GATA6-/-* chimeric lungs (F,G) displayed diminished branching morphogenesis both macroscopically (F) and microscopically (H) when compared to lungs of wild-type littermates (E,G). All pictures are representative for a series of experiments and are at the same magnification. (Arrow indicates area with diminished branching morphogenesis)

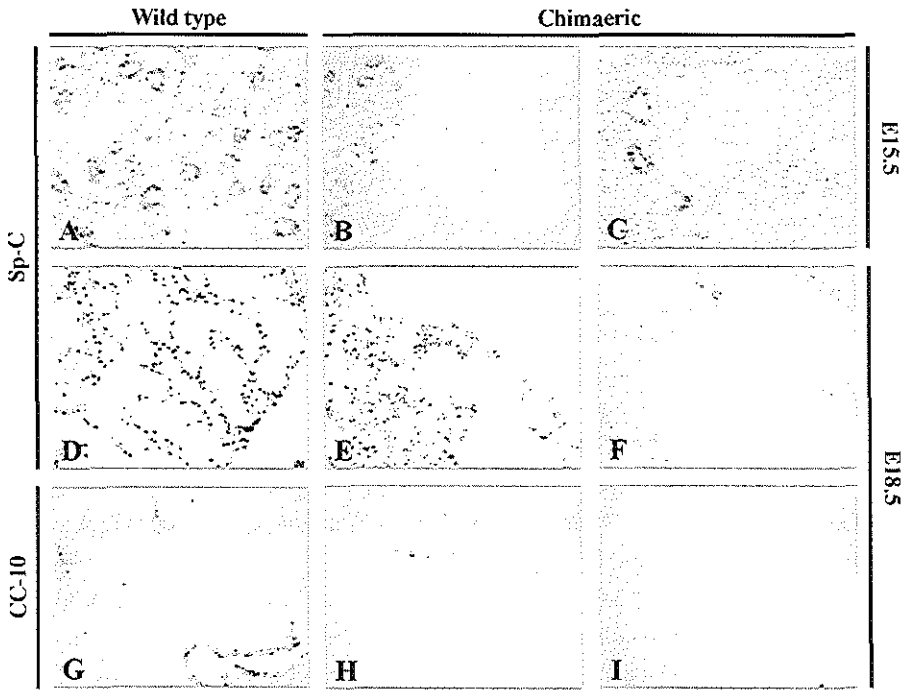

Figure 5
mRNA distribution of molecular markers for distal (surfactant protein-C) (A-F) and proximal (Clara cell marker-10) (G-I) epithelial cell differentiation. In both E15.5 (A-C) and E18.5 (D-F) surfactant protein-C mRNA was expressed in normally branched areas of wild-type (A,D) and *GATA6-/-* chimeric (B,E) lungs. No surfactant protein-C mRNA expression was observed in abnormally branched areas of *GATA6-/-* chimeric lungs (B,C,F). At E18.5 Clara cell marker-10 mRNA was expressed in normally branched areas of both wild-type (G) and *GATA6-/-* chimeric lungs (H), whereas in abnormally branched areas of *GATA6-/-* chimeric lungs no Clara cell marker-10 mRNA expression could be observed. All pictures are at the same magnification, except (B), which is a low power overview of (C). (SP C indicates surfactant protein-C; CC 10 indicates Clara cell marker-10)

Expression of SP C, CC 10 and TTF 1 in *GATA6-/-* chimeric lungs

In order to investigate if cell differentiation was altered in *GATA6-/-* chimeric lungs, non-radioactive in situ hybridization with probes for SP C, a marker for type II cells that indicates distal epithelial cell differentiation, and CC 10, a marker for Clara cells that indicates proximal epithelial cell differentiation, was performed. The promoters of the genes for both these proteins have previously been demonstrated to contain GATA motifs,

indicating that the transcription of these genes may be regulated by GATA transcription factors (Ray et al., 1993; Wert et al., 1993). Another epithelial cell marker which has been shown to be regulated by GATA6 in vitro is TTF 1. Localization of TTF 1 protein was investigated in the *GATA6-/-* chimeric lungs.

At E15.5 (Figs. 5A-C) and E18.5 (Figs. 5D-F), normal SP C expression was observed in *GATA6-/-* chimeric lungs in the areas that appeared to have branched normally (Fig. 5B,C,E). In contrast, no SP C expression was observed in areas with abnormal big sacculi (Fig. 5B,C,F). Normal SP C expression was observed in the wild-type lungs in distal epithelium at E15.5 (Fig. 5A) and in type II cells at E18.5 (Fig. 5D).

At E15.5, no CC 10 expression was observed in either wild-type or *GATA6-/-* chimeric lungs, which was expected, because the gene for CC 10 is not expressed at that stage (results not shown). At E18.5, CC 10 was expressed in the proximal airways of wild-type lungs, as well as in the normally branched parts of the *GATA6-/-* chimeric lungs (Fig. 5G,H). In the abnormally branched parts of the *GATA6-/-* chimeric lungs, no CC 10 expression was observed (Fig. 5I).

At E15.5 and E18.5 TTF 1 protein was localized normally in epithelial cells of both wild-type and *GATA6-/-* chimeric lungs (Figs. 6A-F). No differences were observed between areas with normal or abnormal branching morphogenesis in the *GATA6-/-* chimeric lungs.

Contribution of *GATA6-/-* ES cells in chimeric lungs

In order to identify the origin of the endoderm of the abnormally branched areas, *GATA6-/-* ES cells (white) were injected into ROSA26 blastocysts (blue). In the targeted ES cell clones we used for generation of the chimeras, *GATA6* was inactivated by insertion of a *LacZ* marker gene (Koutsourakis et al., 1999). However, when we investigated β-galactosidase activity in early embryonic lungs, we could not detect activity in developing airways lined with pulmonary endoderm (results not shown). In contrast, β-galactosidase activity was detected in developing major vessels later during lung development (results not shown). Therefore, we did not expect any interference between β-galactosidase activity in ROSA26- and ES cell-derived endoderm.

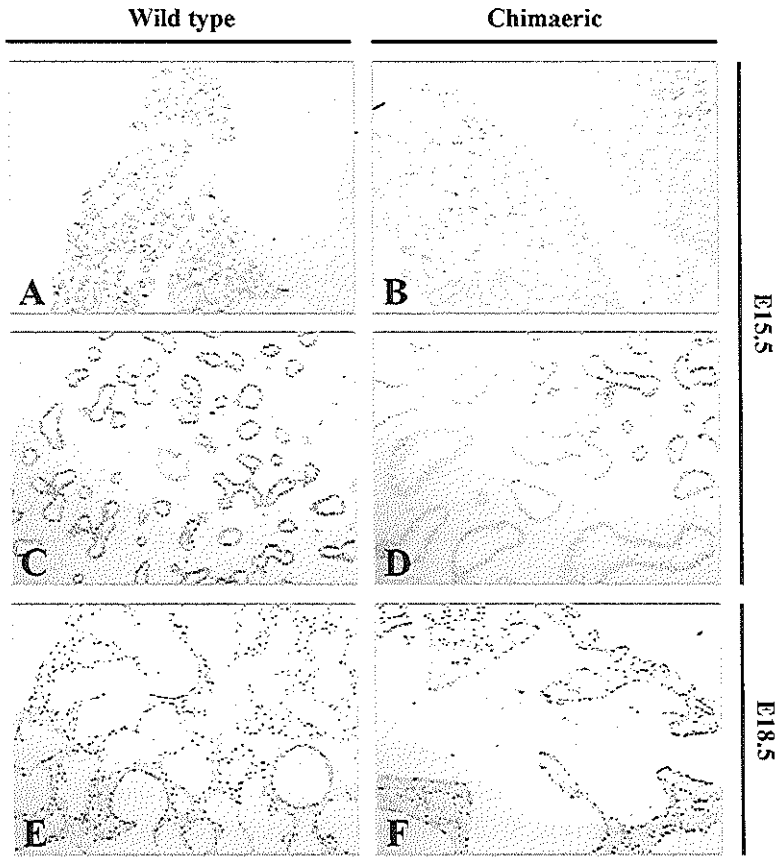

Figure 6
Thyroid transcription factor-1 (TTF 1) protein distribution as a marker for pulmonary endoderm specification. In wild-type (A,C,E) and *GATA6*-/- chimeric (B,D,F) lungs similar patterns of TTF 1 protein distribution were observed at E15.5 (A-D) and E18.5 (E,F). (A,B) are a low power overview of E15.5 and (C-F) are at same magnification.

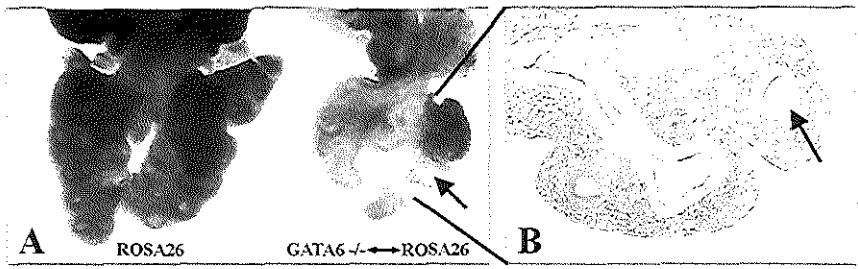

Figure 7
Distribution of *GATA6*-/- ES cell contribution into ROSA26 host lungs. (A) *GATA6*-/- ES cells (white) contribute to both mesenchyme and endoderm of E12.5 chimeric lungs (right), which are smaller and have less branches than lungs from wild-type littermates (left). In a section (B) of the chimeric lung, blue (wild-type ROSA26), mixed white and blue, and white (*GATA6*-/-) endoderm are observed, clearly indicating that *GATA6*-/- ES cells do contribute to pulmonary endoderm (please note, as opposed to the usual experiment, that the white areas are abnormal and the blue areas normal). Arrows indicate white endoderm derived from *GATA6*-/- ES cells.

Using this approach, a similar phenotype of E12.5 lungs was observed upon isolation as in the previous experiments. The *GATA6*-/-↔ROSA26 chimeric lungs were smaller and had developed fewer lung buds (Fig. 7A). More interestingly, after detection of β-galactosidase activity, completely white endodermal buds as well as mixtures of white and blue endodermal cells and completely blue endodermal buds were observed in highly chimeric lungs (Fig. 7B). In E15.5 *GATA6*-/-↔ROSA26 chimeric lungs, the same observations were made (Fig 8A,B). Areas with reduced branching morphogenesis had almost completely white endoderm derived from *GATA6*-/- ES cells (Fig. 8A,B, arrows), whereas areas with normal branching morphogenesis had completely blue endoderm, derived from the wild-type ROSA26 host blastocyst cells (Fig. 8A,B).

3.5 Discussion

Based on experiments using chimeric embryos and extrapolation of results obtained in other organs and model systems, GATA6 was presumed to be essential for the specification of foregut endoderm into pulmonary endoderm (Kalb et al., 1998; Morrisey et al., 1998). Using experiments with antisense oligonucleotides in organotypic explant cultures, and (in particular) using chimeric experiments with *GATA6*-/- ES cells, we provide evidence for a different and more extensive role for GATA6 in fetal pulmonary

development than the previously proposed role of endoderm specification. In lung explants with already specified lung endoderm (wild-type) we could abrogate branching morphogenesis in vitro by culturing the explants in the presence of antisense oligonucleotides against *GATA6*. In agreement with this, highly chimeric lungs displayed diminished branching morphogenesis both in vitro and in vivo at different gestational ages. Proximal and distal epithelial cell differentiation was attenuated in chimeric lungs, as indicated by diminished expression of markers for distal and proximal epithelial cell differentiation, SP C and CC 10 mRNA, respectively. TTF 1, an early pulmonary epithelial cell marker that has also been shown to be regulated by GATA6 in vitro, was expressed as normal in chimeric lungs both at E15.5 and E18.5, indicating that endoderm specification occurred normally. In accordance with this, we found that *GATA6-/-* ES cells contribute to pulmonary endoderm in highly chimeric lungs, and, consequently, the observed phenotype of reduced branching morphogenesis and attenuated epithelial cell differentiation is the result of loss of function of GATA6 in pulmonary endoderm.

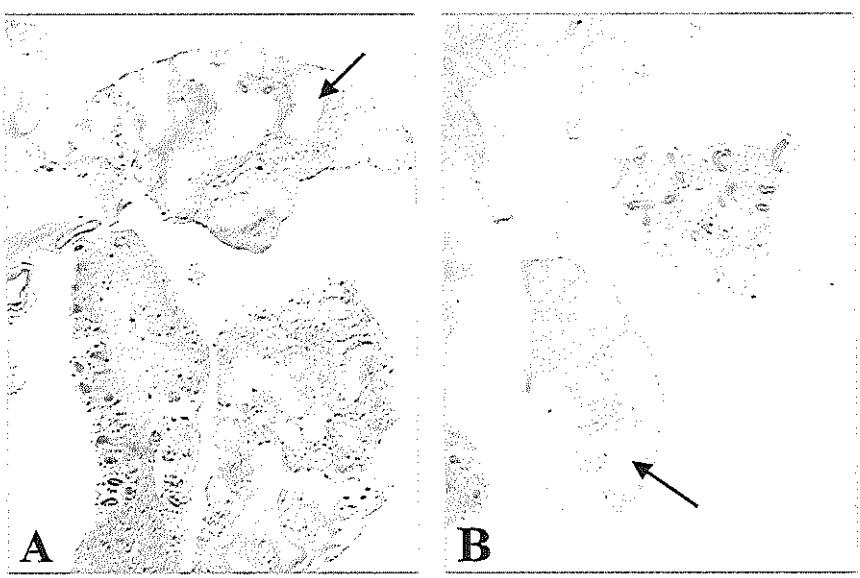

Figure 8
Distribution of *GATA6-/-* ES cell contribution in E15.5 lungs. Both sections of different *GATA6-/-* chimeric lungs contain areas with abnormal branching morphogenesis which have white endoderm (*GATA6-/-*) and areas with normal branching morphogenesis which have blue endoderm coming from the wild-type ROSA 26 blastocyst. (Arrow indicates white endoderm derived from *GATA6-/-* ES cells)

GATA6 is essential for branching morphogenesis

Using both in vitro and in vivo experiments, we demonstrate here that GATA6 plays an essential role during certain stages of branching morphogenesis in the lung. Specification of foregut endoderm into pulmonary endoderm occurred normally in the chimeric lungs, because we obtained highly chimeric embryos with lungs that had undergone morphogenesis. Outgrowth of two endodermally derived primary lung buds initiates the process of lung formation. Thus, highly chimeric lungs successfully completed the earliest phase of lung development, i.e. the specification of pulmonary endoderm, The next event in lung development, formation of four primary lung buds on the right and one primary lung bud on the left in mice also occurred normally in highly chimeric lungs, since all isolated lungs at E12.5, E13.5, E15.5 and E18.5 had four lobes on the right and one lobe on the left side. This indicates that primary branching morphogenesis does not require GATA6. Consequently, the observed phenotype of reduced branching morphogenesis in lungs of highly chimeric embryos is the result of an essential role for GATA6 in secondary branching morphogenesis, the next stage of early pulmonary development. Either by blocking wild-type GATA6 translation with antisense oligonucleotides, or by depleting *GATA6* function in chimeric lung endoderm, we demonstrate that absence of GATA6 gives rise to reduced branching morphogenesis. This resulted in lungs with very big airspaces. However, these lungs appeared no smaller than wild-type lungs, and when a marker for proliferation (Ki-67) was investigated in chimeric and wild-type lungs no differences were observed (results not shown). Thus the observed defect in branching morphogenesis is not the result of growth or proliferation inhibition by GATA6. For future studies it will be very interesting to investigate expression patterns of other molecules that have been demonstrated to play an important role during branching morphogenesis. Endodermally expressed markers like sonic hedgehog (SHH) (Litingtung et al., 1998), bone morphogenetic protein 4 (BMP 4) (Bellusci et al., 1996) and hepatocyte nuclear factor/forkhead homologue 4 (HFH 4) (Chen et al., 1998), as well as mesenchymally expressed markers like fibroblast growth factor 10 (FGF 10) (Sekine et al., 1999) and GATA5 (Morrisey et al., 1997a) are likely candidates.

GATA6 is essential for late epithelial cell differentiation, but not endoderm specification

In highly chimeric lungs, we could not detect expression of SP C and CC 10 mRNA in abnormally branched areas, indicating that both proximal and distal epithelial cell

differentiation is disturbed in these parts of the lungs. In areas that had undergone normal branching morphogenesis SP C and CC 10 were normally expressed. These data provide evidence for a functional role of the GATA motifs in the promoters of the gene for human SP C and murine CC 10 in vivo (Ray et al., 1993; Wert et al., 1993). Another gene that is thought to be regulated by GATA6 on the basis of in vitro studies is TTF 1 (Shaw-White et al., 1999). In addition, TTF 1 alone has been demonstrated to regulate transcription of SP C and CC 10 (Kelly et al., 1996; Zhang et al., 1997). Nevertheless, we could not detect any differences in protein expression of TTF 1 between chimeric and wild-type lungs using immunohistochemistry with an antibody against TTF 1. This indicates that downregulation of the expression of the late epithelial markers SP C and CC 10 does not function through down-regulation of TTF 1, which suggests that if GATA6 and TTF 1 regulate SP C and CC 10 gene transcription, they do it through different pathways. The fact that we observed normal expression of TTF 1 protein, a marker for lung endoderm specification, indicates that specification of foregut endoderm into pulmonary endoderm occurred normally in the chimeric lungs. Hence, we conclude that GATA6 is not involved in pulmonary endoderm specification, as has been suggested before (Morrisey et al., 1998). This conclusion corroborates with recent data obtained in experiments with *Xenopus* in which both GATA4 and GATA5 were demonstrated to induce early endodermal marker genes, whereas GATA6 was demonstrated not to induce these endodermal markers (Weber et al., 2000).

***GATA6-/-* ES cells do contribute to pulmonary endoderm**

Using ROSA26 blastocysts (blue) as host blastocysts for *GATA6-/-* ES cells (white), we were able to demonstrate contribution of *GATA6-/-* ES cells to pulmonary endoderm. Abnormally branched areas in E15.5 chimeric lungs had consistently white endoderm (*GATA6-/-*) and normally branched areas blue endoderm (wild-type) and therefore we conclude that reduced branching morphogenesis in *GATA6-/-* chimeric lungs is the result of defective GATA6 mutant endoderm. Since we observed different areas with either blue, white or mixed white and blue endodermal cells and not lungs or lobes that were completely white or blue, it is likely that the lungs originate from a substantial number of cells from the foregut. Our observations are in contrast with data presented by another group, who demonstrated that *GATA6-/-* ES cells do not contribute to the bronchial epithelium in chimeric lungs, and concluded that GATA6 is required for establishment of the endodermally derived bronchial epithelium (Morrisey et al., 1998). The contradiction

in observations can be explained by the different approach that we used in order to obtain animals that were highly chimeric. Whereas regular protocols suggest the injection of nine to 12 ES cells into blastocysts to generate chimeric embryos (Hogan et al., 1994), we used double the number of ES cells to obtain highly chimeric embryos. GPI electrophoresis revealed that most chimeric embryos were derived from at least 50% *GATA6-/-* ES cells and only chimeric embryos that were derived from more than 50% *GATA6-/-* ES cells were used in our experiments. If we assume that selection by competition between wild-type and mutant cells occurs during the earliest stages of lung development in favor of wild-type cells then the regular amount of *GATA6-/-* ES cells (as used by Morrisey et al.) may not have progeny in pulmonary endoderm. However, by using more *GATA6-/-* ES cells, mutant cells were able to survive competition and to contribute to pulmonary endoderm. To overcome any interference that resulted from the percentage of wild-type cells present during early pulmonary specification of these chimeric lungs, it will be of interest to generate embryos exclusively derived from *GATA6-/-* ES cells. However, in all our experiments we never found embryos that were close to 100% chimeric (based on GPI electrophoresis), although a number of implantation sites with resorbed embryos were present. Based on the fact that GATA6 is not only expressed in extra-embryonic tissues, but also in embryonic tissues (Koutsourakis et al., 1999; Morrisey et al., 1996; Morrisey et al., 1998) it is likely that embryos exclusively derived from *GATA6-/-* ES cells do not survive to later stages of gestation. Hence, it is unlikely that the use of methods such as the generation of tetraploid embryo chimeras would answer this question. It will probably require the generation of conditional knockout mice.

Role for GATA6 in epithelial-mesenchymal interactions

Since branching morphogenesis is highly dependent on epithelial-mesenchymal interactions (Hogan and Yingling, 1998), it is tempting to speculate which tissue layer causes the defect that reduces branching morphogenesis. Since the spatial-temporal distribution of GATA6 mRNA was predominantly observed in developing lung endoderm, it is most logical to postulate defective endoderm as the site where the primary defect is localized. Defective mutant *GATA6-/-* endoderm appears unable to undergo normal branching morphogenesis and late epithelial cell differentiation resulting in the observed phenotype in the chimeric lungs. This would lead to the conclusion that the observed phenotype is based on a cell-autonomous defect in *GATA6-/-* endodermal cells. However, there is an alternative explanation. Specification of pulmonary endoderm and

primary branching morphogenesis occurs normally in a *GATA6*-/- environment, but at the initiation of subsequent branching morphogenesis, as yet unidentified signals produced by the endoderm are not processed normally by the mesenchyme, which in turn sends abnormal or no signals to the (up till that moment normal) endoderm. From then on, the endoderm would be instructed incorrectly by the mesenchyme, and would not undergo normal branching morphogenesis, resulting in big airspaces and attenuated epithelial cell differentiation. As a consequence, the observed phenotype is based on a cell-nonautonomous defect. Whichever scenario proves right, further investigation is warranted, and studies using transgenic and conditional knockout mice would be invaluable. Another important question not addressed in this study is what the early target genes for GATA6 in pulmonary development are. It is of interest whether known "master" genes of branching morphogenesis such as *BMP 4* (Bellusci et al., 1996), *SHH* (Litingtung et al., 1998) and members of the fibroblast growth factor family (Post et al., 1996; Sekine et al., 1999) are also transcriptionally regulated by GATA6. Future studies using immunoprecipitation, yeast two-hybrid screens and DNA microarray techniques, as well as detailed promoter analysis, should bring more insight.

GATA6 function during pulmonary development: a hierarchical model

Based on the results of our experiments, a reconsideration of the position of GATA6 in the hierarchy of factors involved in pulmonary development is warranted. To date *GATA6* was considered to be one of the master genes in lung development (Whitsett and Tichelaar, 1999). Together with HNF 3β (Ang and Rossant, 1994) and TTF 1 (Kimura et al., 1996), GATA6 was positioned high up in the hierarchy because all three factors were thought to be essential for the specification of foregut endoderm into pulmonary endoderm. However, in this study, we provide evidence that GATA6 is not essential for pulmonary endoderm specification, but plays an important role in branching morphogenesis and late epithelial cell differentiation. Therefore, we postulate that GATA6 functions (in concert with TTF 1) in the process of branching morphogenesis and late epithelial cell differentiation.

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Chapter 4

Branching and differentiation defects in pulmonary epithelium with elevated GATA6 expression

Based on:
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4.1 Abstract

The transcription factor GATA-6 is expressed in the fetal pulmonary epithelium of the developing mouse lung and loss of function studies strongly suggested that it is required for proper branching morphogenesis and epithelial differentiation. We have further investigated the role of GATA-6 in this process by utilizing a pulmonary epithelium specific promoter to maintain high levels of GATA-6 protein during fetal lung development. Transgenic mice expressing a myc-tagged *GATA-6* cDNA under the control of the human *Surfactant Protein-C* (*SP-C*) promoter were generated and their lungs were analyzed during fetal stages. Transgenic lungs exhibit branching defects as early as embryonic day (E) 14.5 and molecular analysis just before birth (E18.5) shows a lack of distal epithelium differentiation whereas proximal epithelium is unaffected. Electron microscopic analysis and glycogen staining confirm the lack of differentiation to mature Type II cells. Thus, elevated levels of GATA-6 protein affect early lung development and in analogy to other GATA factors in other tissues, GATA-6 also plays a crucial role in the terminal differentiation in this case of the distal pulmonary epithelium.

4.2 Introduction

The complexity of the lung tissue with respect to morphogenesis and cell type constitution reflects the complexity in the control of its development. Early specification (around embryonic day 9.5 in the mouse) is far from understood and a molecule like HNF-3β that is thought to be involved, has a more general role in foregut and midgut formation (Ang and Rossant, 1994; Dufort et al., 1998). For the initial lung bud outgrowth mesenchymal-epithelial interactions are crucial since genetic studies have shown that molecules like FGF-10, Gli-2 and Gli-3, all expressed in the lung mesenchyme, are key factors (Min et al., 1998; Motoyama et al., 1998). Mesenchymal-epithelial interactions are also important throughout branching morphogenesis and in the current concept of lung budding, localized high expression of *FGF-10* induces the endoderm to bud (Hogan, 1999; Weaver et al., 2000). This pre-programmed process is reiterated and leads to the establishment of an extensive respiratory tree which, by the end of the pseudoglandular stage of lung development (E16.5), consists of proximal and distal airways. At this stage, epithelial cells undergo extensive differentiation to give rise to a number of distinct cell types (ciliated, nonciliated secretory, goblet, basal, alveolar Type I and Type II cells), which will establish a functional lung. Although some studies describe these different cell types morphologically and in terms of

surfactant protein expression (Ten Have-Opbroek, 1991; Ten Have-Opbroek et al., 1988; Ten Have-Opbroek et al., 1990) their differentiation program remains obscure at the molecular level.

BMP-4 expression in the endoderm is thought to contribute to the acquisition of cell fate with distal epithelium resulting from exposure to high growth factor concentration (Weaver et al., 1999). A number of other molecules including FGFs, glucocorticoids and some transcription factors like TTF-1, HFH-4 and GATA-6 have been implicated in epithelial cell differentiation and surfactant protein production (Perl and Whitsett, 1999; Warburton et al., 2000; Whitsett and Tichelaar, 1999). TTF-1 was shown to positively influence BMP-4 levels and its absence results in a lack of distal structures and the lack of surfactant proteins expression (Minoo et al., 1999). The HNF3/forkhead homologue-4 (HFH-4) has been associated with induction of proximal fate and specifically the appearance of ciliated cells (Tichelaar et al., 1999). GATA-6, a member of the GATA DNA binding family of zinc finger transcription factors, is expressed in the early branching endoderm and it was thought to be essential for bronchial endoderm specification (Morrisey et al., 1998). However, recent studies have shown that the protein is not required for early specification but for normal branching morphogenesis and epithelial differentiation. Lung endoderm depleted of GATA-6 fails to branch normally and to acquire either distal or proximal fate (Keijzer et al., 2001). Furthermore, GATA binding sites have been identified in the promoters of Nkx2.1, Clara cell marker-10 and Surfactant Protein-A and –C (Bruno et al., 2000; Shaw-White et al., 1999; Wert et al., 1993).

GATA proteins have been shown to play key roles in the differentiation of a number of distinct cell lineages and their levels can influence cell choices between differentiation and proliferation (Molkentin, 2000). GATA-1 functions in erythrocytes and alteration of its levels results in a block of differentiation (Pevny et al., 1991; Whyatt et al., 1997). Overexpression of GATA-2 blocks differentiation of early haematopoietic progenitors (Briegel et al., 1993) and overexpression of GATA-3 is involved in the differentiation of T-cells (Hendriks et al., 1999; Zheng and Flavell, 1997). *In vivo* loss of function studies in mice have shown distinct roles for GATA-4, -5 and –6 during development and both GATA-4 and –6 primarily function in the extraembryonic visceral endoderm (Koutsourakis et al., 1999; Kuo et al., 1997; Molkentin et al., 1997; Morrisey et al., 1998). *In vitro* studies have implicated GATA-6 in the differentiation program of vascular smooth muscle cells, VSMCs (Mano et al., 1999), and more recently in glomerular mesangial cells, GMCs (Nagata et al., 2000). In Xenopus embryos, it has been demonstrated that decreasing *GATA-*

6 expression is associated with differentiation of cardiac precursors. When GATA-6 levels were sustained high, the cardiac differentiation program was blocked (Gove et al., 1997).

In this study we utilized a previously characterized promoter/enchancer from the human *Surfactant Protein-C* (*SP-C*) gene (Wert et al., 1993) to keep *GATA-6* expression levels elevated in the pulmonary epithelium. Transgenic embryos were generated and their lungs were analyzed morphologically at various stages. Just before birth molecular as well as Electron Microscopic analysis were performed to evaluate the status of differentiation of the lung epithelium. Our results parallel data from other GATA factors and show that elevated *GATA-6* expression in the lung epithelium, *in vivo*, not only affects its normal branching pattern but also impairs the differentiation program in the distal airways.

4.3 Materials and methods

Construction of the GATA-6 transgene

The full *GATA-6* cDNA was constructed by joining the two *Eco*RI fragments which were isolated from a mouse E11.5 library (CLONETECH) (Brewer et al., 1999; Koutsourakis et al., 1999). 3' UTR was eliminated by PCR from the unique *PstI* site to the stop codon (144bp) and a unique site was introduced in the 3' PCR primer in order to clone the last non-coding exon, intron and polyA (PA) (2.8 kb) from the human *β-globin* gene (Drabek et al., 1997). PCR between the first ATG and the unique *Not*I (520bp) site was used to eliminate 5' sequences. In the 5' PCR primer the myc epitope (EQKLISEEDL) was introduced immediately after the ATG of *GATA-6* (Elefanty et al., 1996). The sequence of all PCR generated clones was confirmed before further cloning. The complete (1.7 kb) myc-tagged *GATA-6* cDNA was transfected in COS-1 cells and the overexpressed protein was immunoreactive in situ with both the anti-myc (9E10) and the anti-GATA-6 (SantaCruz) antibodies (data not shown). The human SP-C promoter (Wert et al., 1993) was cloned as a 3.7 kb blunted *Nde*I-*Sal*I fragment in blunted *Sal*I of pBS and the *myc-GATA-6-globinPA* as an *Xho*I fragment. The transgene was released with *Cla*I between the pBS polylinker and the *Cla*I that was present at the end of the *PA*. The 8.2 kb fragment was gel purified with CONCERT™ Matrix (GIBCO BRL) and passed through ELUTIP-D column (Schleicher & Schuell).

Generation of transgenic embryos

Transgenic mouse embryos were generated by pronuclear injection of FVB/N oocytes with transgene concentration 2-3 ng/μl according to standard protocols (Hogan et al., 1994). Day of injection and transfer to pseudopregnant females was considered as day 0.5 for the subsequent staging of the dissected embryos and lungs. Part of the tail of the dissected embryos was used for southern blot genotyping using the human *β-globin PA* as a probe.

In situ hybridization and immunohistochemistry

Fetal or newborn lungs were dissected out and fixed in 4% PFA overnight at 4°C before processing for paraffin embedding according to routine protocols. 5-7μm thick sections were used for H&E staining, RNA in situ hybridization (Motoyama et al., 1998) and immunohistochemistry (Keijzer et al., 2000). Digoxigenin labeled RNA probes were made from cDNA fragments of 1.5kb for *GATA-6*, 0.33 kb for *SP-C* and 0.315 kb for *CC-10* according to the protocol supplied by Roche Diagnostics. For TTF-1, a monoclonal antibody (Neomarkers, CA, USA) was used in 1:100 dilution and the sections were microwave boiled in citrate buffer for 15 min. The same dilution was used for the monoclonal anti-myc antibody (9E10) but for antigen retrieval, trypsin treatment (0.6mg/ml for 5 min at RT) was used.

Electron Microscopy

The caudal lobes of E18.5 fetal lungs were fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer pH 7.3 at 4°C for a few days until the genotypes were known. One trangenic and one wild type littermate lobe were washed in 0.1 M cacodylate buffer and postfixed in 1% OsO_4 in 0.1 M cacodylate buffer and finally embedded in epon as has been previously described (De Bruijn and Den Beejen, 1975). Ultrathin sections were contrasted with Uranyl-acetate and Lead-citrate prior to Electron Microscopic analysis (Hanaichi et al., 1986).

PAS staining

From the epon-embedded E18.5 lungs, semithin (1μm) sections were used for periodic acid Shiff's staining as previously described (Nevalainen et al., 1972).

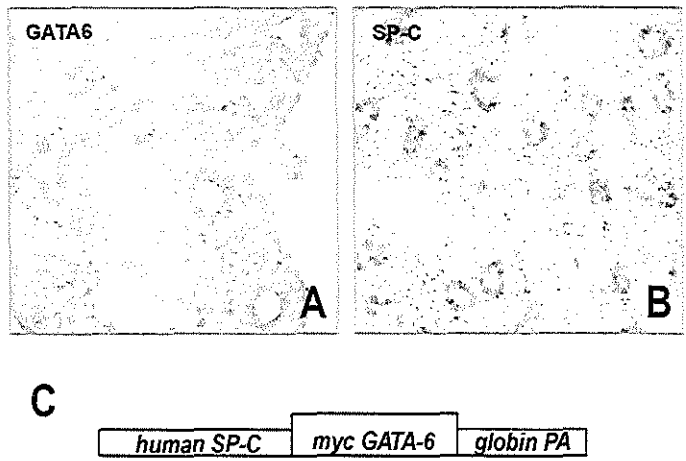

Figure 1
Distribution of *GATA-6* (A) and *SP-C* (B) mRNA in E15.5 lungs. Both genes, although at a different level, are expressed in cells lining the distal epithelium (blue is the detected mRNA and the tissue is counterstained with methyl-green). In C the transgene used to overexpress *GATA-6* in the pulmonary epithelium is schematically depicted. A myc-tagged version of the complete *mGATA-6* cDNA is under the control of the 3.7 kb human *SP-C* promoter and it is followed by the human *β-globin* last intron and polyadenylation signal.

4.4 Results

GATA-6 expression and over-expression in the pulmonary epithelium

Expression of *GATA-6* in the developing embryonic bronchial epithelium has been described previously (Morrisey et al., 1996). In a recent study (Keijzer et al., 2001), a more extensive analysis of *GATA-6* expression revealed that initially it is expressed at the tips of the growing buds (E10.5) and by E12.5 it is expressed in the entire endoderm lining the developing buds. By E15.5, *GATA-6* mRNA is predominantly expressed in the endoderm lining the growing airways and to a lower extent in parts of the surrounding mesenchyme (Fig. 1A). This expression pattern, although not at a comparable level, is quite reminiscent to that of *SP-C* at the same developmental stage (Fig. 1B). This observation led us to employ the 3.7kb promoter/enhancer of the human *SP-C* gene (Wert et al., 1993) in order to sustain high *GATA-6* expression levels in the developing epithelium *in vivo*. The complete mouse GATA-6 cDNA was cloned downstream of the *SP-C* promoter and was followed by the

human *β-globin* last exon, intron and polyA for mRNA stability. Sequences coding for the myc epitope were introduced in frame with the first ATG of the gene (Fig. 1C). The *SPC-mycGATA-6* transgene was injected into fertilized eggs and lungs were isolated from embryos at different developmental stages, E14.5, E16.5 and E18.5. Embryos were genotyped by Southern blotting (data not shown) and transgenic lungs were identified. All the analysis was performed in F_0 transgenic founder embryos. Transgenic lungs were not significantly different in size from their wild type littermates (data not shown). Only after careful examination of the whole transgenic lungs, a difference in the texture was noticed, especially in the affected lungs at E18.5 (data not shown). At this stage, out of 5 transgenic founder, 3 were expressing the transgene and they all had a comparable phenotype (Fig. 2F and 4H). Sections from the same transgenic E18.5 lung were used for both morphological (Fig. 2F) and transgene expression analysis (Fig. 3B&D). Expression analysis of molecular markers (Fig. 4) and EM (Fig. 5) were performed on sections from a different transgenic E18.5 lung.

Branching morphogenesis in the SP-C/GATA-6 transgenic fetal lungs

Starting at E14.5, the transgenic lungs looked similar in size to the wild type but they had undergone less extensive branching judged by the fewer terminal buds (Fig. 2B). Two days later, at E16.5, the defect in branching morphogenesis was more pronounced because of the presence of grossly dilated terminal buds separated by excess of mesenchyme (Fig. 2D). Just before birth, at E18.5, the phenotype was comparable to that seen at E16.5 and the overall growth of the tissue was not severely affected (Fig. 2F). The mesenchyme appears to be thickened although it is at present not clear whether this is due to an actual increase in the number of mesenchymal cells or the lack of being intersected by branching endoderm. Postnatally, although the remains of a transgenic pup were found subsequent to its perinatal death, few transgenic pups were born alive and they were breathing normally. These transgenic pups were left to breathe for a few hours before they were sacrificed for genotyping and histologic analysis. In figure 2H a section of an abnormal lung among the transgenic pups is shown with a phenotype similar to the clinical centro-acinar aeration pattern. The severity of the phenotype was less than that seen in E18.5 lungs (Fig. 2F) most likely due to the lower expression levels of the transgene. Expression of the transgene in the affected E18.5 lungs was confirmed by immunostaining for the myc epitope (Fig. 3B) and *GATA-6* mRNA, transgenic and endogenous, was detected by in situ hybridization (Fig. 3C and D).

Molecular analysis of differentiation in the GATA-6 over-expressing epithelium

Since extensive differentiation and the initiation of surfactant production start at the saccular stage of lung development (E17.5), which extends even after birth (to P5), we decided to investigate epithelium differentiation in E18.5 transgenic lungs. Thyroid Transcription Factor-1 (TTF-1 or Nkx2.1) protein, a marker for specified pulmonary endoderm (Minoo et al., 1999), was abundantly present in transgenic lungs (Fig. 4B and C). However, the staining in all cells lining the dilated terminal buds is more intense than the characteristic staining for this stage of development as seen in wild type E18.5 lungs (Fig. 4A). *Clara Cell marker-10* mRNA (Ray et al., 1996) was expressed in transgenic lungs at a normal level indicating the presence of non-ciliated sectretory cells in the proximal epithelium (Fig. 4D-F). In the distal epithelium, topologically represented by the dilated buds, *Surfactant Protein-C* (Kalina et al., 1992) expressing cells could be detected among the cells lining the epithelium (Fig. 4H and I) although the number of cells and their distribution were very different from that observed in wild type lungs (Fig. 4G). *SP-C* is expressed in the Type II cells which function mainly in surfactant production and they can further differentiate into Type I pneumocytes, the functional cells for gas exchange.

Morphological analysis of differentiation in the GATA-6 over-expressing epithelium

From morphological studies (Ten Have-Opbroek, 1991; Ten Have-Opbroek et al., 1988) it was shown that before maturation, Type II cells have an excess of glycogen present in their cytoplasm and their nucleus is less round. We therefore stained the epithelium of the transgenic lungs (E18.5) for glycogen (Fig. 4J-L) and used Electron Microscopy to examine the ultrastructure of the cells lining the dilated buds (Fig. 5). Periodic acid Shiff's staining (PAS) for glycogen revealed that a very high percentage of cells lining the distal epithelium of the transgenic lungs is positive for glycogen in their cytoplasm (Fig. 4K and L). Normally at this stage, due to advanced differentiation, glycogen positive cells are hardly detectable in the lining of the distal epithelium (Fig. 4J). The caudal lobe of a transgenic (Fig. 4) and a wild type littermate lung were processed for Electron Microscopy. In the epithelium of the wild type lung, normal Type II cells could be easily identified by the cuboidal shape, the almost round nuclei and the presence of several multilamellar bodies (Fig. 5C). Squamous Type I cells were also observed surrounding capillaries (Fig. 5E, arrows indicate capillaries).

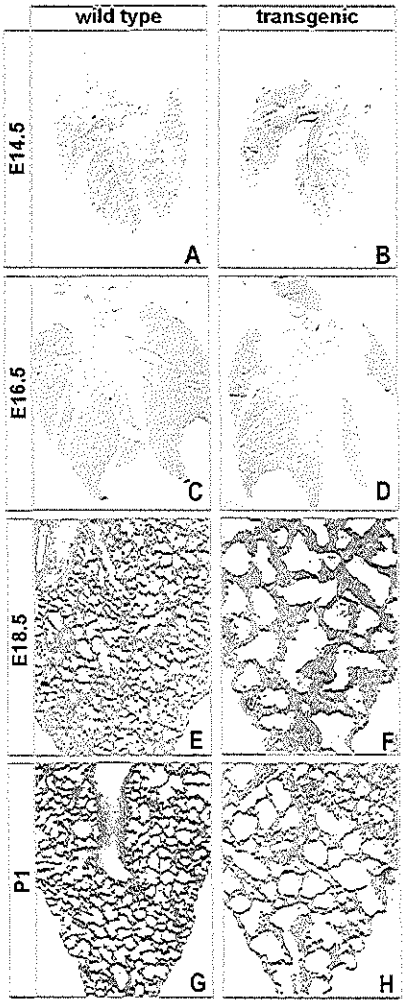

Figure 2

Histological analysis of *SP-C/GATA-6* transgenic lungs during fetal lung development. At E14.5, transgenic lungs (B) are smaller than wild type (A) and they have fewer terminal buds. Two days later, at E16.5 the branching defect is more prominent with big dilated distal buds present in the transgenic lungs (D) while fine branching is already apparent in wild type littermates (C). This phenotype is more dramatic just before birth, at E18.5, with the presence of abnormally shaped and sized distal alveoli (F) in contrast to normal alveolization that can be seen in wild type lungs (E). A few transgenic pups were born alive and the most severely affected one (H) had a milder branching phenotype when compared to most of the E18.5 transgenic lungs (F). This phenotype is similar to the clinical central-acinar aeration pattern. All pups were sacrificed few hours after birth.

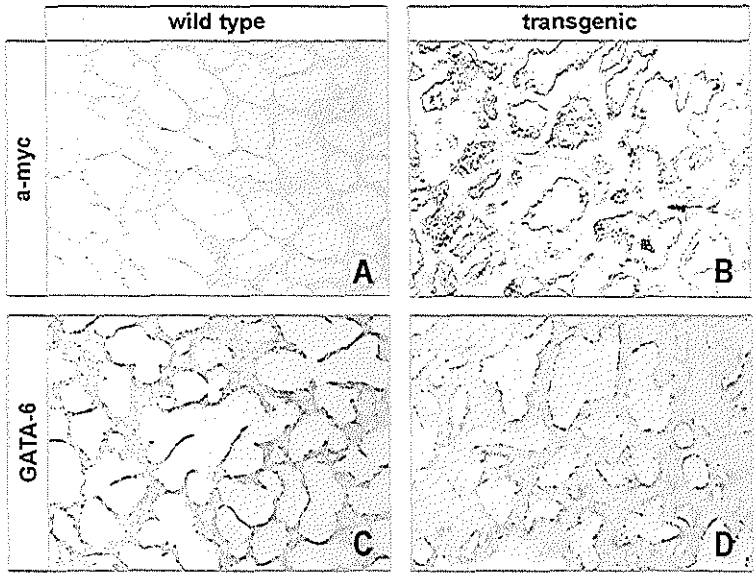

Figure 3
Expression of the *SP-C/GATA-6* transgene in E18.5 lungs. The anti-myc antibody was used to detect chimeric protein in transgenic lungs (B). Intense staining can be seen in the epithelium lining the abnormally dilated alveoli (brown). Wild type lungs show no staining (A). *GATA-6* mRNA was detected in both wild type and transgenic lungs by in situ hybridization. In C, normal *GATA-6* expression in parts of the epithelium and in the surrounding mesenchyme can be seen in wild type E18.5 lungs (blue). In transgenic lungs (D) the signal for RNA expression (blue) is very intense in the lining of the epithelium as seen with the anti-myc antibody staining (B). For this analysis, sections from the lung that is shown on Figure 2F were used.

Both Type II and I cells were represented in every alveoli that was examined. In contrast, examining the epithelial lining in the dilated alveoli in the transgenic lung we were not able to find any typical Type II or I cells. Instead, a number of cells with more irregular nuclei and numerous glycogen fields in their cytoplasm were found (Fig. 5D, glycogen fields are indicated by arrowheads). Although formation and localization of capillaries appeared normal, the squamous cells present around them had different morphology than that of a typical Type I cell and some even had glycogen in their cytoplasm (Fig. 5F, arrow indicates capillary and arrowhead glycogen). Proximal epithelium appeared normal and ciliated cells were observed as in the wild type epithelium (data not shown).

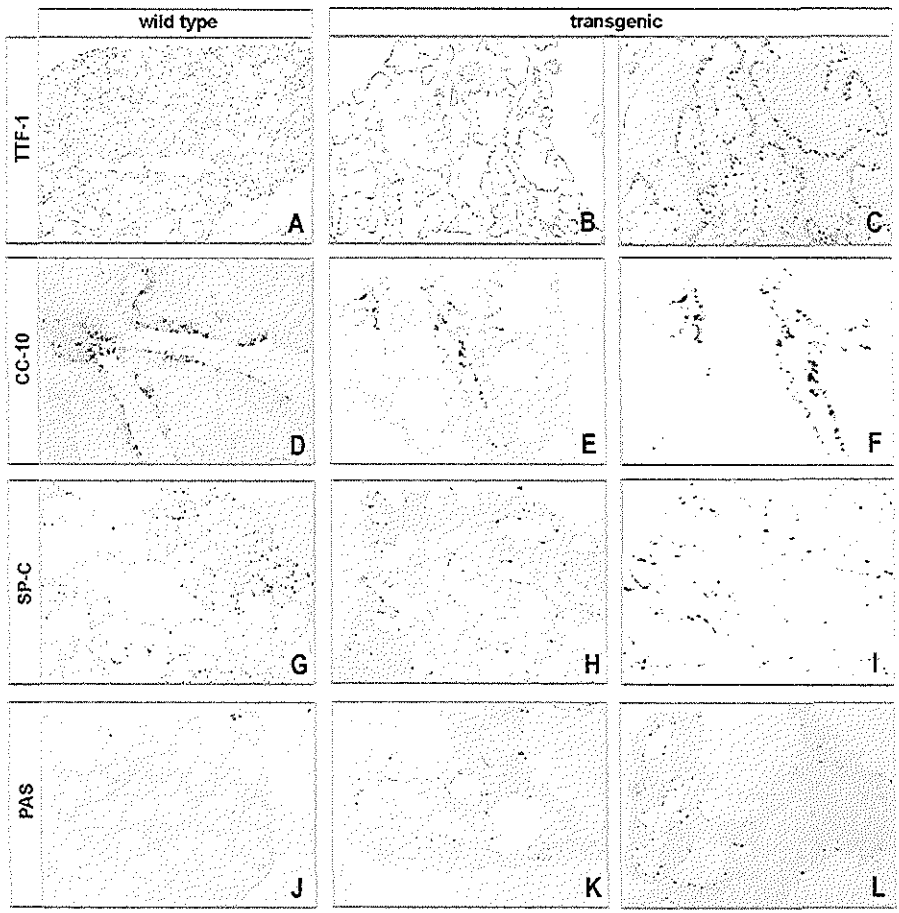

Figure 4
Molecular analysis of differentiation in the transgenic epithelium at E18.5. TTF-1 protein localization in wild type (A) and transgenic (B and C) epithelium shows the abnormally, for this stage, TTF-1 positive epithelium which lines the complete transgenic alveoli (brown). In D and E, F the normal expression of *CC-10* mRNA, a marker for proximal non-ciliated cells, is shown in both wild type and transgenic lungs, respectively (blue). In the distal epithelium, expression of endogenous *SP-C*, a marker for Type II cells, can be seen in a wild type lung (G) and the decreased levels with the different distribution in the transgenic lung (H and I) (blue). Periodic Acid Shiff's staining for glycogen (purple) revealing that in contrast to the wild type lung (J), the transgenic lung has an alveolar epithelium which is lined almost exclusively by glycogen positive cells (K and L).

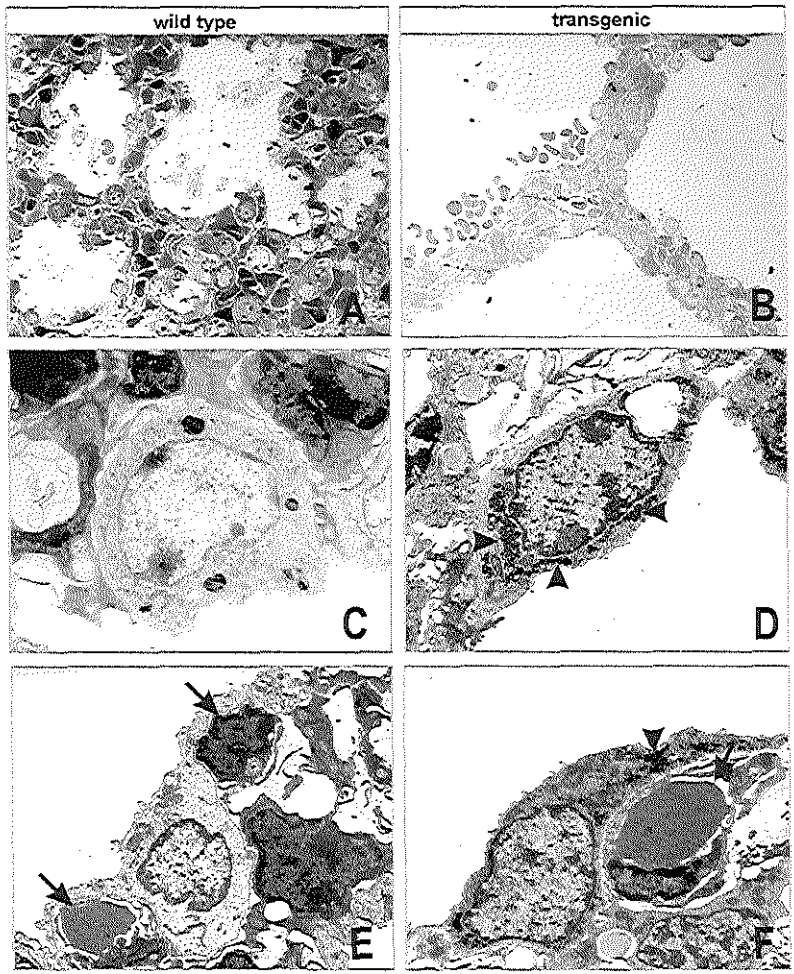

Figure 5
Electron Microscopic analysis of the distal epithelium at E18.5. A low power overview of a wild type and a transgenic lung is shown in A and B, respectively. In the distal epithelium of a wild type lung both typical Type II cells, with multilamellar bodies in their cytoplasm (C) and flat Type I pneumonocytes, around capillaries, can be detected (E). In the transgenic lung atypical cells lining the alveoli are detected and the presence of glycogen in their cytoplasm is a common feature (D). Flat cells are present around the capillaries (F) although they look distinct from wild type Type I cells and some of them still have glycogen in their cytoplasm (arrowheads point to glycogen fields and arrows indicate capillaries; magnifications: A and B X620, C-F X5800).

4.5 Discussion

Depletion of *GATA-6* expression in the pulmonary endoderm, both *in vitro* and *in vivo*, resulted in lack of branching morphogenesis and failure of the epithelium to differentiate (Keijzer et al., 2001). To better understand the role of this transcription factor in lung development we used the *SP-C* promoter to artificially maintain high levels of *GATA-6* expression in the fetal pulmonary epithelium *in vivo*. This alteration also resulted in defective branching morphogenesis and epithelial differentiation. However, the branching phenotype was manifested later during development and in a less severe form probably due to the late onset of the transgene expression (Wert et al., 1993). The promoter is active after pulmonary endoderm specification, initial budding and lobe determination but before the differentiation program starts at the pseudoglandular stage (E14.5) of lung development (Perl and Whitsett, 1999; Warburton et al., 2000). Hence, *GATA-6* expression in the *SP-C/GATA-6* lungs is already elevated in the primordial epithelium before any proximal or distal fate had been acquired. Nevertheless, this overexpression affected only distal epithelium where the gene is normally expressed. Thus, this study shows that GATA-6 has a specific regulatory role in the differentiation program of the distal epithelium and also confirms that GATA-6 protein levels are crucial during both lung morphogenesis and cell type specification (Keijzer et al., 2001)

Branching morphogenesis in the SP-C/GATA-6 transgenic lungs

The interaction between epithelium and mesenchyme is a determining factor in lung development starting from the initial budding and continuing during branching morphogenesis involving well-studied molecules like FGF-10, BMP-4 and SHH (Hogan, 1999; Weaver et al., 2000). The localized high expression of *FGF-10* in the mesenchyme promotes the endoderm to bud and to express high levels of *BMP-4*. Subsequently, BMP-4 together with SHH downregulates *FGF-10* to prevent new budding at the same position while allowing the newly formed bud to grow. When a certain distance has been reached, the high expression domains of *FGF-10* present on either side or both sides of the growing bud induce new budding resulting in lateral or dichotomous branching, respectively. *GATA-6* expression in the endoderm coincides with branching morphogenesis but unlike *BMP-4* expression (Weaver et al., 2000), there are no detailed *GATA-6* expression data during the induction and growth of a bud. In E10.5 lungs *GATA-6* mRNA has been localized to the growing tips of the initial buds (Keijzer et al., 2001). At that stage, the *SP-C* promoter

becomes active resulting in high expression of *GATA-6* in all SP-C expressing cells throughout the transgenic endoderm. This alteration of *GATA-6* expression results in the branching defects in the transgenic lungs which could be explained in two ways. Either by distorting a molecular pathway intrinsic to the branching endoderm or by interrupting a pathway involved in processing an inductive signal from the mesenchyme.

In cardiac development, FGFs and BMPs have been placed upstream of GATAs (Schultheiss et al., 1997). Cardiac induction involves GATA-4 and its co-factor Nkx 2.5 and they are both necessary to establish a cardiac phenotype (Durocher et al., 1997). An analogous regulatory pathway appears to operate in the lung endoderm. TTF-1 (or Nkx 2.1), a gene from the same Nkx homeobox family and GATA-6 are expressed in the lung endoderm from early pulmonary specification and both are required for branching morphogenesis (Lazzaro et al., 1991). It has been suggested that TTF-1 expression may be downstream and depends on GATA-6 expression (Shaw-White *et al.*, 1999), however, TTF-1 expression is normal in *GATA-6 -/-* endoderm showing that TTF-1 expression is independent of GATA-6 (Keijzer et al., 2001). Unfortunately it is not known whether GATA-6 is still expressed in *TTF-1 -/-* embryos (Minoo *et al.*, 1999)

GATA-6 levels and pulmonary epithelium differentiation

Expression of *GATA-6* starts in the primordial epithelium and it is restricted to the distal epithelium by E15.5. The levels of its expression are difficult to quantitate by in situ hybridization. By expressing *GATA-6* under the control of *SP-C*, a constant high level of the protein was present from the onset of differentiation resulting in a distal epithelium attenuated of any terminal differentiation. Unlike overexpression of *TGF-β1* (Zhou et al., 1996) and *HNF-3β* (Zhou et al., 1997) that resulted in endodermal arrest at the primordial or pseudoglandular stages, *GATA-6* overexpression did not affect initiation of the differentiation program. The pathway of proximal epithelium differentiation was unaffected since both ciliated cells, seen in the EM analysis, and non-ciliated secretory cells, visualized by *CC-10* expression, were detected as normal. Along the distal epithelium no mature Type II cells were present and all the cells were still expressing *TTF-1*. Normally the presence of numerous glycogen fields in the cells lining the distal airways is indicative of their differentiating status (Sorokin, 1965). This has been described in detail in an extensive morphological study of the features of Type II alveolar epithelial cells by Ten Have-Opbroek (1988). Later during lung development, before birth, a multi-step formation of multilamellar bodies (MLB) is thought to compartmentalize glycogen (Ten Have-Opbroek

et al., 1990). Our data show a continued presence of glycogen in the distal epithelial cells suggesting that GATA-6 is involved in a particular stage of this maturation process. When the levels are elevated, the cells do not initiate the final differentiation step which is characterized by multilamellar bodies formation. Instead, most of the cells lining the alveolar epithelium have glycogen in their cytoplasm, as visualized by periodic acid Shiff's staining (Fig. 4K and L), indicating a block in the Type II differentiation pathway. The flat Type I cells which function in gas exchange, are thought to originate from further differentiation of Type II cells although it is not clear whether they are derived from a mature Type II cell or from one of the intermediate stages during their maturation. In the *GATA-6* overexpressing lungs, flat epithelial cells surrounding the capillaries were observed and their shape and localization suggest that they are Type I cells. However, some of them still had glycogen fields, they were not as flat as normal Type I cells and their nucleus had an irregular shape. At this final step, differentiation is thought to be driven by growth and particularly by the intercalation of the forming capillaries with the epithelium (Ten Have-Opbroek et al., 1988). Thus, the apparent Type I-like cells we see, could be the same precursors of Type II cells as discussed above, except that they are in close proximity with the capillaries and have less glycogen fields. This would suggest that elevated levels of GATA-6 would prevent the formation of fully differentiated Type I cells even when in contact with capillaries. In conclusion, our data demonstrate that GATA-6 plays an important role in lung organogenesis and especially during the multi-step process of maturation of Type II cells. Elevated GATA-6 protein levels result in a block of terminal differentiation to mature Type II and Type I pneumocytes.

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Chapter 5

Apoptosis and proliferation in a cell recombinant model of fetal rat lung

Based on:
Keijzer, R., Deimling, J.F., Liu, J., Tseu, I., Tibboel, D., and Post, M. Apoptosis and proliferation in a cell recombinant model of fetal rat lung. (submitted)

5.1 Abstract

It is well established that lung morphogenesis is dependent on epithelial-mesenchymal interactions, however, the exact molecular mechanisms guiding lung organogenesis have yet to be determined. Cell proliferation has been recognized to be a crucial modulator of lung development. Recently, it has been suggested that programmed cell death, apoptosis, is another important mechanism involved in remodeling of both the prenatal and postnatal lung. Utilizing an *in vitro* cell recombinant model, we investigated cell proliferation and apoptosis during lung morphogenesis *in vitro* and the influences of epithelial-mesenchymal interactions on these processes. Embryonic day (E) 13 and E19 mesenchymal and endodermal pulmonary cells were isolated from fetal rat lungs using standard primary culture techniques. The cells were then recombined in various combinations and cultured in a semi-dry system. In these recombinants, proliferating cells were identified using a proliferating cell marker and apoptosis was determined by TUNEL assay. We observed more proliferating cells in homotypic E13 recombinants than in E19 recombinants. The proliferation characteristics of the different tissues appeared to be intrinsic. Massive apoptosis was observed in fibroblasts of E19 homotypic recombinants. Surprisingly, the apoptosis of E19 fibroblasts was diminished after heterotypic recombination with E13 epithelial cells. The massive apoptosis appeared to be due to a loss of inhibition by E19 epithelial cells. In conclusion, both proliferation and apoptosis are developmentally regulated during morphogenesis *in vitro*. In addition, the apoptosis of late gestation fibroblasts appears to be regulated by a loss of inhibition of the neighboring epithelial cells.

5.2 Introduction

Organogenesis of the lung is, like organogenesis of other glandular-like organs, highly dependent on epithelial-mesenchymal interactions as well as cell-matrix interactions [for review see (Hogan, 1999)]. Several tissue recombination studies have demonstrated that for normal branching morphogenesis of the lung to occur, interaction between lung epithelium and lung mesenchyme is absolutely required (Masters, 1976; Shannon et al., 1998; Spooner and Wessells, 1970). It has long been recognized that branching morphogenesis of lung epithelium is guided by "instructions" coming from the surrounding mesenchyme. Interestingly, proximal tracheal epithelium, which is normally

not able to branch, can in this manner be forced to undergo branching morphogenesis when it is recombined with distal lung mesenchyme (Alescio and Cassini, 1962). In contrast, distal lung epithelium, which is normally undergoing extensive branching morphogenesis during pulmonary development, can be inhibited by recombining it with proximal tracheal lung mesenchyme (Wessells, 1970). Additional experiments demonstrated that epithelial differentiation is dependent on the type of mesenchyme used for recombination (Shannon et al., 1998). These epithelial-mesenchymal interactions are modulated by a well orchestrated interplay between hormones, growth factors and extracellular matrix proteins [for review see (Keijzer and Post, 1999)]. The factors involved have just recently been revealed as was shown by experiments with isolated mesenchyme-free pulmonary epithelium. In this system isolated epithelium could be stimulated to undergo branching morphogenesis when it was cultured in the presence of a specified mix of certain growth factors and extracellular matrix proteins (Shannon et al., 1999). All these experiments have demonstrated an important role for the mesenchyme in instructing the developing epithelium. However, the influence of epithelium on development of the mesenchyme is less well understood and has not been studied extensively thus far.

Cell proliferation has been recognized as a crucial modulator not only for lung growth, but also branching morphogenesis (Goldin and Wessells, 1979; Mollard and Dziadek, 1998). Outgrowth of terminal lung buds is associated with higher numbers of proliferating cells. Recently, it has been suggested that programmed cell death, apoptosis, is another important mechanism involved in remodeling of both the prenatal and postnatal lung (Kresch et al., 1998; Scavo et al., 1998). Apoptosis was demonstrated to occur predominantly in interstitial cells, in particular during the perinatal period (Kresch et al., 1998; Scavo et al., 1998). This is the period when a huge thinning of the pulmonary mesenchyme has to occur in order to enable gas exchange over the pulmonary epithelium. During this process, apoptosis is known to play a significant role (Kresch et al., 1998; Scavo et al., 1998). Taken together, a balanced interplay between cell proliferation on the one hand and apoptosis on the other is required to help a primary lung bud early in embryonic development to become the postnatal gas exchanging organ which has undergone a huge surface expansion.

The aim of this study was to investigate cell proliferation and apoptosis into more detail in a cell recombinant model. Isolated fetal rat lung embryonic day (E)13 mesenchymal cells or E19 fibroblasts were recombined with E13 endodermal cells or E19

epithelial cells. The cells were recombined in various combinations (Figure 1) and the cell mixtures were cultured for 5 days. Subsequently, cell proliferation and apoptosis were investigated in the recombinants using immunohistochemistry with an antibody for Proliferating Cell Nuclear Antigen (PCNA) and Terminal deoxyribonucleotidyl transferase dUTP Nick End-Labeling (TUNEL) assay, respectively. The cell recombinant model enabled us to dissect out the developmental and tissue-specific influences that the various recombinations of cells have on cell proliferation and apoptosis. Our results indicate that both proliferation and apoptosis are developmentally regulated during morphogenesis of lung epithelial cells and fibroblasts *in vitro*. In addition, epithelial cells appear to be less susceptible to apoptosis than fibroblasts in this tissue cell recombinant model, which corroborates with previously published *in vivo* studies (Kresch et al., 1998; Scavo et al., 1998). Finally, we demonstrate that apoptosis of fibroblasts which occurs during thinning of the mesenchymal tissue mass late in gestation can be inhibited by endodermal cells of early gestation, but cannot be induced by epithelial cells of late gestation.

5.3 Materials and methods

Animals

Female (200-250 g) and male (250-300 g) Wistar rats were obtained from Charles River (St. Constant, Quebec, Canada). The animals were kept in a controlled light-dark cycle and food and water were supplied ad libitum. Rats were mated overnight and the finding of a sperm-positive vaginal smear was designated day 0 of gestation. At embryonic day (E) 13 and E19 (term = E22) timed-pregnant rats were killed by exposure to an overdose of diethyl ether. The fetuses were delivered by Caesarian section using aseptical surgical techniques.

Cell preparations and recombinations

Using microsurgical techniques, lungs were dissected from embryos at E13 and transferred to Hanks Balanced Salt Solution (-) (HBBS(-)) from Gibco (Grand Islands, NY). Subsequently, the lungs were treated with 20% (v/v) Dispase and 100 $\mu g.ml^{-1}$ DNAse for 45 minutes at 37°C in order to "loosen" the tissue layers. Following neutralization of enzyme activity with fetal calf serum (FCS), the endodermal and mesenchymal components were separated under a dissection microscope, using

microsurgical techniques. The separated rudiment layers were broken up in single cells by gentle mechanical agitation.

At E19, epithelial cells and fibroblasts were separated using standard primary culture techniques, which were previously described (Caniggia et al., 1991). After separation of epithelial cells and fibroblasts, E19 epithelial cells were recombined with either E19 fibroblasts or E13 mesenchymal cells. E19 fibroblasts were also recombined with E13 endodermal cells (see figure 1). In order to recombine the cells, individual cell populations of late gestation (i.e. E19) were first trypsinized from tissue culture flasks and counted by a Coulter counter. Approximately 3 x 10^6 endodermal or epithelial cells were mixed with 3 x 10^6 mesenchymal cells or fibroblasts and the cell mixtures were then centrifuged at 420 x g for 5 minutes. Subsequently, excess medium was removed and the cell aggregates were incubated at 37°, in air with 5% CO_2 for 1 hour. Aggregates were then gently loosened by stirring with the tip of a micro-pipette and transferred to porous membrane inserts (4 μm pore size) from Millipore (Bedford, MA) in 24-well tissue culture plates from Nunc (Intermed, Denmark). Membranes were presoaked in MEM (Gibco, Grand Islands, NY) for 1 hour before addition of the cells. 200 μl Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Islands, NY) + 5% (v/v) FCS was added to each well so that the medium just moistened the surface of the cells, but the aggregates were not submerged (i.e. a semi-dry culture). The cell recombinants were cultured at 37°C in an atmosphere of 5% CO_2 in air for 5 days.

Tissue preparation

All recombinants were fixed in 4% (v/v) paraformaldehyde in phosphate buffered saline (PBS) for 16-18 hours at 4°C, dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraplast (Oxford, Labware, St. Louis, MO). Sections of 5 μm were cut and mounted on Superfrost slides (Fisher Scientific, Unionville, ON). After de-waxing, the tissue sections were rehydrated in a graded series of ethanol.

Epithelial-Mesenchymal Recombinants

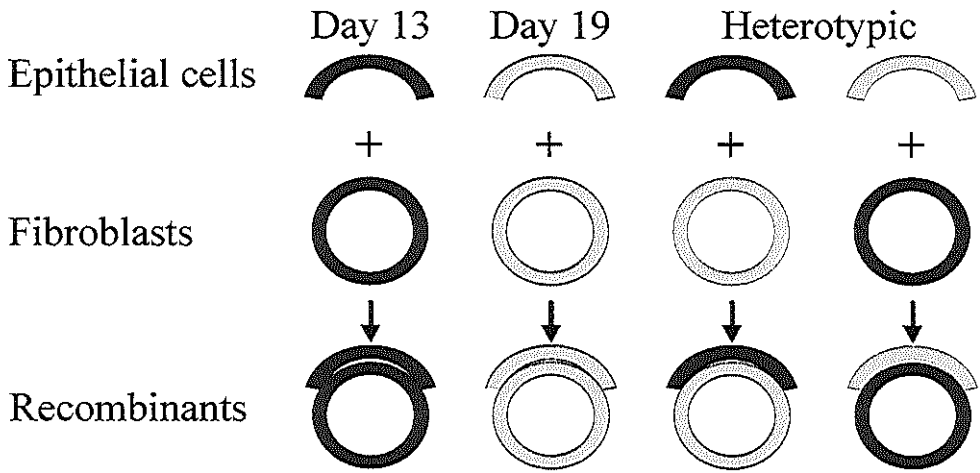

Figure 1
Schematic representation of the various combinations that were used in the recombination experiments. Following separation of lung tissues, E13 mesenchymal cells (black) were recombined with E13 endodermal cells (black) in homotypic recombinants. The same procedure was used in making the homotypic recombinants of E19 fibroblasts (grey) and E19 epithelial cells (grey). In heterotypic recombinants, E13 endodermal cells (black) were recombined with E19 fibroblasts (grey) or E19 epithelial cells (grey) were recombined with E13 mesenchymal cells (black).

Immunohistochemistry

Immunohistochemistry was essentially carried out as described before (Keijzer et al., 2000). Following pretreatments, sections of the recombinants were incubated for 16-18 hours at 4°C with 1:5 diluted primary monoclonal antibody to cytokeratin (Boehringer Mannheim, Laval, QC), or undiluted primary monoclonal antibody to vimentin (Serotec, Missisauga, ON) or 1:1000 diluted primary monoclonal antibody to proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA). Terminal Deoxyribonucleotidyl Transferase dUTP Nick-End labeling (TUNEL) Assay was carried out exactly as described before (Keijzer et al., 2000).

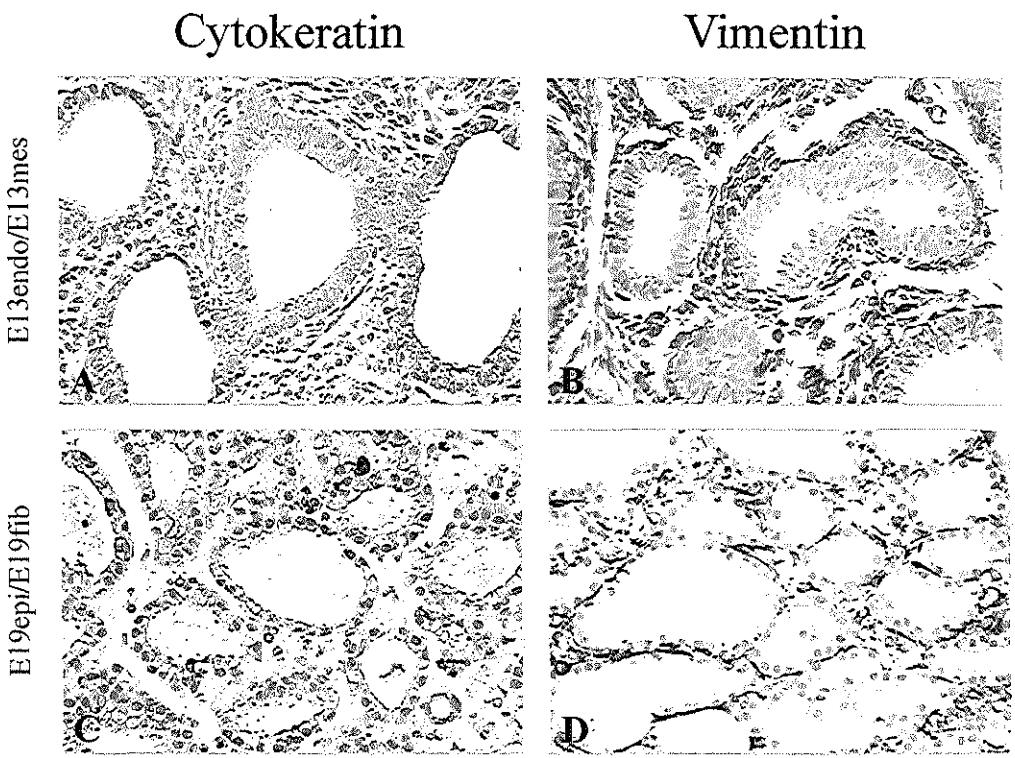

Figure 2
Morphogenesis occurred spontaneously in the recombinants during the culture period. Antibodies against cytokeratin (A,C) and vimentin (B,D) were used to distinguish between endodermal and mesenchymal cells, respectively. In the E13 homotypic recombinants large airspaces developed with a lining of cuboidal endodermal cells (cytokeratin positive) surrounded by mesenchymal cells (vimentin positive) (A,B). In the E19 homotypic recombinants, alveolar-like structures formed consisting of a nice circle lining of epithelial cells (cytokeratin positive) and a thin interstitial layer of fibroblasts (vimentin positive) (C,D).

5.4 Results

Morphogenesis in the recombinants

Approximately 72 hours after putting the recombined cell mixtures into culture, morphogenesis started to occur in the till then unorganized clump of cells (results not shown). In the E13 homotypic recombinants large airspaces developed with a lining of cuboidal endodermal cells surrounded by mesenchymal cells (Figure 2A,B). We used the

molecular markers cytokeratin (Figure 2A,C) and vimentin (Figure 2B,D) to distinguish between endodermal and mesenchymal cells, respectively. In the E19 homotypic recombinants, alveolar-like structures formed consisting of a nice circle lining of epithelial cells and a thin interstitial layer of fibroblasts (Figure 2C,D). In the recombinant of E13 endodermal cells and E19 fibroblasts, large airspaces with a thick lining of cuboidal endodermal cells were formed (Figure 3C). Mesenchymal cells surrounded the endodermal lining. The structure of this recombinant resembled the structure of the E13 recombinant (Figure 2A,B). In the recombinant of E19 epithelial cells and E13 mesenchymal cells more alveolar-like structures were observed (Figure 3D), albeit smaller than in the E19 homotypic recombinant (Figure 2C).

Proliferation in the recombinants

Using an antibody against proliferating cell nuclear antigen (PCNA), a cell cycle associated protein, patterns of proliferation were investigated in the different recombinants. Massive cell proliferation, between mesenchymal and endodermal cells, was observed in E13 homotypic recombinants (Figure 3A). In contrast, proliferating cells were mainly observed in the epithelial lining of cells in the E19 homotypic recombinants (Figure 3B) and much less in fibroblasts. In the heterotypic recombinants, a comparable pattern of proliferation was observed. In the recombinant of E13 endodermal cells and E19 fibroblasts, proliferating cells were predominantly localized in the endodermal cells, whereas proliferation appeared to be attenuated in the fibroblasts when compared to the E13 homotypic recombinants (Figure 3C). In the recombinant of E19 epithelial cells and E13 mesenchymal cells, proliferation appeared to be less pronounced, but evenly distributed between epithelial cells and mesenchymal cells (Figure 3D). (For a summary of results see also table 1)

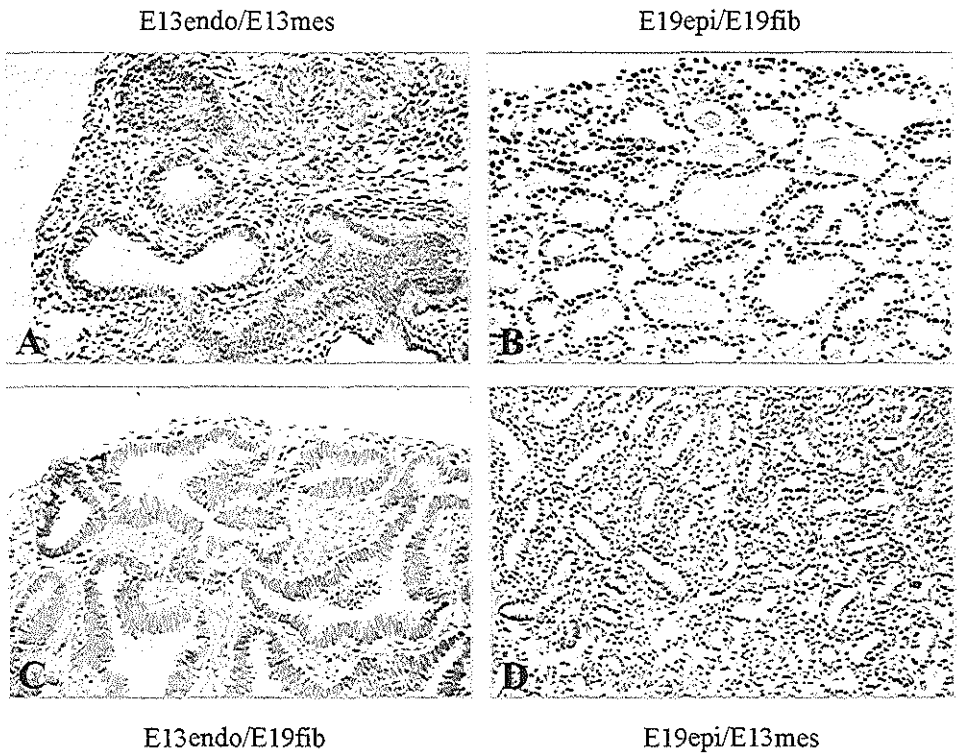

Figure 3
An antibody against PCNA was used to investigate cell proliferation in the recombinants. Highest numbers of proliferating cells, mesenchymal and endodermal cells, were observed in E13 homotypic recombinants (A). In E19 homotypic recombinants (B) proliferating cells were mainly observed between epithelial cells and much less between fibroblasts. In the recombinants of E13 endodermal cells and E19 fibroblasts, proliferating cells were predominantly localized in the endodermal cell lining (C). In the recombinant of E19 epithelial cells and E13 mesenchymal cells, proliferation was less pronounced, but evenly distributed over both cell layers (D).

Apoptosis in the recombinants

Using the TUNEL assay, apoptosis was investigated in all recombinants. Apoptotic cells were only observed in the mesenchymal cells or fibroblasts. In the E13 homotypic recombinant, TUNEL positive cells were sporadically observed in the mesenchymal cells (Figure 4A). In sharp contrast with this observation, almost every fibroblast was TUNEL positive in the E19 homotypic recombinant (Figure 4C). In both heterotypic recombinants, a similar picture as in the E13 homotypic recombinant was observed: hardly any mesenchymal cell or fibroblast was TUNEL positive, whereas no TUNEL-

positive endodermal or epithelial cells were observed (Figure 4E,G). All nuclei were stained with DAPI to visualize the tissue structure of the recombinants (Figure 4B,D,F,H). (For a summary of results see also table 1)

Table 1

Summary of proliferation and apoptosis results in the recombinants.

	Proliferation		Apoptosis	
Recombinant	Endodermal/ Epithelial cells	Mesenchymal cells/Fibroblasts	Endodermal/ Epithelial cells	Mesenchymal cells/Fibroblasts
E13 endo/E13 mes	++	+	-	+/-
E19 epi/E19 fib	+	+/-	-	++
E13 endo/E19 fib	++	+/-	-	+/-
E19 epi/E13 mes	+	+	-	+/-

++ indicates high number of cells; + indicates medium number of cells; +/- indicates low number of cells; - indicates no cells.
E indicates Embryonic day; endo indicates endodermal cells; mes indicates mesenchymal cells; epi indicates epithelial cells; fib indicates fibroblasts

5.5 Discussion

Morphogenesis of the lung has long been recognized to be highly dependent on epithelial-mesenchymal interactions (Hogan and Yingling, 1998). Most studies have focused on branching morphogenesis and demonstrated that these epithelial-mesenchymal interactions occur mainly into one direction, considering a pivotal role of the mesenchyme over the epithelium. The regulation of pulmonary endoderm branching morphogenesis is thereby modulated by factors produced by the surrounding mesenchyme (Keijzer and Post, 1999; Warburton et al., 2000). Using a cell recombinant model of fetal rat lung we provide here evidence for regulation of mesenchymal morphogenesis by its surrounding epithelium. Our results indicate that proliferation in both pulmonary fibroblasts and epithelial cells occurs cell-autonomously during pulmonary development. Highest numbers of proliferating cells were observed between E13 endodermal and mesenchymal cells. Recombining these cells with E19 epithelial cells or fibroblasts did not alter the distribution and number of proliferating cells.

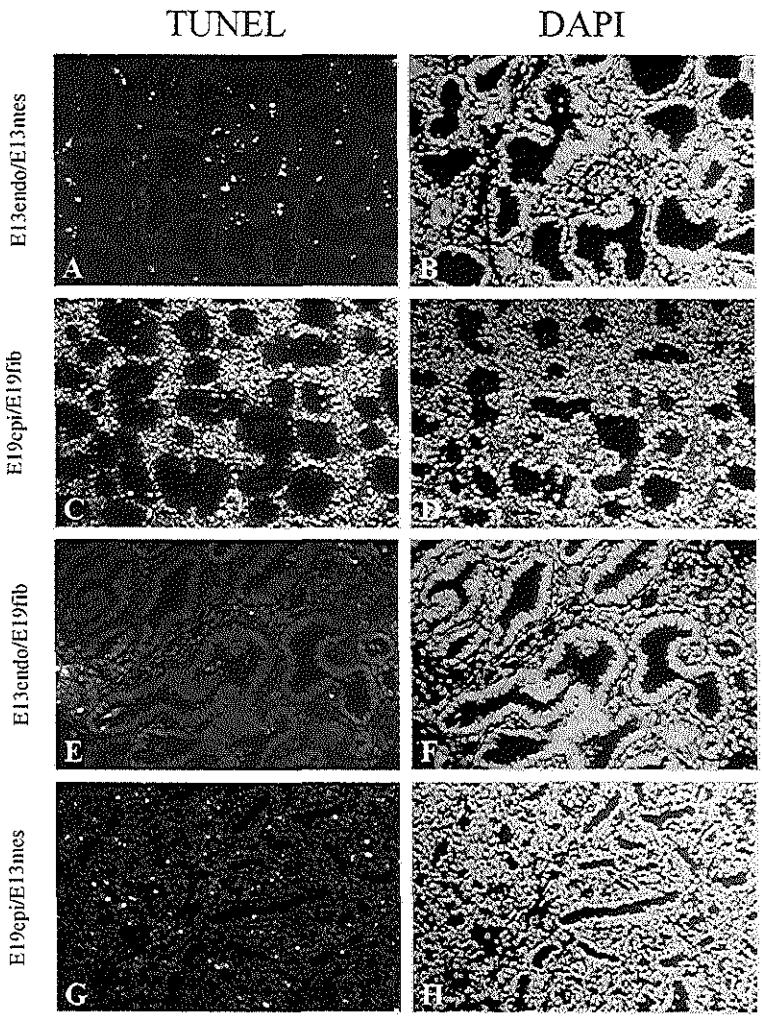

Figure 4
Programmed cell death, apoptosis, was determined in the recombinants using TUNEL assay (A,C,E,G). All nuclei were stained with DAPI to visualize the tissue structure of the recombinants (B,D,F,H). In the E13 homotypic recombinant only a few TUNEL positive cells were observed in the mesenchymal cells (A). The same holds true for both the heterotypic recombinant of E13 endodermal cells and E19 fibroblasts (E) and the heterotypic recombinant of E19 epithelial cells and E13 mesenchymal cells (G). In contrast, massive apoptosis was observed between the E19 fibroblasts in the E19 homotypic recombinant (C).

In contrast to these observations, apoptosis of E19 fibroblasts was demonstrated to be developmentally regulated by epithelial cells. Massive apoptosis of E19 fibroblasts occurs when they are recombined with E19 epithelial cells, but this can be inhibited when E19 fibroblasts are recombined with E13 endodermal cells. On the other hand, E13 mesenchymal cells could not be forced to undergo apoptosis by recombining them with E19 epithelial cells, indicating, that E13 endodermal cells inhibit apoptosis of E19 fibroblasts in contrast to induction of apoptosis of E19 fibroblasts by E19 epithelial cells. Thus, we conclude from these results that thinning of pulmonary interstitial tissue through apoptosis occurs because of a loss of inhibition by the surrounding epithelial cells. This is in accordance with the distribution of apoptotic cells *in vivo* during rat pulmonary development. Apoptotic cells were only observed in the mesenchyme and predominantly during the later stages of pulmonary development (Kresch et al., 1998; Scavo et al., 1998).

Our studies indicate that proliferation is a cell intrinsic feature of both fibroblasts and epithelial cells when they are cultured in close contact to each other. E13 endodermal cells remained in a high proliferative state when recombined with E19 fibroblasts that proliferate much less. On the other hand, the E19 fibroblasts could not be induced to proliferate more when recombined with the E13 endodermal cells. Proliferation characteristics of heterotypic recombinants of E19 epithelial cells and E13 mesenchymal cells also did not change when compared to their homotypic counterparts. Thus, E13 mesenchymal cells, E19 fibroblasts, E13 endodermal cells and E19 epithelial cells all displayed intrinsic proliferation characteristics, which were not influenced by recombination with cells from a heterotypic tissue. This result is in contrast to results obtained with studies using conditioned medium from epithelial cells which were demonstrated to elaborate a hydrophobic polypeptide that inhibits fetal lung fibroblast proliferation *in vitro* (Caniggia et al., 1995). However, in this study the fibroblasts were not in close contact to the epithelial cells, and this might be an essential condition for normal proliferation to occur.

The situation was somewhat different in case of apoptosis characteristics. We observed massive apoptosis in E19 fibroblasts in homotypic E19 recombinants. This is possibly a physiological phenomena which is required for normal thinning of mesenchymal tissue in late pulmonary development. The fact that we and others observed much less apoptosis *in vivo* than *in vitro* during late gestation can be explained by the fact that we recombined equal amounts of fibroblasts and epithelial cells whereas *in vivo* there

are already much less fibroblasts than epithelial cells at the investigated stage of pulmonary development. Apoptosis characteristics appeared to be non cell-autonomous, since they changed depending on the recombination that was used. Massive apoptosis was observed in E19 homotypic recombinants. This could be the result of either cell intrinsic apoptosis of E19 fibroblasts, induction of E19 fibroblast apoptosis by epithelial cells or loss of inhibition of apoptosis of E19 fibroblast by epithelial cells. The latter situation seems to be the case in our studies, because E19 fibroblasts did not undergo apoptosis when recombined with E13 epithelial cells ruling out the first possibility. Moreover, E13 fibroblasts were not induced to undergo apoptosis when recombined with E19 epithelial cells ruling out the second possibility. This leaves loss of inhibition of E19 fibroblast apoptosis by E19 epithelial cells as the only explanation for our observations. One could argue that E13 mesenchymal cells cannot yet be induced to apoptosis by E19 epithelial cells, because they are not sensitive yet for the inductive signals coming from the epithelial cells. However, in E13 homotypic recombinants we demonstrate that E13 mesenchymal are indeed able to undergo apoptosis.

Taken together, our results indicate that thinning of mesenchymal tissue late in pulmonary development is possibly due to a loss of inhibition of apoptosis by epithelial cells. This is one of the first studies providing direct evidence of modulation of mesenchymal morphogenesis (through apoptosis) by its surrounding epithelium. This adds to the already extensively studied influence of mesenchyme on branching morphogenesis of the endoderm (Alescio and Cassini, 1962; Hogan and Yingling, 1998; Masters, 1976; Shannon et al., 1998; Spooner and Wessells, 1970; Wessells, 1970). In addition, our study demonstrates again the essential role of epithelial-mesenchymal interactions in lung morphogenesis through a balanced interplay between cell proliferation and mesenchymal apoptosis. Future studies revealing the factors involved in these epithelial-mesenchymal interactions, and in particular in modulating mesenchymal apoptosis through loss of epithelial inhibition are warranted.

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Chapter 6

Developmental changes in the expression of the thyroid hormone receptors alpha and beta in rat tissues, evidence for extensive post-transcriptional regulation.

Based on:
Keijzer, R., Blommaart, P.J.E., Bakker, O., Labruyère, W.T., Vermeulen, J.L.M., Tibboel, D., and Lamers, W.H. Developmental changes in the expression of the thyroid hormone receptors alpha and beta in rat tissues, evidence for extensive post-transcriptional regulation. (submitted)

6.1 Abstract

The significance of thyroid hormone for normal development is undisputed, yet the signal-transduction pathways that mediate its effects have remained elusive. Although the involvement of thyroid-hormone receptors as ligand-dependent transcription factors seems likely, the respective roles of the TRα and TRβ isoforms are far from clear. Using in situ hybridization and immunohistochemistry as visualization techniques, and RT-PCR and Western blotting as quantification techniques, the TRα isoform was found to be expressed in the mesenchymal tissues and the TRβ isoform in the epithelial tissues of liver, lung, bone, and kidney. Furthermore, we observed extensive posttranscriptional control. A $TR\beta_2$-like protein of 47 kD, i.e. 11 kD less than that predicted by the reported open reading frame, was abundantly expressed in many tissues, but the corresponding mRNA concentration was vanishingly low. Tissue $TR\alpha_2$, $TR\beta_1$ and $TR\beta_2$ protein levels reached the adult level at 5 days before birth, whereas $TR\alpha_1$ protein reached its highest level only after birth. The distinct time-course of $TR\alpha_1$ and TRβ suggests an initiating, TRβ-mediated signaling from the epithelium, followed by a TRα-mediated response of the mesenchyme. Extensive co-expression of TRα and TRβ was observed in the developing brain and intestinal epithelium. These organs were characterized by a very low TRα (brain, intestine) and TRβ (intestinal epithelium only) protein/mRNA ratio, implying a low translational efficiency of the TR mRNA or a high turnover of TR protein. Furthermore, the $TR\beta_2$ protein levels in brain and intestine were the highest measured. These data indicate that the TR-dependent regulatory cascades function differently in organs with a complementary expression pattern and those with co-expression of the TRα and TRβ genes. The developmental delay in the expression of $TR\beta_2$ protein in the pituitary may serve to accommodate the perinatal rise in circulating thyroid hormones in rodents.

6.2 Introduction

It is generally accepted that thyroid hormones play a crucial role in controlling growth, development, differentiation and metabolism of virtually all tissues of vertebrates. In particular, studies in amphibians have shown that thyroid hormones are involved in metamorphosis and remodeling certain tissues and organs [for review see (Tata, 1993)]. The effects of thyroid hormones on gene expression are mediated via (nuclear) thyroid-hormone receptors (TRs), which are members of the steroid/thyroid-hormone receptor superfamily of

ligand-dependent transcription factors (Tsai and O'Malley, 1994; Zhang and Lazar, 2000). There are two thyroid hormone-receptor genes, TRα and TRβ, which are located in humans on chromosomes 17 and 3, respectively (Dayton et al., 1984; Weinberger et al., 1986). Due to alternative splicing, three different thyroid hormone-binding receptors are generated, namely $TR\alpha_1$, $TR\beta_1$ and $TR\beta_2$, of which the latter is thought to have the most limited distribution. In addition, three proteins that do not bind thyroid hormone are formed: $TR\alpha_2$ and $TR\Delta\alpha_1$ and $TR\Delta\alpha_2$. These proteins are thought to function as antagonists of thyroid hormone-mediated gene expression (Koenig et al., 1989; Lazar et al., 1989a; Lazar et al., 1989b; Weinberger et al., 1986). In the liganded condition, TRs bind to DNA as homodimers, or as heterodimers in combination with another member of the superfamily, preferentially the 9-*cis* retinoic-acid receptor (RXR) (Wahlstrom et al., 1992; Yen et al., 1994; Yen et al., 1992). Binding to thyroid–hormone-response elements (TREs) in regulatory DNA regions leads to activation or inhibition of gene transcription. The validity of this model of thyroid hormone-mediated gene transcription has been confirmed both at the structural and the functional level (Brent, 1994; Rastinejad et al., 1995).

Despite the detailed insights into the molecular action of thyroid hormones, such elaborate insight is not yet available for the developmental effects of these hormones. A prototypical example of the developmental significance of thyroid hormones, the induction of amphibian metamorphosis, is known for 90 years now (Gudernatsch, 1912; Etkin, 1968). The beneficial effects of thyroid hormones on perinatal development and maturation, especially true for brain, have also been established (DeLong, 1989; Legrand, 1986; van Wassenaer et al., 1997). In addition, it is now firmly established that thyroid hormones can pass the placenta of rodents and do accumulate in embryonic cells to a functionally relevant concentration well before the embryonic thyroid starts to function on its own (Feldman et al., 1961; Morreale de Escobar et al., 1990; Obregon et al., 1984; Porterfield and Hendrich, 1992; Vulsma et al., 1989). However, a specific hypothesis for the mechanism of action of thyroid hormones on prenatal organ development is not yet available.

In chickens, the TRα gene is ubiquitously expressed, that is, in brain, lung, kidney, heart, liver, eye, intestine, spleen and muscle tissue, from early developmental stages onward (Forrest et al., 1990). In rat, the TR mRNA was demonstrated by Northern blot analysis to be expressed in liver, brain, kidney, heart and spleen tissue from 18 days of development onward (Strait et al., 1990). In chicken, expression of the TRβ gene is more restricted and confined to brain, eye, lung and kidney, whereas in developing mammals, TRβ mRNA is found in the same organs that also express TRα. However, TRβ expression starts late in

embryonic development and increases to highest levels just after birth, thereby marking the onset of the so-called hormone-sensitive period, suggesting that TRβ equates with perinatal hormonal responsiveness (Forrest et al., 1990). Bradley et al. (Bradley et al., 1992) reported a specific spatial and temporal expression of the TRα and TRβ mRNAs in the developing rat nervous system. The TRα gene is widely expressed, but highest levels were found in the fetal neocortical plate, that is, the site of cortical neuronal differentiation. Expression of the TRβ gene is restricted to zones of neuroblast proliferation such as the germinal trigone and the cortical ventricular layer.

In order to obtain a better insight into the role of thyroid hormones on organogenesis, the spatial and temporal distribution of the mRNAs and proteins encoding the TRs was investigated by in situ hybridization and immunohistochemistry. Since our in situ probes did not differentiate between the two isoforms, quantitative polymerase chain reaction (PCR) techniques were used to determine the tissue levels of $TR\alpha_1$ and $TR\alpha_2$. Similarly, tissue levels of $TR\beta_1$ and $TR\beta_2$ mRNA were determined, even though it is assumed that in the adult, $TR\beta_2$ is only expressed in the pituitary gland (Weinberger et al., 1986). Since there are good indications that there may not be a simple direct relationship between TR mRNA level and nuclear thyroid hormone-binding capacity (i.e. functional TR protein) in some tissues (Strait et al., 1990), we also investigated tissue TR protein level by Western-blot analysis and immunohistochemistry. We report a highly specific, but heterogeneous spatial and temporal expression pattern of the TRα and TRβ mRNAs, as well as extensive posttranscriptional regulation in the developing rodent fetus and neonate.

6.3 Materials and methods

Animals

Adult Wistar rats were obtained from the Broekman Institute B.V. in Someren, The Netherlands. The animals were kept in a controlled light/dark cycle and food and water were supplied ad libitum. Females in heat were selected and mated at the end of the morning. The next day was designated day 1 of gestation or embryonic day 1 (ED1). Fetuses were delivered by Caesarian section at ED13, 14, 15, 16, 17, 18, 19 and 20, or were born naturally and sacrificed 1 day after birth (ND1), ND4, or as an adult.

Tissue isolation and preparation

ED13-17 fetuses were fixed in toto. Older fetuses were decapitated, their skin was removed and their body cavities were opened for optimal penetration of the fixative. From ED20 onward, the head and lower part of the body were removed. From all newborns, organs were isolated and fixed separately. All tissues were fixed at 4°C for 16-18 hours in phosphate-buffered 4% formaldehyde for in situ hybridization and in methanol:acetone:water [2:2:1 (v/v)] for immunohistochemistry, dehydrated in a graded series of ethanol, cleared with isobutanol and embedded in Paraplast Plus (Monoject, Kildare, Ireland). 7 μm serial frontal sections were cut and mounted onto RNAse-free 3-aminopropyltriethoxysilane- (Sigma A3648) coated slides.

In situ hybridization

As probes for the TRα and TRβ mRNA detection, EcoRI-HindIII cDNA fragments of the clones rc-erbA-α (nucleotide -29 to nucleotide +1821) and rc-erbA-β (nucleotide -37 to nucleotide +2165) were used, respectively. The fragments contain the coding region of the respective genes and recognize both the $TR\alpha_1$ and $TR\alpha_2$ and $TR\beta_1$ and $TR\beta_2$ isoforms. Both cDNA fragments were obtained from H.C. Towle (Bradley et al., 1992). The [α-^{35}S]UTP labeled antisense probes for both the TRα and TRβ were generated with Sp6 RNA polymerase, after linearization of the plasmids with HindIII, while the sense probes were generated with T7 RNA polymerase after linearization with EcoRI. All plasmids were linearized by enzyme digestion using enzymes of Gibco/BRL (Life Technologies B.V., The Netherlands). Radioactively labeled RNA transcripts were obtained using either T3, T7 (Gibco/BRL) or SP6 (HT Biotechnology Ltd., Cambridge, England) RNA polymerase in the presence of 50 μCi [α-^{35}S]UTP (Amersham, United Kingdom). After degradation of the full-length transcripts by alkaline hydrolysis, the fragments were purified by phenol-chloroform extraction and ethanol precipitation. In situ hybridization was carried out exactly as described (Moorman et al., 2000).

Quantitative PCR

At ED15, ED18, ND1, and at adult age, mRNA concentrations of $TR\alpha_1$, $TR\alpha_2$, $TR\beta_1$ and $TR\beta_2$ were determined in brain, lung, liver, kidney, and intestines, using quantitative PCR. For each stage, minimally 3 rats were assayed. PolyA$^+$ RNA was isolated from 50-250 mg tissue with the PolyATtract® System 100 (Promega Corporation, Madison, WI, USA).

Absorbance at 260nm was used as a measure for the amount of polyA$^+$ RNA and the ratio between the absorbance at 260nm and 280nm as a measure for purity.

TRα mRNA was estimated by competitive RT-PCR. The sense primer was chosen in the common part of the TRα_1 and TRα_2 mRNA sequence, whereas the antisense primers were located in the specific part of the sequences. For TRα_1, a 343bp fragment was formed (nt 1219-1562 of the mRNA) and for TRα_2 a 242bp fragment (nt 1411-1653) (primers: Gibco/BRL). Messenger RNA (0.5 μg) was reverse transcribed with 100 units Superscript^{TM} II (Gibco/BRL) for 60 min at 42°C in the presence of 0.5 μg oligo $(dT)_{12\text{-}18}$ primer (Gibco/BRL) and 25 μg BSA in 75 mM Tris/HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 1.25 mM $MgCl_2$, 0.01% Tween 20, 1 mM DTT and 100 μM dNTPs. As a control for DNA contamination, the reaction was also carried out without the addition of Superscript II. One tenth of the RT-reaction was used for the PCR. After an initial denaturation of 2 min at 94 °C, the cDNA was amplified with 0.25 units *Taq* polymerase (Goldstar; Eurogentec, Seraing, Belgium), 0.8 μM primers, 3 mM $MgCl_2$ (buffer and dNTP concentration are the same as for the RT-reaction) for a total of 35 cycles according to a 'touch-down' protocol in which the mixture was denatured for 30 s at 94 °C at the beginning of each cycle, annealed for 30 s at 62 °C (this temperature was lowered 0.5 °C each cycle during the initial 20 cycles), and extended for 1 min at 68 °C. The concentration of TRα_1 mRNA in 50 ng total RNA was estimated by titrating 8-5,000.10^{-4} amol of the TRα_1 PCR fragment without a 82 bp Hinc II fragment. The concentration of TRα_2 in 50 ng total RNA was estimated by titrating 4-2,500.10^{-3} amol of the TRα_2 PCR fragment missing the same 82 bp Hinc II fragment as TRα1. The competimer or mimic fragments were quantified by including a known amount of [^{35}S]dATPαS during the large-scale PCR amplification of these fragments. Fragments were separated on 1.5% pronarose MS-8 (Hispanagar, Burgos, Spain) in 0.5x TBE buffer (0.09 M Tris, 0.89 M H_3BO_3, 0.02 M EDTA, pH 8.0) and quantified with the Eagle Eye II system (Stratagene, La Jolla, CA, USA). TRα mRNA concentration in the sample was calculated by plotting the logarithm of the TRα/competimer ratio as a function of the logarithm of the competimer concentration (Möller et al, 1997).

TRβ mRNA levels were measured by real-time RT-PCR. The antisense primer was chosen in the common part of the TRβ_1 and TRβ_2 sequences, whereas the sense primers were in the specific part. For TRβ_1, a 185bp fragment was formed (nt 437-622 of the mRNA) and for TRβ_2, a 244bp fragment (nt 343-587 of the mRNA). For the RT-reaction

the same protocol was used as for the TRαs. Real-time PCR was performed using one twentieth of the RT-reaction (25 ng mRNA) for both the $TR\beta_1$ and the $TR\beta_2$ with the LightCycler™ DNA Master SYBR Green I kit in the LightCycler™ (Roche Molecular Biochemicals, Mannheim, Germany), as prescribed by the manufacturer. The final concentrations in the reaction mixture were 1x DNA Master SYBR Green I, 3 mM $MgCl_2$, 0.5 μM primers and 10 pg/μl tRNA in a final reaction volume of 20 μl. After an initial denaturation step at 95 °C for 30 s, amplification was performed using 50 cycles of denaturation (95 °C for 0 s), annealing (52 °C for 5 s) and extension (72 °C for 10 s) and a transition rate of 20 °C/s for all steps. Fluorescence (530 nm) was measured at the end of each extension step to monitor amplification. After amplification was complete, a melting curve analysis was done. For the measurement of the concentration of TRβ mRNA 5-50,000.10^{-4} amol of a DNA fragment was used to generate a standard curve. The standard curve is created by plotting the cycle numbers at which the fluorescent signals enter the log phase versus the log of the concentrations of the standard. The mimic fragments were made by PCR with the $TR\beta_1$ and $TR\beta_2$-specific sense primers and an antisense megaprimer internally missing 33bp of the mRNA sequence, and were quantified by including a known amount of [α-^{32}P]dATP during the large-scale PCR amplification of the fragment. $TR\beta_2$ mRNA levels are also measured with the LightCycler™ DNA Master Hybridization Probes (Roche Molecular Biochemicals). Amplification primers and hybridization probes were designed and synthesized by Tip MolBiol (Berlin, Germany). With the amplification primers a 316bp fragment was formed (nt 241-556 of the $TR\beta_2$ mRNA). The LightCycler Red640-labeled probe was located at nt 264-290 and the fluorescein-labeled probe at nt 292-316. The final concentrations in the reaction mixture were 1x DNA Master Hybridization Probes, 4 mM $MgCl_2$, 0.25 μM amplification primers and 0.2 μM hybridization probes in a final reaction volume of 20 μl. After an initial denaturation at 95 °C for 30 s, amplification was performed using 45 cycles of denaturation (95 °C for 0 s), annealing (55 °C for 10 s) and extension (72 °C for 13 s). Fluorescence (530 and 640 nm) was measured after each annealing step. For the measurement of the $TR\beta_2$ mRNA concentration, dilutions of pituitary gland mRNA with a known $TR\beta_2$ mRNA concentration were used to generate a standard curve.

The efficiency of the reverse-transcription step was measured using the TRβ mimic DNAs. Large-scale mRNA synthesis was performed with the MEGAshortscript™ T7 Kit (Ambion, Austin, Texas, USA) and a known amount of [α-^{35}S]CTP. In addition, different

amounts of the $TR\beta_1$ and $TR\beta_2$ RNA transcripts were used. The transcription efficiency was calculated by plotting the mRNA input concentration against the DNA output concentration and amounted to approximately 80% for the $TR\beta_1$ and 100% for the $TR\beta_2$ mRNA.

Western blotting and immunohistochemistry

At ED17, ND4 and in the adult, extracts of brain, liver, trachea, lung, heart, kidneys, adrenals, intestines, spleen, muscle, pituitary gland, bladder, thyroid, epididymal fat pad and testis were analyzed by Western blotting, using antisera that discriminate between the TRα isoforms $TR\alpha_1$ and $TR\alpha_2$ and the TRβ isoforms $TR\beta_1$ and $TR\beta_2$. These antisera were raised in rabbits against oligopeptides, coupled to thyroglobulin or keyhole-limpet hemocyanin. The antibodies recognized proteins of 47 kDa ($TR\alpha_1$), 55 kDa ($TR\alpha_2$), 57 kDa ($TR\beta_1$), and 47 kDa ($TR\beta_2$). The extracts were prepared in 0.25 M sucrose, to which DTT, SDS, EDTA, TrisHCl, and glycerol were added to 40 mM, 2%, 1 mM, 62.5 mM, and 10%, respectively. The proteins in the extracts were separated on 10% (w/v) polyacrylamide gels in the presence of SDS and subsequently blotted on a 0.45 μm polyvinylidene fluoride membrane (Immobilon-P, Millipore Corporation, Bedford, MA) in 25 mM ethanolamine/glycine buffer (pH 9.5), 20% methanol. After staining with amidoblack to assure similar protein loading, the membrane was stained for the presence of TR protein by incubation for 6 hrs in 10 mM Tris.HCl (pH 8.0), 150 mM NaCl, 0.5% Tween20, and 5% non-fat dried milk powder (Natrinon, Nutricia, Zoetermeer, The Netherlands), followed by an overnight incubation in a 1:1,000 dilution of the rabbit antisera. Antibody binding was quantified using a chemiluminescent substrate based on dioxetane/alkaline phosphatase (AP) (CDP-*Star*™ Roche), in combination with a LUMI-imager™ F1 (Roche) (Bakker, 1998).

Immunoperoxidase staining was performed on dewaxed tissue sections. Antibody binding on sections was visualized by the indirect unlabelled antibody peroxidase anti-peroxidase (PAP) method (Sternberger et al., 1970) with 0.05% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (Sigma) as chromogen and 0.01% (v/v) H_2O_2 as peroxidase substrate. Color was developed for 5-7 minutes in 25 mM imidazole, 1 mM EDTA, pH 7.0. PAP immunocomplexes were purchased from Nordic (Nordic, Tilburg, The Netherlands).

Image recording

Digital optical density images were recorded using a Photometrics (Tucson, AZ, USA) cooled CCD camera attached to an Axioplan microscope (Carl Zeiss, Oberkochen, Germany), a stabilized power supply and an infrared blocking filter. mRNA distribution patterns were recorded using white light, whereas protein distribution patterns were recorded using blue light. The obtained images were corrected for inhomogeneity of illumination.

6.4 Results

Specificity of the antisense RNA probes and antisera

The specificity of the hybridization-signal of the TRα and TRβ antisense RNA probes was shown by the absence of a hybridization signal with TRα and TRβ sense RNA probes in sections of an ED18 fetus (results not shown). Furthermore, the respective radioactively labeled antisense RNA probes produced highly characteristic and often mutually complementary hybridization signals, which constitute important tissue-intrinsic specificity.

Western-blot analysis of extracts of organs at different gestational ages was carried out to test the specificity of the respective rabbit antisera. For all antibodies, specific bands were detected at the anticipated M_r of the corresponding receptors, with the exception of the antiserum against the $TR\beta_2$ protein, where a band of 47 kD instead of 58 kD was detected. However, inspection of the $TR\beta_2$ cDNA sequence (Hodin et al., 1989) indicated that the 117 aa downstream ATG codon that produces the 47 kD protein, is in a much more favourable Kozak context than the one giving rise to the 58 kD form. In liver and kidney extracts, extra bands at both higher and lower M_r than found in other organs were observed for $TR\alpha_1$ and $TR\beta_1$. These patterns were also found if only these organs were isolated and analyzed immediately. The lower bands were probably due to protein degradation. It is unlikely that the higher bands represent aggregates, as the protein extracts were denatured in the presence of 40 mM DTT.

Distribution of the TRα and TRβ

Although the focus of this study is on non-neural tissue, we include a brief description of some prominent structures in the central nervous system to make this study comparable to earlier ones, which have focused on that organ (Bradley et al., 1992). For the same reason, we include a brief description of our findings in the pituitary gland.

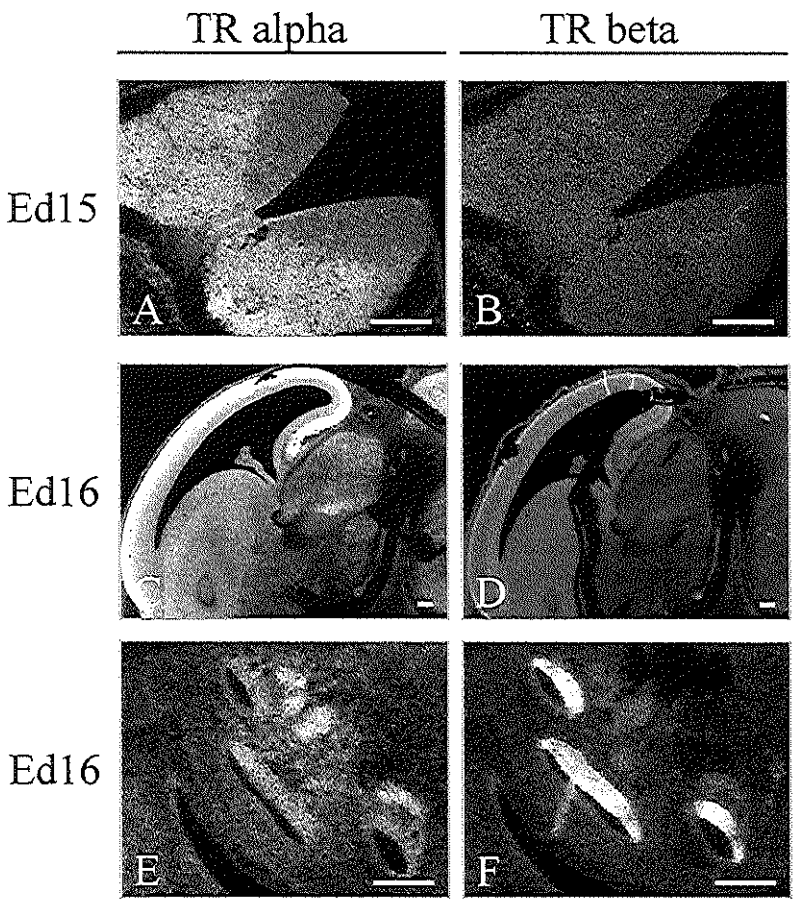


Figure 1
Thyroid-hormone-receptor-mRNA expression in fetal central nervous system. TRα mRNA was observed in the basal plate of the ED15 neural tube (A), whereas no TRβ mRNA was present at this stage (B). In the ED16 brain, high TRα expression was observed in the cortical plate (C). At this stage TRβ expression was also present in the cortical plate, albeit at a much lower cellular concentration (D). In contrast, a very intense TRβ signal was observed in the cochlear sensory epithelium (F), whereas TRα could only weakly be detected in the cochlear nerve (E). (Scalebar represents magnification level)

Central nervous system

In the early fetus (ED15), a high TRα mRNA expression was observed in the basal plate of the neural tube (Fig. 1A), whereas TRβ mRNA was not yet expressed (Fig. 1B). Similarly, a high TRα expression in the cortical plate of the brain was observed at ED16. TRβ mRNA expression in the telencephalon coincided with that of TRα, but its cellular concentration was much lower (Fig. 1C,D). In accordance with earlier observations (Bradley et al., 1992), quantitative PCR demonstrated that the $TR\alpha_2$ isoform concentration exceeded that of $TR\alpha_1$ more than 20-fold and, hence, is responsible for the observed TRα expression pattern (Fig. 2). The expression of both isoforms was highest at ND1 (Fig. 2). $TR\beta_1$ expression followed a similar temporal pattern as $TR\alpha_2$, except that the observed mRNA levels were approximately twenty times lower (Fig. 2). $TR\beta_2$ mRNA levels in whole brain extracts were below the limit of detection (results not shown). Whereas co-expression of TRα and TRβ was observed at many sites in central nervous tissue, this was not the case in the sensory epithelium of the inner ear: at ED16, we observed a strong and very specific expression of TRβ mRNA in the cochlear sensory epithelium and only a very weak signal in the cochlear nerve, whereas the staining pattern of TRα mRNA was just opposite (Fig. 1E,F).

Both before and shortly after birth, the $TR\alpha_1$ and $TR\alpha_2$ protein concentration in the brain was very low compared to that in the other organs investigated (Fig. 3A,B). In contrast, $TR\beta_1$ protein was already present in fetal brain (ED17) and increased to a higher level at four days after birth (Fig. 3C). Similarly, the $TR\beta_2$ protein was already present at a reasonable level around the ventricles before birth (Fig. 3D, 4D) and at a higher level shortly after birth (Fig. 3D), when it accumulates mainly in the choroid plexus (Fig. 4H).

Comparing mRNA and protein levels, we observed a very low protein/mRNA ratio for $TR\alpha_1$ and, in particular $TR\alpha_2$, whereas this ratio was much higher for $TR\beta_1$ and, in particular, for $TR\beta_2$.

Pituitary gland

Protein for all four receptors was observed in extracts of adult pituitary glands (Fig. 3A-D). The TRβ isoforms were more highly expressed than the TRα isoforms. Interestingly, $TR\beta_1$ protein was present in the ED18 pituitary, but $TR\beta_2$ protein not yet (Fig. 5C,D). In ND4 pituitaries, $TR\beta_1$ and $TR\beta_2$ protein were both present (Fig. 5 E,F), whereas the proteins of both TRα isoform could not be detected (Fig. 5G,H).

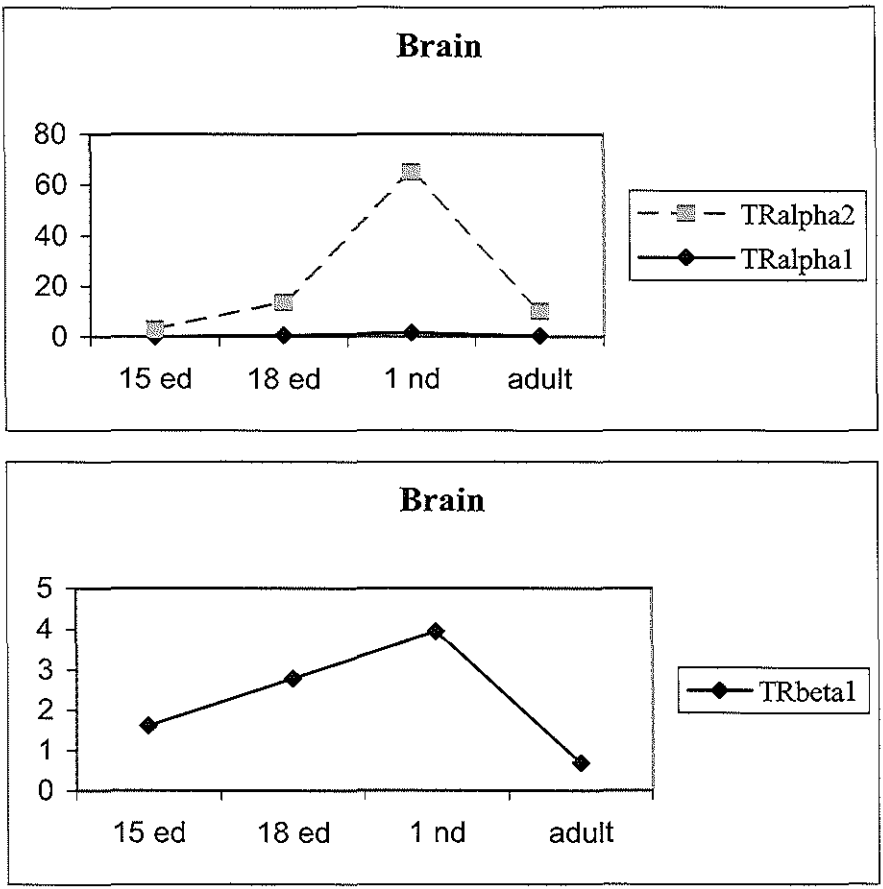


Figure 2
Thyroid-hormone-receptor-mRNA levels during brain development. $TR\alpha_1$ and $TR\alpha_2$ and $TR\beta_1$ mRNA levels during brain development of the rat. $TR\alpha_2$ levels exceed $TR\alpha_1$ levels more than 20-fold. All three isoforms reach maximum levels at ND1.

Lung

The lung primordium, an outgrowth of foregut endoderm that appears at ED10-11 in the rat, develops by dichotomous branching. The endodermally derived pulmonary epithelium is initially surrounded by a conspicuous mass of mesenchyme. Expression of TRα mRNA was observed in the mesenchyme surrounding the developing lung buds from ED13 onwards (ED15 shown in Fig. 6A), whereas only a faint expression of the TRβ mRNA was detected in the pulmonary epithelium up to ED15 (Fig. 6B). During the canalicular stage of pulmonary development (ED16-18 in the rat), the expression of TRβ mRNA became increasingly strong in the epithelium (Fig. 6D), whereas expression of TRα mRNA remained confined to the mesenchyme (Fig. 6C). During the alveolar stage of lung development (>ED19), the prominence of the interstitial tissue decreases. In this period, TRβ mRNA disappeared from the pulmonary epithelium (Fig. 6F), but TRα mRNA remained present in the pulmonary mesenchyme (Fig. 6E). Because of attenuation of the walls of the airways due to expansion of the gas-exchange surface, the complementary distribution was more difficult to discern. TRβ mRNA was also present in pulmonary arteries at all time points (Fig. 6D), whereas expression of the TRα mRNA was continuously observed in the myocardium of the pulmonary veins (Fig. 6C). Quantitative PCR demonstrated that $TR\alpha_2$ mRNA levels were approx. 10-fold higher than $TR\alpha_1$ mRNA levels (Fig. 7). $TR\alpha_2$ mRNA levels increased from ED15 to peak at ED18 and ND1 (Fig. 7). These data indicate that the TRα mRNA observed in the in situ hybridization experiments actually represented $TR\alpha_2$ mRNA. The TRβ signal in the in situ hybridization experiments represented $TR\beta_1$, because no $TR\beta_2$ mRNA could be detected upon PCR amplification (results not shown). $TR\beta_1$ mRNA levels were approximately 3-fold lower than $TR\alpha_2$ mRNA levels and peaked at ED18 (Fig. 7).

Western blot analysis revealed a band of the expected size of $TR\alpha_1$ in extracts at ED17, which had markedly increased in intensity at ND4 (Fig. 3A). At the same time points, the intensity of the $TR\alpha_2$-protein band was relatively weak and even appeared to decrease with development (Fig. 3B). $TR\beta_1$ protein levels were similar at ED17 and ND4 (Fig. 3C). $TR\beta_2$ protein levels were very modest both pre- and postnatally (Fig. 3D). Despite the positive Western blots, we were not able to demonstrate $TR\alpha_1$ or $TR\alpha_2$ protein by immunohistochemistry, indicating that the proteins were present at low levels in many cells (Fig. 8A,B,E,F). $TR\beta_1$ protein was predominantly and $TR\beta_2$ protein was exclusively expressed in lung epithelium (Fig. 8C,D,G,H).

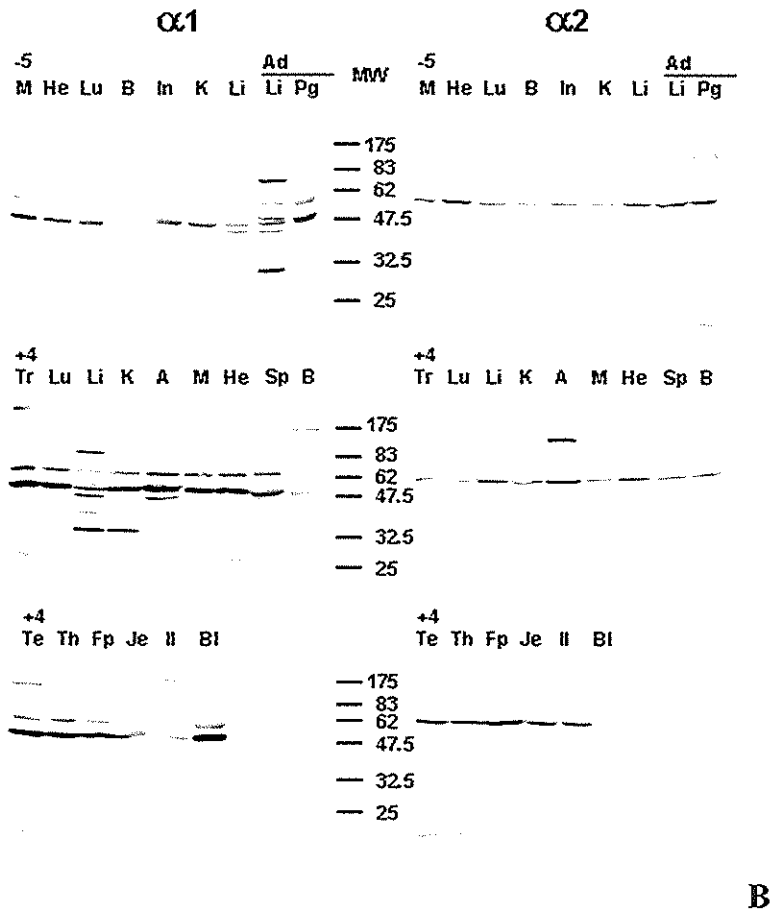


Figure 3

Thyroid-hormone-receptor-protein levels in various organs during development. Western blotting experiments for all four TR isoforms in organs of ED17 (muscle, heart, lung, brain, intestines, kidneys, liver), ND4 (trachea, lung, liver, kidneys, adrenals, muscle, heart, spleen, brain, testis, thyroid, fat pad, jejunum, ileum and bladder) and adult animals (liver, pituitary gland). See text for details.

M= muscle; He=heart; Lu=lung; B=brain; In=intestine; K=kidneys; Li=liver; Pg=pituitary gland; Tr=trachea; A=adrenal gland; Sp=spleen; Te=testis; Th=thyroid gland; Fp=fat pad; Je=jejunum; Il=ileum; Bl=bladder

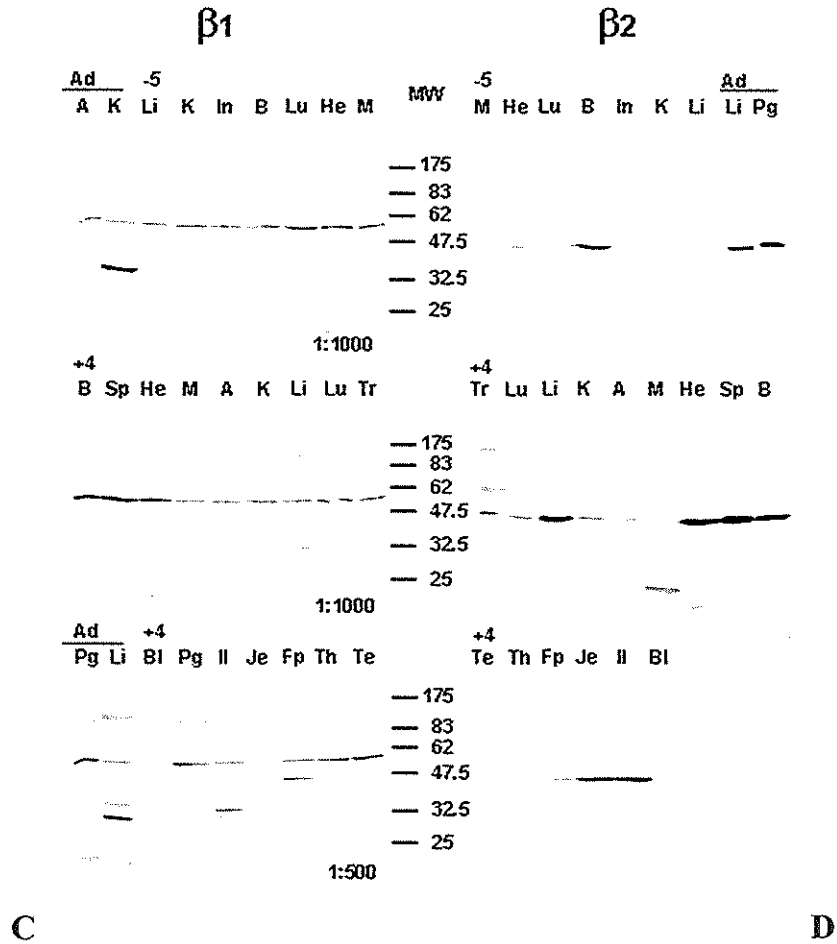


Figure 3 continued

Thyroid-hormone-receptor-protein levels in various organs during development. Western blotting experiments for all four TR isoforms in organs of ED17 (muscle, heart, lung, brain, intestines, kidneys, liver), ND4 (trachea, lung, liver, kidneys, adrenals, muscle, heart, spleen, brain, testis, thyroid, fat pad, jejunum, ileum and bladder) and adult animals (liver, pituitary gland). See text for details.

M= muscle; He=heart; Lu=lung; B=brain; In=intestine; K=kidneys; Li=liver; Pg=pituitary gland; Tr=trachea; A=adrenal gland; Sp=spleen; Te=testis; Th=thyroid gland; Fp=fat pad; Je=jejunum; Il=ileum; Bl=bladder

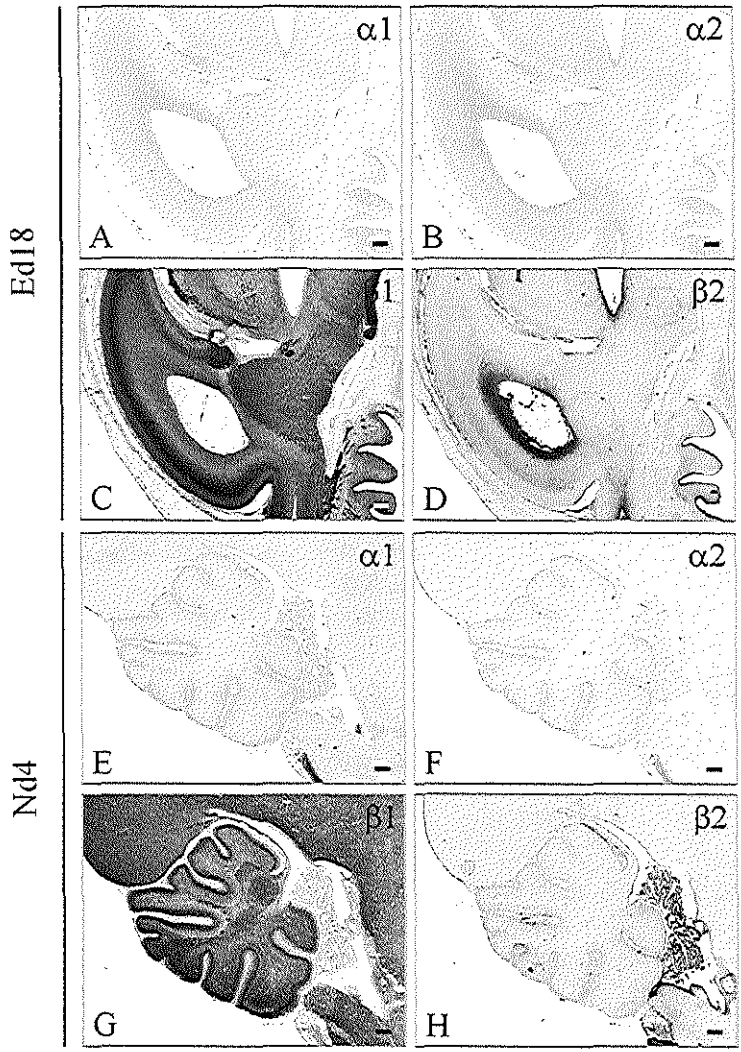


Figure 4
Thyroid-hormone-receptor-protein distribution during brain development. At ED18 and ND4, protein expression of both TRα isoforms is very low (A,B,E,F). In contrast, $TR\beta_1$ protein is highly expressed in various structures of the brain (C,G), whereas $TR\beta_2$ protein is confined to the choroid plexus (D,H). (Scalebar represents magnification level)

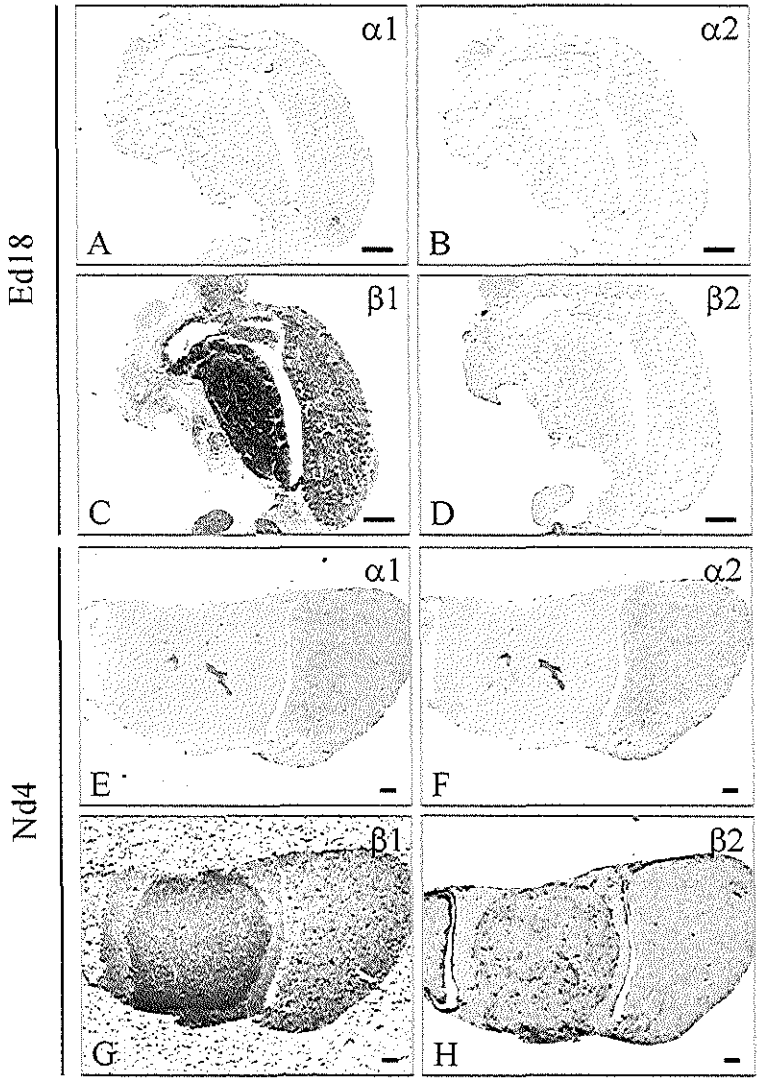


Figure 5
Thyroid-hormone-receptor-protein distribution during pituitary gland development. $TR\beta_1$ is the only isoform of which protein is expressed at ED18 (C), whereas the other isoforms do not express any protein at this stage (A,B,D). At ND4, both TRα isoforms do not express any protein (E,F), whereas both $TR\beta_1$ and $TR\beta_2$ protein are observed at this stage in the pituitary gland. (Scalebar represents magnification level)

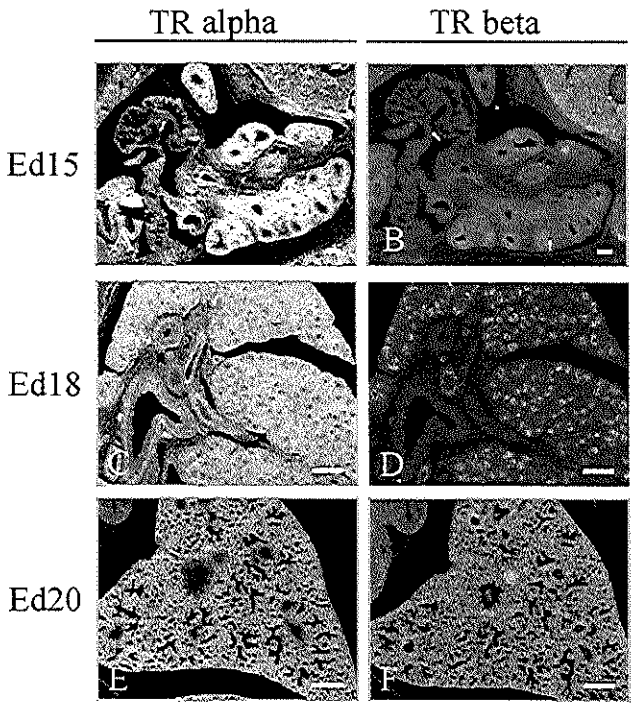


Figure 6
Thyroid-hormone-receptor-mRNA expression during fetal lung development. TRα mRNA is observed in the mesenchyme of the developing lung from ED15 onwards (A,C,E). TRβ mRNA is only faintly expressed in the pulmonary epithelium at ED15 (B), but the expression is more pronounced at ED18 (D). Expression of TRβ mRNA in the epithelium decreases near term (F). (Scalebar represents magnification level)

The protein/mRNA ratio increased for $TR\alpha_1$, and remained more or less constant for $TR\alpha_2$ and $TR\beta_1$ in development. Since $TR\beta_2$ mRNA concentration was undetectably low, this ratio could not be determined.

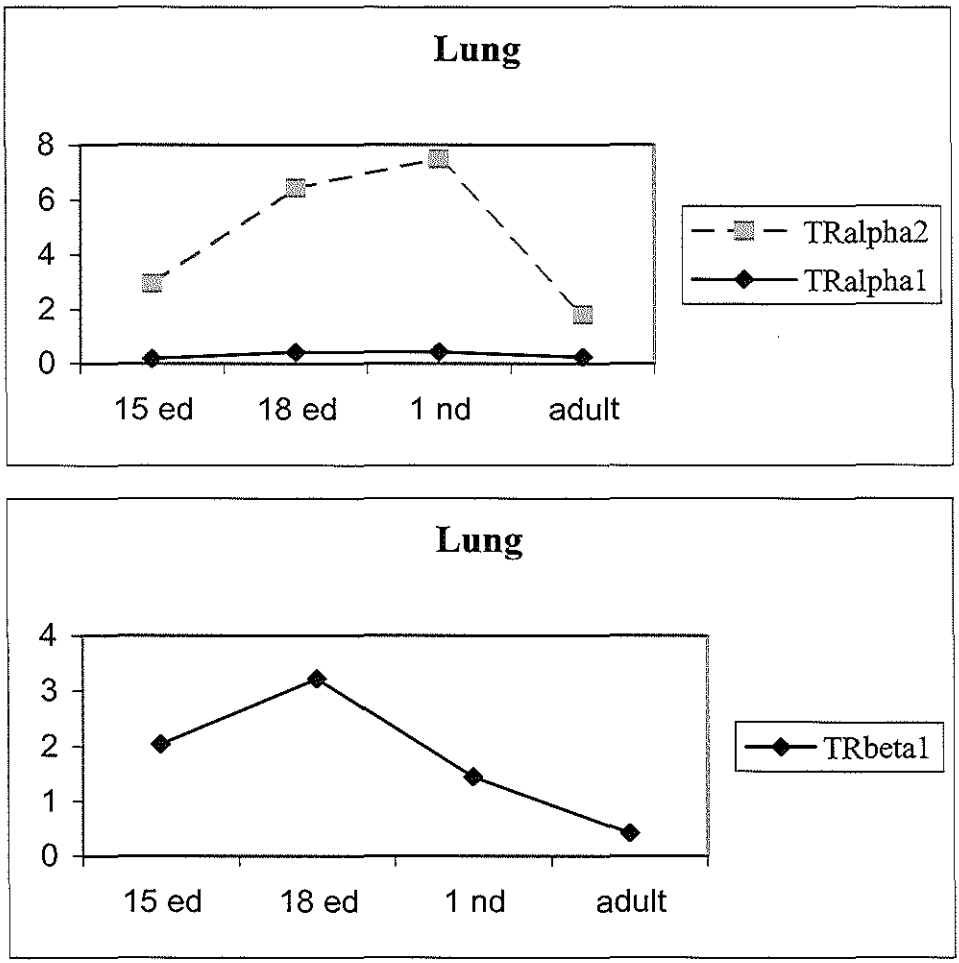


Figure 7
Thyroid-hormone-receptor-mRNA levels during pulmonary development. $TR\alpha_2$ mRNA levels are approximately 10-fold higher than $TR\alpha_1$ mRNA levels. $TR\alpha_2$ mRNA levels peak at ED18 and ND1, whereas $TR\beta_1$ mRNA levels peak at ED18 and are approximately 3-fold lower than $TR\alpha_2$ mRNA levels.

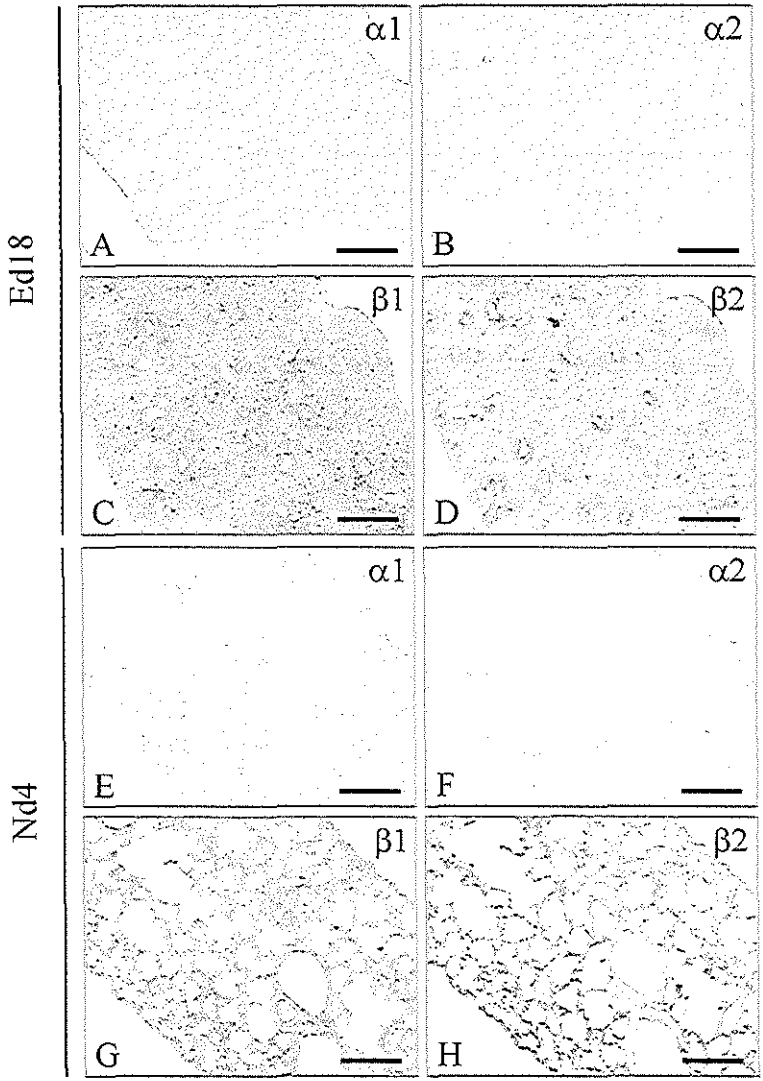


Figure 8
Thyroid-hormone-receptor-protein distribution during pulmonary development. Despite positive Western blots for both TRα isoforms, no protein is seen in the developing lung (A,B,E,F). In contrast, TRβ protein is easily detectable, with $TR\beta_1$ being predominantly and $TR\beta_2$ exclusively expressed in lung epithelium at ED18 (C,D), and ND4 (G,H). (Scalebar represents magnification level)

Liver

The liver, like the lungs, is a ventral outgrowth of the caudal foregut. Expression of TRα mRNA was first observed in the liver at ED15 (Fig. 9A). From 17 days of gestation onwards, this expression could be located in the stromal tissue of the liver, including the wall of the veins (Fig. 9C,E). Expression of TRβ mRNA was observed from ED15, declined during the next 2 days to undetectable levels, and reappeared in the parenchymal tissue only at ED19 (Fig. 9B,D,F). At ED20, the reciprocal expression of TRα in the stromal tissue and of TRβ in the parenchymal tissue became very striking (Fig. 9E,F). The identity of portal and central veins was verified by the expression pattern of carbamoylphosphate synthetase and glutamine synthetase in the surrounding hepatocytes, respectively (Christoffels et al., 1999)(results not shown). The concentration of $TR\alpha_1$ and, in particular, $TR\alpha_2$ mRNA, was very low in comparison to other tissues during all investigated stages (Fig. 10). As a result, the $TR\alpha_1/TR\alpha_2$ ratio was the highest observed in any of the tissues investigated and was more than 5 times as high as in the brain. $TR\beta_1$ mRNA levels were higher than those of $TR\alpha_2$ and peaked at ED18 (Fig. 10). $TR\beta_2$ mRNA levels were below the limit of detection (results not shown).

The $TR\alpha_1$ antibody detected a band at the expected size in extracts of liver, but in addition, bands at higher and lower *M*$_r$s. $TR\alpha_1$ levels increased between ED17 and ND4, when levels were comparable to those obtained in the adult (Fig. 3A). $TR\alpha_2$ protein did not change in development (Fig. 3B). $TR\beta_1$ levels were similar at 5 days before and 4 days after birth, but appeared to be higher in the adult, with a prominent band at 30 kD (Fig. 3C). No band was detected prenatally with the $TR\beta_2$ antibody, but very intense bands appeared in postnatal liver (Fig. 3D). Immunohistochemical staining revealed weak staining of $TR\alpha_1$ protein in parenchymal cells surrounding the central veins, but after birth only, whereas $TR\alpha_2$ protein had then become detectable in isolated liver cells in a wide region surrounding the portal veins (Fig. 11A,B,E,F). In fetal rat liver, $TR\beta_1$ protein was observed faintly in the parenchyma of the liver (Fig. 11C). After birth, expression had become much stronger, but only in hepatocytes surrounding the central veins (Fig. 11G). In contrast, $TR\beta_2$ protein was difficult to localize (Fig. 11D,H).

The protein/mRNA ratios for $TR\alpha_1$, $TR\alpha_2$ and $TR\beta_1$ did not change appreciably with development.

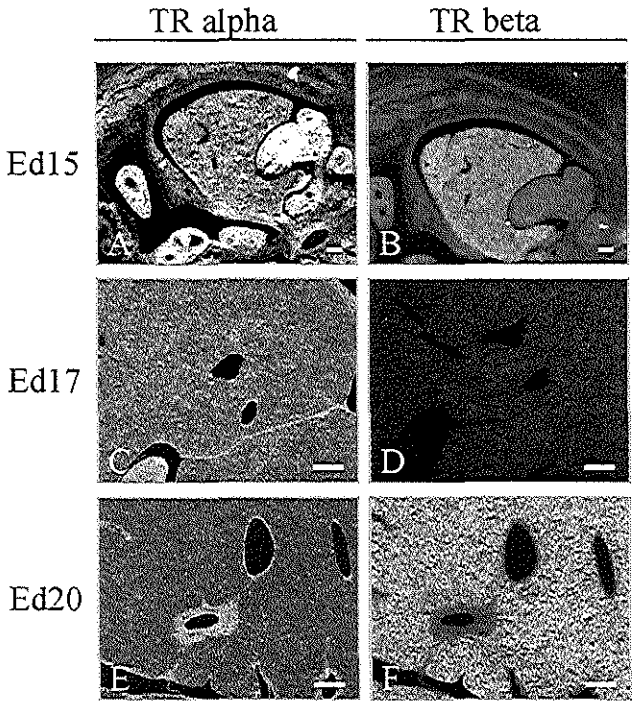


Figure 9
Thyroid-hormone-receptor-mRNA expression during fetal liver development. TRα mRNA is expressed in the liver at ED15 (A). At ED17 the TRα mRNA is located in the stromal tissue, including the walls of the veins (C). TRβ mRNA was observed from ED15 (B), has disappeared at ED17 (D), but reappears at ED19. At ED20, TRα mRNA is expressed in the stromal tissue (E), and TRβ mRNA in the parenchymal tissue (F). (Scalebar represents magnification level)

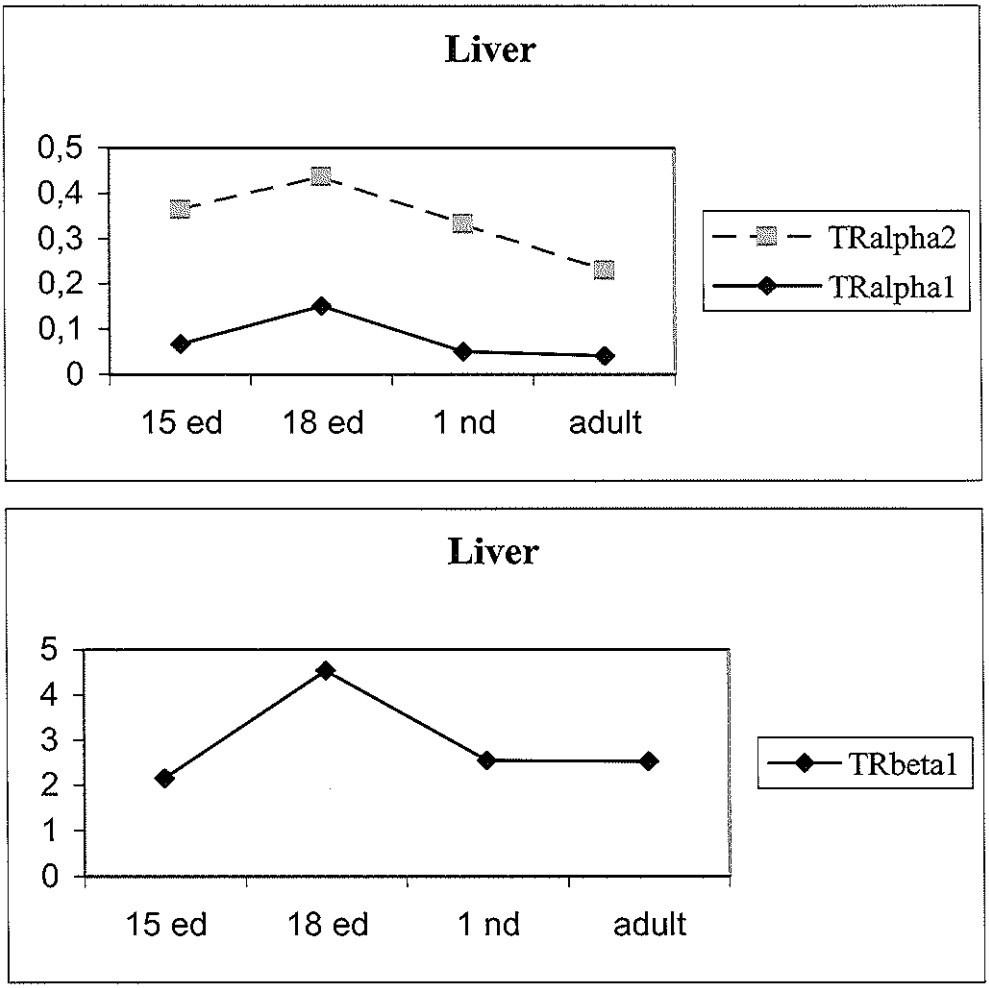


Figure 10
Thyroid-hormone-receptor-mRNA levels during liver development. Both $TR\alpha_1$ and $TR\alpha_2$ mRNA levels are very low during liver development, when compared to other tissues. In liver, $TR\beta_1$ mRNA levels are higher than those of $TR\alpha_2$ and peak at ED18.

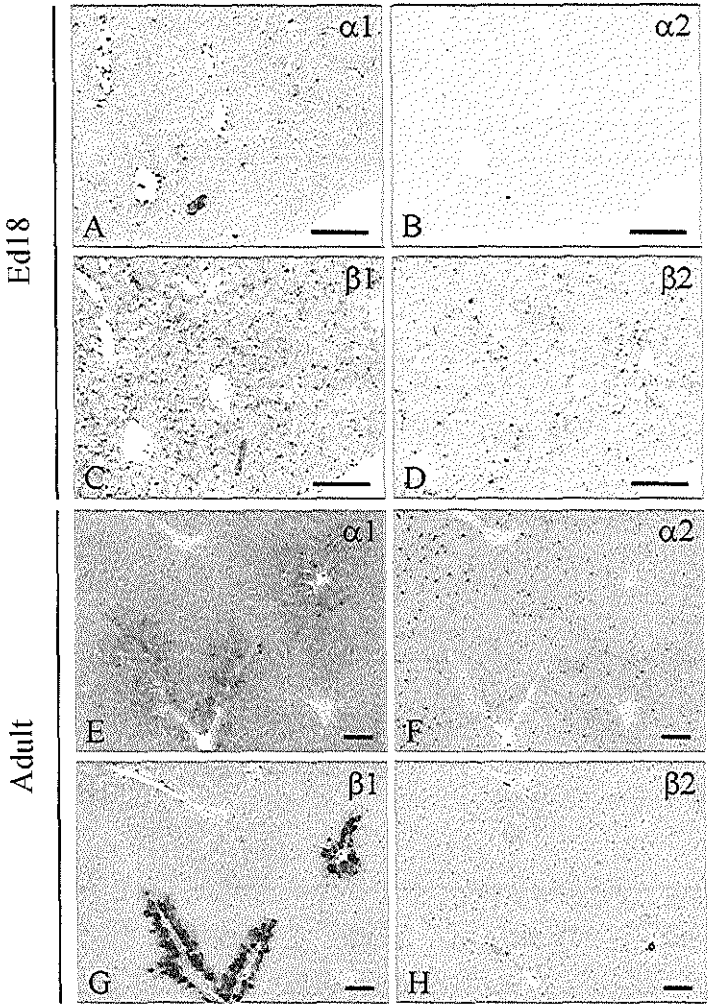


Figure 11
Thyroid-hormone-receptor-protein distribution during liver development. $TR\alpha_1$ protein can be detected only after birth in parenchymal cells surrounding central veins (E). At that stage $TR\alpha_2$ protein is observed in isolated liver cells in a wide region surrounding the portal veins (F). No $TR\alpha$ isoform protein is observed before birth (A,B). In contrast, $TR\beta_1$ protein is observed in the parenchyma of the liver before birth (C), but very strong protein expression is observed after birth in hepatocytes surrounding the central veins (G).$TR\beta_2$ protein is difficult to localize (D,H). (Scalebar represents magnification level)

Kidney

The definitive kidney (metanephros) starts to develop when the ureteric bud penetrates the metanephric mass. From ED16 onwards, metanephric tubules fuse with collecting tubules (originating from the ureteric bud) to form the nephrons. TRβ mRNA was found to be expressed in the developing nephrons from ED16 onwards (Fig. 12B,D,F), whereas TRα mRNA then became expressed in the ureteric bud and surrounding mesenchyme (Fig. 12A,C,E). PCR analysis showed that $TR\alpha_2$ mRNA levels were more than ten times higher than $TR\alpha_1$ levels (Fig. 13). Both $TR\alpha_1$ and $TR\alpha_2$ mRNA concentrations peaked perinatally and were lower in adulthood (Fig. 13). $TR\beta_1$ mRNA peaked just after birth with a level that was almost 3 times higher than that of $TR\alpha_2$ (Fig. 13). $TR\beta_2$ mRNA concentration was below the limit of detection of our assay.

$TR\alpha_1$ protein levels increased perinatally, with a band at approximately 30 kD that is also present in liver extracts, becoming more pronounced (Fig. 3A). In adult kidney, this band has become even stronger than the 47 kD species (Fig. 3A). At ED18, $TR\alpha_1$ protein was weakly present in the collecting tubules (Fig. 14A), but had disappeared at ND4 (Fig. 14E). $TR\alpha_2$ protein was not detectable in any cell type in the kidney (Fig. 14B,F). $TR\beta_1$ protein was detected in the glomeruli and the tubules at ED18, with particularly high levels in the collecting tubules (Fig. 14C). However, after birth, the glomeruli were no longer positive, but the tubules still stained homogeneously. $TR\beta_2$ protein expression was limited to the collecting tubules before birth (Fig. 14D), but present in all tubules after birth (Fig. 14H).

If the 30 kD species is a proper $TR\alpha_1$ product, the protein/mRNA ratio for $TR\alpha_1$ increases with development. The protein/mRNA ratio for $TR\alpha_2$ did not change appreciably in development. The protein/mRNA ratio for $TR\beta_1$ declined when comparing prenatal with postnatal stages, but in the adult, a 30 kD band that was also seen in liver, became very prominent. If this is a bona fide $TR\beta_1$ gene product, the ratio is substantially higher in the adult.

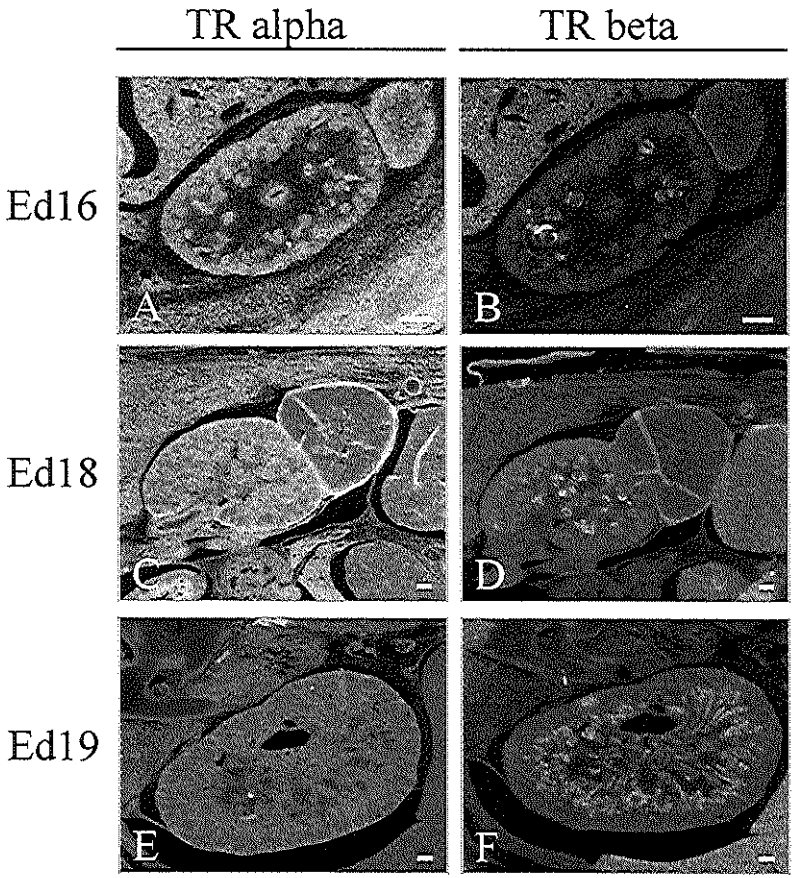


Figure 12
Thyroid-hormone-receptor-mRNA expression during fetal kidney development. TRβ mRNA is expressed in the developing nephrons from ED16 onwards (B,D,F), whereas TRα mRNA is expressed in the surrounding mesenchyme (A,C,E). TRα mRNA is weakly present in the entire adrenal at ED16 (A), but only in a few cells at ED18 (C). TRβ mRNA is only weakly seen in the capsule of the adrenals (B,D). (Scalebar represents magnification level)

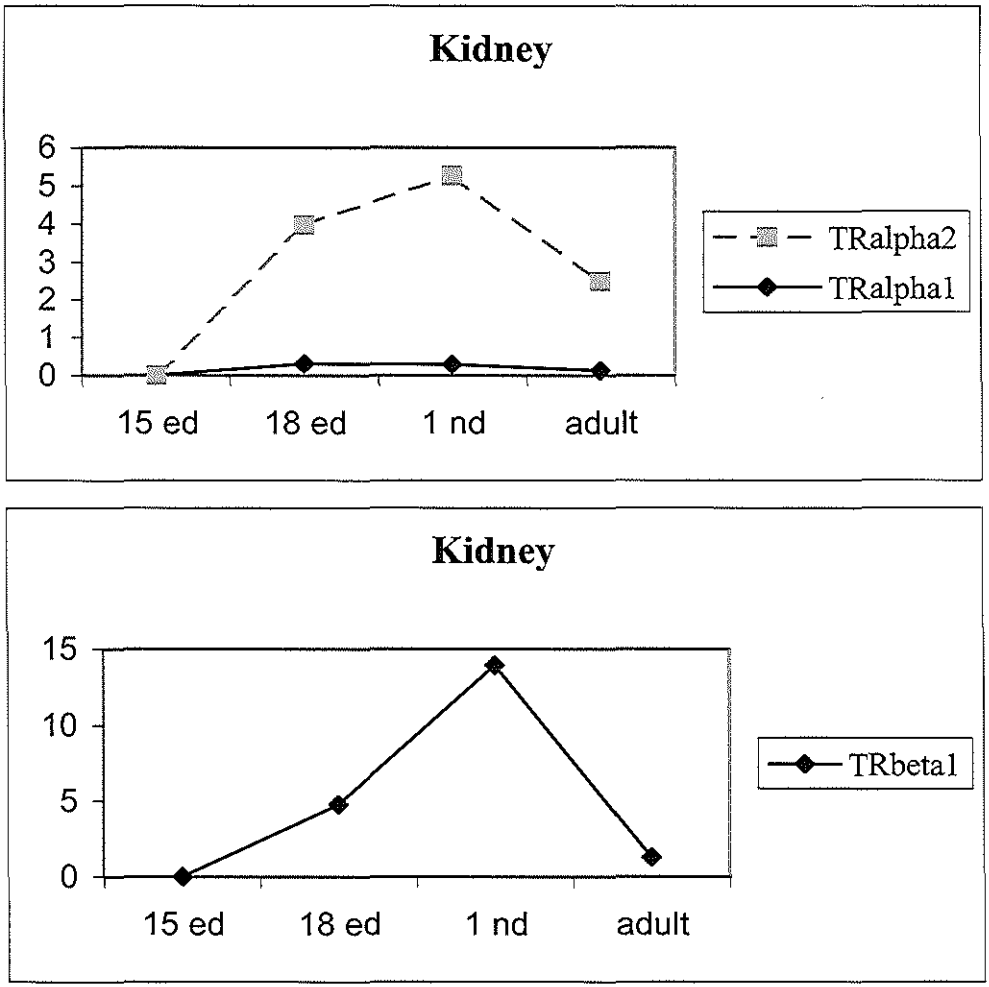


Figure 13
Thyroid-hormone-receptor-mRNA levels during kidney development. $TR\alpha_2$ mRNA levels are approximately 10-fold higher than $TR\alpha_1$ mRNA levels. mRNA levels of both isoforms peak perinatally. $TR\beta_1$ mRNA levels peak after birth, and are approximately 3 times higher than $TR\alpha_2$ mRNA levels.

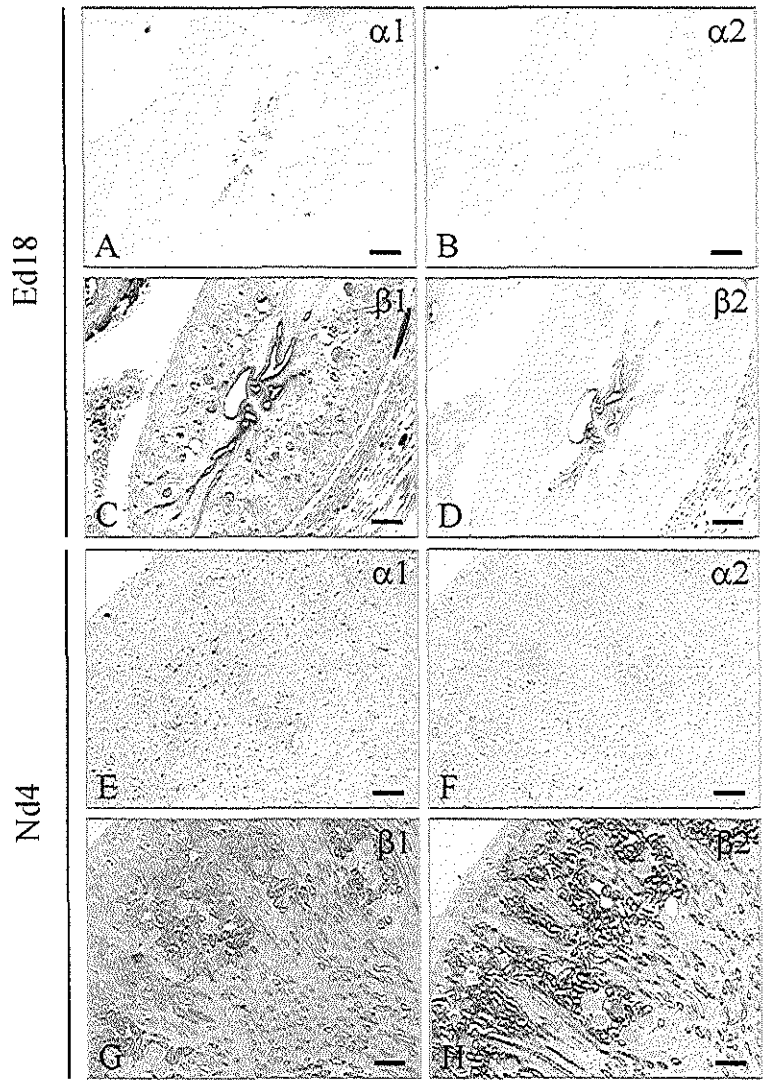


Figure 14
Thyroid-hormone-receptor-protein distribution during kidney development. TRα_1 protein is present at ED18 in the collecting tubules (A) and disappears at ND4 (E). No TRα_2 protein could be detected at both stages (B,F). At ED18, TRβ_1 protein is detected in the glomeruli and collecting tubules (C). After birth TRβ_1 protein disappears from the glomeruli (G). TRβ_2 protein is only detected in the tubules, both before (D) and after birth (H). (Scalebar represents magnification level)

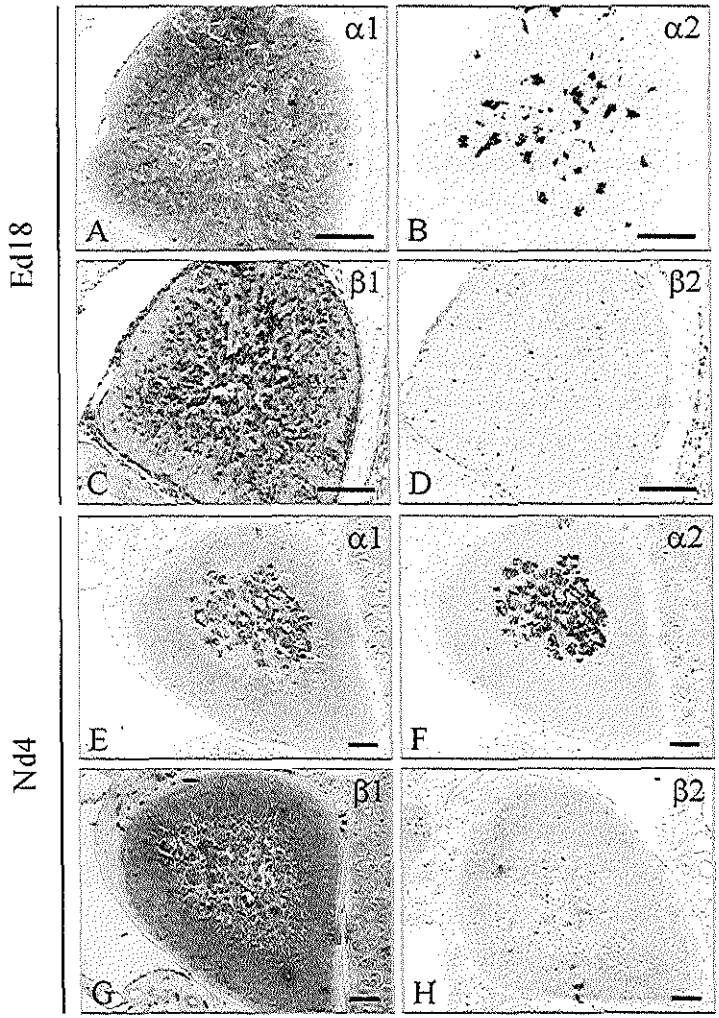


Figure 15
Thyroid-hormone-receptor-protein distribution during adrenal development. $TR\alpha_1$ protein is diffusely distributed at ED18 (A), but is confined to the adrenal medulla at ND4 (E). $TR\alpha_2$ protein is confined to islets of cells at ED18 (B) and becomes also confined to the adrenal medulla after birth (F). $TR\beta_1$ protein is seen in the adrenal and its capsule at ED18 (C), but becomes confined to the adrenal cortex after birth (G). No $TR\beta_2$ proteins is observed in the adrenal. (Scalebar represents magnification level)

Adrenals

In situ hybridization revealed a weak TRα signal in the entire adrenal at ED16, which had become confined to dispersed islands of cells and the capsule at ED18 (Fig. 12A,C). TRβ mRNA levels were low and confined to the capsule (Fig. 12B,D). PCR analysis was not performed. Western blot analysis showed prominent bands for both $TR\alpha_1$ and $TR\alpha_2$ after birth (Fig. 3A,B). In addition, a prominent 100 kD $TR\alpha_2$ band was observed. This band was also observed in human (not shown), but not rat pituitary gland. Only weak bands were observed for $TR\beta_1$ and $TR\beta_2$ protein after birth (Fig. 3C,D). Immunohistochemistry revealed a highly characteristic distribution of the TR proteins. $TR\alpha_1$ protein was diffusely distributed at ED18 (Fig. 15A), but the protein had become confined to the adrenal medulla at ND4 (Fig. 15E). $TR\alpha_2$ protein was already confined to well-defined islets of cells at ED18 (Fig. 15B) and became also concentrated in the medulla after birth (Fig. 15F). $TR\beta_1$ protein was diffusely distributed in the adrenal tissue and capsule at ED18 (Fig. 15C), but became localized to the adrenal cortex after birth (Fig. 15G). No $TR\beta_2$ protein was observed in the adrenals (Fig. 15D,H).

Although we have no PCR data for the adrenals, the near absence of TRβ mRNA and the easily detectable levels of $TR\beta_1$ protein indicate a high protein/mRNA ratio for this gene product. The expression pattern of TRα mRNA and $TR\alpha_2$ protein coincided.

Intestines

Expression of both TRα and TRβ mRNA was observed in the epithelial layer of the intestines and in the parenchymal cells of the pancreas from ED16 onward (Fig. 16A-F). The small and large intestine were pooled for PCR analysis before birth. $TR\alpha_1$ mRNA levels peaked just after birth, but were 6-8 times lower than those of $TR\alpha_2$ (Fig. 17). $TR\alpha_2$ mRNA levels were highest perinatally and were lower in the adult (Fig. 17). $TR\beta_1$ mRNA concentration followed a similar developmental pattern as $TR\alpha_2$, but the observed levels were two to three times higher (Fig. 17).

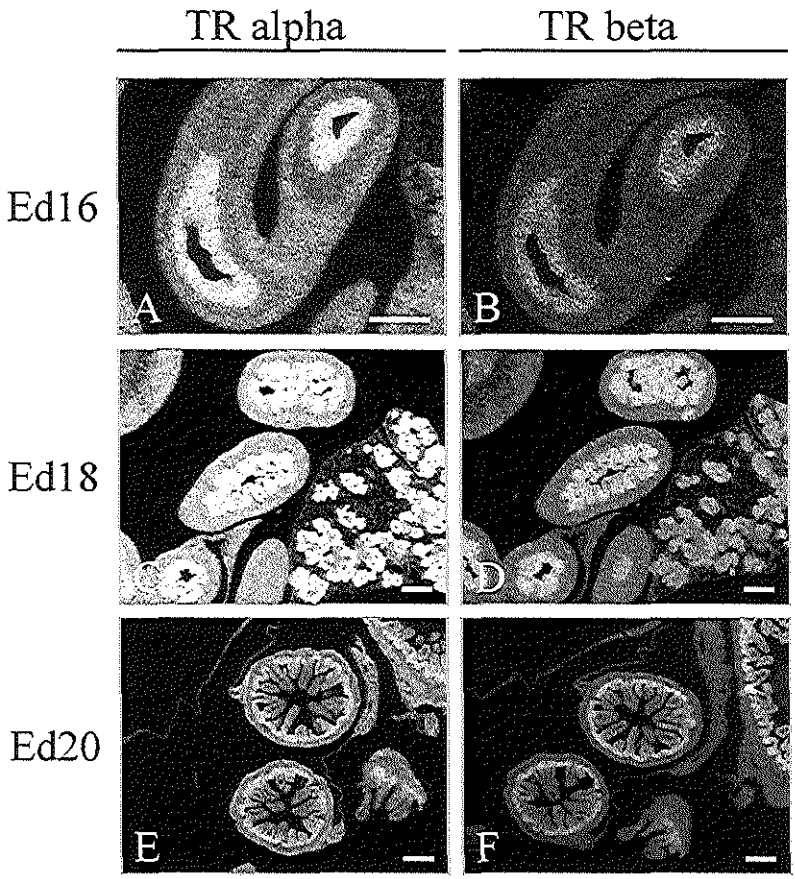
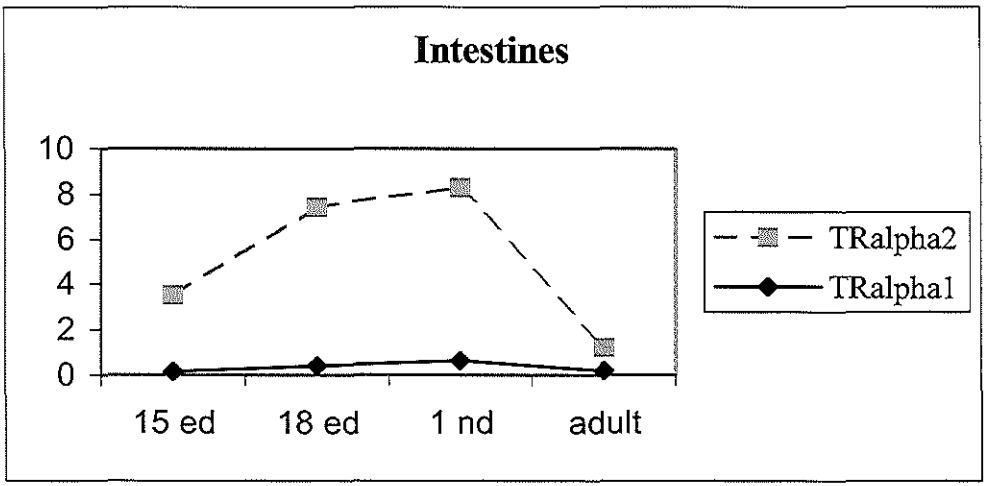


Figure 16

Thyroid-hormone-receptor-mRNA expression during fetal intestinal development. In contrast to the other investigated organs, both TRα and TRβ mRNA are co-expressed in the epithelial layer of the intestines from ED16 onwards (A-F). (Scalebar represents magnification level)



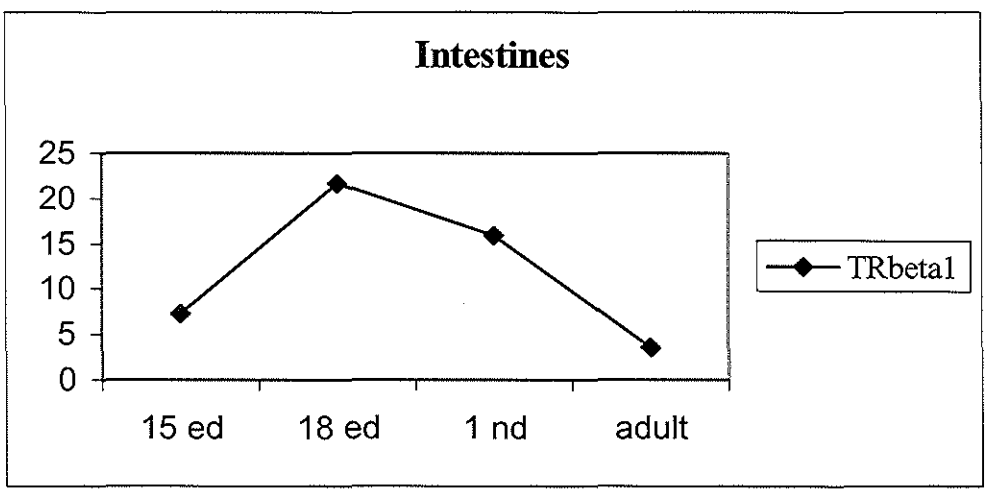


Figure 17

Thyroid-hormone-receptor-mRNA levels during intestinal development. Both $TR\alpha_1$ and $TR\alpha_2$ mRNA levels peak after birth, although $TR\alpha_2$ mRNA levels are 6-8 times higher. $TR\beta_1$ mRNA levels peak also after birth, but the observed levels are 2-3 times higher than $TR\alpha_2$ mRNA levels.

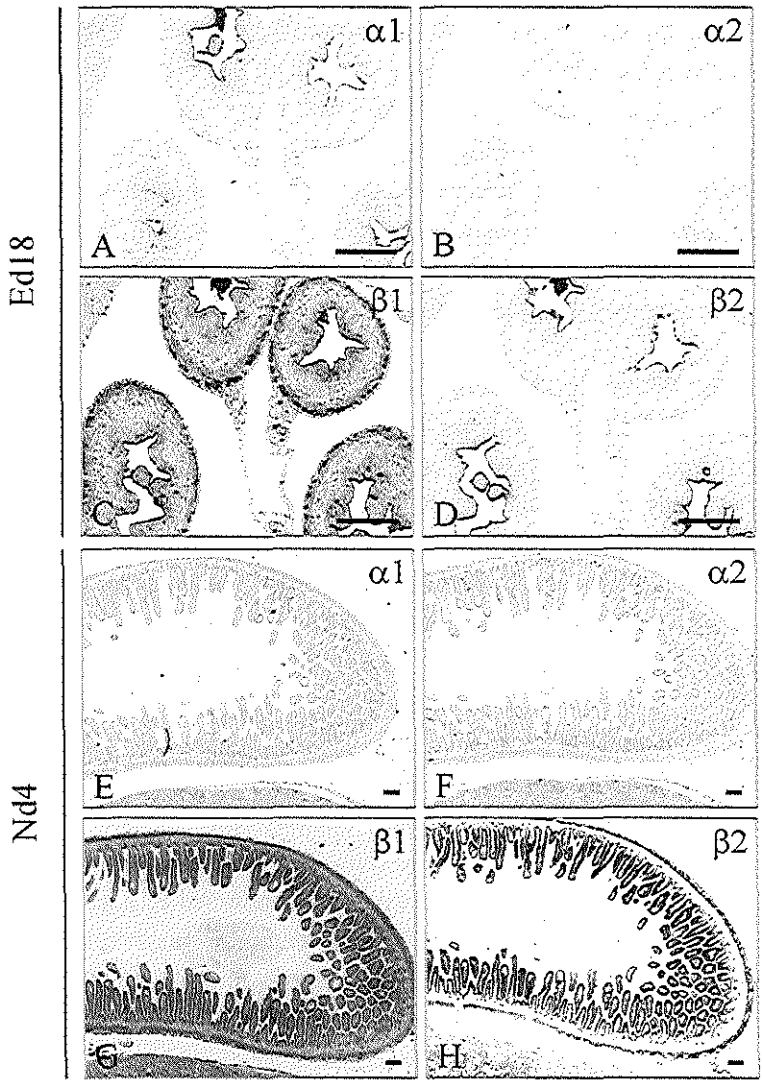


Figure 18

Thyroid-hormone-receptor-protein distribution during intestinal development. No $TR\alpha_1$ or $TR\alpha_2$ protein can be detected in the intestines immunohistochemically (A,B,E,F). $TR\beta_1$ protein is seen in the smooth muscle layer of the intestine at ED18 (C), and in the mucosa as well postnatally (G). After birth $TR\beta_2$ protein is present in the same cells. (Scalebar represents magnification level)

Western blotting showed that TRα_1 protein levels decreased between 5 days before and 4 days after birth (Fig. 3A). The opposite was true for TRα_2 protein (Fig. 3B). Relatively low levels of TRβ_1 protein were found both pre- and postnatally (Fig. 3C). TRβ_2 protein could not be detected prenatally, but high levels were observed postnatally (Fig. 3D). TRα_1 and TRα_2 protein could not be detected in the intestines immunohistochemically (Fig. 18A,B,E,F). In contrast, TRβ_1 protein was observed in the smooth muscle layer of the intestine at ED18 and, in addition, in the mucosa postnatally (Fig. 18C,G). TRβ_2 protein was localized to the same cells as TRβ_1 protein postnatally, but was undetectable prenatally (Fig. 18H).

Remarkable differences between the TR mRNA and protein distribution were observed. Thus, TRα and TRβ mRNA levels were high to very high in the mucosa at ED16 and ED18 and retracted to the crypts at ED20. However, these mRNAs were hardly translated in these cells, since the protein was undetectable in sections before and after birth (at the latter age for TRα only). At the same time, high levels of TRβ_1 protein were present in the muscle layer without corresponding mRNA. These data suggest that the protein/mRNA ratio for TRα and TRβ (before birth only) is relatively low, whereas the protein/mRNA ratio for TRβ in the smooth muscle layer is high.

Comparison of TR protein in organs

Except for the organs discussed, we demonstrated TRα_1 and TRα_2 protein in Western blots of ND4 trachea (including the thyroid), muscle, heart, thoracic cage (bone and muscle), testis, epididymal fat pad, and blood cells. The concentrations TRα_1 protein were similar to those found in lung and kidney, except for brain and to a lesser extent spinal cord, as well as small intestine, where the TRα_1 concentration was low, and blood, where TRα_1 was twice as high. TRα_2 levels were relatively high in adrenal and fat pad, relatively low in lung, trachea and muscle, and very low in blood. The resulting TRα_1/TRα_2 ratio, a parameter for tissue thyroid hormone responsiveness, was relatively low for brain, adrenal, pituitary gland, liver, prenatal heart, thoracic cage, approx. 10-fold higher for postnatal lung and trachea, and extremely high for blood. The TRβ_1 concentration was relatively high in heart and testis, whereas TRβ_2 concentration was relatively high in brain, spinal cord, liver, intestine, and heart.

Table 1. Expression of TRα and TRβ mRNA during development of the rat fetus. ++, +, +/- and – indicate high, medium, low and no mRNA expression, respectively.

Organ	Age (days of gestation)	TRα	TRβ
Lungs	13	+	-
	14	+	-
	15	+	+/-
	16	+	+
	17	++	++
	18	++	++
	19	+	+/-
	20	+	-
Liver	13	-	+
	14	-	+
	15	-	+
	16	+	+
	17	+	+
	18	++	++
	19	++	++
	20	++	++
Kidneys	13	+	-
	14	+	-
	15	+	-
	16	+	+
	17	+	++
	18	+	++
	19	+	++
	20	+	++
Intestines	13	-	-
	14	-	-
	15	+/-	-
	16	++	+
	17	++	+
	18	++	+
	19	+	+
	20	+	+

Table 2. Expression of $TR\alpha_1$, $TR\alpha_2$, $TR\beta_1$ and $TR\beta_2$ protein expression during development of the rat fetus.
++, +, +/- and – indicate high, medium, low and no protein expression, respectively.

Age	Organ	$TR\alpha_1$	$TR\alpha_2$	$TR\beta_1$	$TR\beta_2$
18 days of gestation	Trachea	-	-	-	-
	Lungs	-	-	+	+
	Liver	+/-	-	+/-	-
	Kidneys	+/-	-	++	+
	Adrenals	+	+	++	-
	Pituitary gland	-	-	+	-
	Brain	-	-	++	+
4 neonatal days	Lungs	-	-	+	+
	Liver	+	-	++	-
	Kidneys	-	-	+	++
	Adrenals	+	++	++	-
	Pituitary gland	-	-	+	+
	Brain	-	-	++	-

Comparison of the protein/mRNA ratio between organs

The protein/mRNA ratio in organs with a similar tissue distribution of mRNA and protein was compared, based on the PCR and Western-blot data. The protein/mRNA ratio for $TR\alpha_1$ was by far the lowest for brain, followed by the small intestine, and then lung, kidney and liver, which were similar. The protein/mRNA ratio for $TR\alpha_2$ was again by far the lowest in brain, whereas liver, kidney and lung were similar. The protein/mRNA ratio for $TR\beta_1$ was highest for brain, followed in descending order by liver, lung, and kidney. In intestine, the mRNA and protein distribution only partly overlapped, precluding a direct correlation based on analysis of extracts.

Table 3. Thyroid-hormone receptor-protein levels in organs, expressed as LUMI-imager light units. Aliquots of tissue extracts containing 50 μg of protein, were separated on a denaturing polyacrylamide gel, blotted unto PDMF membrane and tested for the presence of $TR\alpha_1$, $TR\alpha_2$, $TR\beta_1$, and $TR\beta_2$.

	$TR\alpha_1$			**$TR\alpha_2$**		
	-5	*+4*	*adult*	*-5*	*+4*	*adult*
Liver	8.0	13.0/10.3	4.0/11.3	2.4	3.8	2.7
Lung	9.0	35.2		1.8	0.7	
Trachea		53.1			1.0	
Muscle	14.3	30.9		1.9	1.4	
Kidney	9.4	27.2/20.5	21.8	1.2	2.3	
Adrenal		40.1	16.3		6.4	
Heart	10.3	34.8		3.7	2.1	
Spinal cord		23			1.9	
Brain	1.5	6.2		1.2	1.9	
Testis		36.5			5.3	
Thorax		34.5			4.3	
Fat pad		30.8			10.8	
Jejunum	11.7*	11.7/4.9		1.5*	4.7	
Ileum	11.7*	2.7/12.6		1.5*	4.4	
Blood		63.8			0.2	
Pit. gland		20.2	22.1/29.1			0.3

	$TR\beta_1$			**$TR\beta_2$**		
	-5	*+4*	*adult*	*-5*	*+4*	*adult*
Liver	10.5	15.7/9.6	3.8/7.6	2.3	16.5/12.7	11.8/10.5
Lung	18.0	18.7		3.9	4.1	
Trachea		20.4			4.0	
Muscle	18.3	14.4		2.7	3.8	
Kidney	14.6	17.0/14.3	11.0/3.6	4.6	6.3/0.4	1.0
Adrenal		16.3	13.7/4.6		2.6	1.0
Heart	21.5	28.5		4.6	29.7	
Spinal cord		45.3			37.7	
Brain	20.7	37.2		14.9	21.5	
Testis		8.3			1.2	
Thorax		4.7			2.1	
Fat pad		4.1			7.0	
Jejunum	13.1*	1.6/9.8		4.7*	16.0/29.8	
Ileum	13.1*	3.4/11.2		4.7*	22.5/15.1	
Blood		0.7			1.4	
Pit. gland		7.3/16.4	7.1/6.4		0.2	14.8/5.3

6.5 Discussion

Although the role of thyroid hormone in organogenesis is undisputed (Oppenheimer and Samuels, 1983; Tata, 1993), the respective roles of the TRα and TRβ receptors that mediate the effects of thyroid hormone have remained elusive. Similarly, it is likely that epithelial-mesenchymal interactions, a time-honored and important aspect of organ development, play a role, because mesenchymal cells are thought to be primary determinants of differentiation, growth and morphogenesis of the epithelial cells towards the formed organ (Brard, 1990). Using in situ hybridization and immunohistochemistry as visualization techniques, and RT-PCR and Western blotting as quantification techniques, we observed a highly specific distribution pattern of the TR isoforms. Expression of the TRα isoform was observed in the mesenchymal tissues and of the TRβ isoform in the epithelial tissues of many organs. As far as we know, this generalization has not been made so far. In addition, we observed extensive pre- and posttranscriptional regulation of the expression of the TRs, with differences between organs and between different developmental stages of the same organ [cf. also (Lane et al., 1991; Rodd et al., 1992; Schwartz et al., 1992; Strait et al., 1990; Weiss et al., 1998)].

The most pronounced complementary expression pattern of TRα and TRβ was observed in the developing lung, liver, kidney and inner ear. Since most epithelia cannot differentiate when separated from their associated mesenchyme (Birchmeier and Birchmeier, 1993; Masters, 1976; Saxen et al., 1986; Saxen and Thesleff, 1992), it is thought that epithelial differentiation is under control of its surrounding mesenchyme. Due to extensive posttranscriptional control, the respective expression patterns were much more pronounced at the mRNA than at the protein level. Although both mRNA and protein levels in the organs mentioned reached adult levels perinatally, it is of interest that for those organs for which paired samples were available, tissue $TR\alpha_2$, $TR\beta_1$ and $TR\beta_2$ protein levels had already reached the adult level at 5 days before birth, whereas $TR\alpha_1$ protein levels only reached its highest levels after birth. These protein data therefore strongly indicate distinct roles of the respective receptor isoforms during the fetal phase of organ development [cf. also (Forrest et al., 1991)]. The distinct time-course of TRα and TRβ also suggests an initiating, TRβ-mediated role of the epithelium, followed by a TRα-mediated response of the mesenchyme.

Extensive co-expression of TRα and TRβ was observed in the developing brain and in the intestinal epithelium. Despite the high level of $TR\alpha_1$ and particularly $TR\alpha_2$ mRNA in

these organs, $TR\alpha_1$ protein levels were much lower than, and $TR\alpha_2$ protein levels similar to those in the other organs investigated. In other words, the organs with co-expression seem to be characterized by a very low TR protein/mRNA ratio. Thus, the protein/mRNA ratio for $TR\alpha_1$ was more than 10-fold lower in perinatal brain than in perinatal liver, lung, or kidney and that of $TR\alpha_2$ more than 50-fold lower in perinatal brain and more than 10-fold lower in perinatal lung and kidney than in perinatal liver. In contrast, the protein/mRNA ratio for $TR\beta_1$ was similar in these 4 organs in the perinatal period, but was very low in intestinal epithelium, especially before birth. This implies that in organs with co-expression of TRα and TRβ, the translational efficiency of one of the TR mRNAs is much lower, and/or that the turnover of TR protein is much higher than that in cells without co-expression. Finally, the $TR\beta_2$ protein levels in brain and intestine are the highest measured (the $TR\beta_2$ mRNA levels were below the detection limit of our assays). These data indicate that the TR-dependent regulatory cascades function differently in those organs in which the TRα and TRβ genes are characterized by a complementary expression pattern and those in which they are co-expressed.

We observed an apparent discrepancy between the relatively poor visibility of TRα proteins in histological sections of all organs except the adrenal, and their strong presence, in particularly $TR\alpha_1$, on Western blots. The performance of the antibodies on the Western blots, as well as on sections of the adrenals, shows that they were of similar quality as those for the TRβs. Therefore, the TRαs are either not well accessible in the tissue sections of most organs or, more likely, they are distributed over many more cells, such that the cellular concentration remains too low to strongly stain these tissues. The latter possibility is supported by their presence in the stroma of organs such as liver, lung, bone and kidney. As stated before, the low staining intensity in brain and intestine is due to the low protein levels in these organs.

The antibody raised against the $TR\beta_2$-specific oligopeptide recognized a protein of 47 kD, i.e. 11 kD less than that predicted by the reported open reading frame (Hodin et al., 1989). We observed an identical apparent size of the $TR\beta_2$ with 3 other antisera that were raised against the same $TR\beta_2$-specific oligopeptide. Although the size of the $TR\beta_2$-like protein corresponds with the recently discovered $TR\beta_3$ isoform (Williams, 2000), the oligopeptide against which the antisera were raised, is located in an exon that is present in the $TR\beta_2$, but not the $TR\beta_3$ isoform. More likely, the discrepancy should therefore be explained by the less than optimal Kozak sequence (Kozak, 1992) that surrounds the first

in-frame AUG of the $TR\beta_2$ cDNA when compared to the one surrounding the AUG 351 nucleotides further downstream. Usage of a more downstream AUG is also suggested by studies aiming to delineate the transcription initiation site of $TR\beta_2$ (Wood et al., 1994). Unfortunately, no Western blot data of the $TR\beta_2$ oligopeptide-specific antisera that were reported in the literature (Lechan et al., 1993; Schwartz et al., 1994), are available for comparison. As part of the efforts to validate the specificity of our antiserum, we determined the isoelectric point of the protein that was recognized (not shown). As expected from the amino-acid sequence of $TR\beta_2$, the pI of the $TR\beta_2$-like protein in liver extracts was 7.6, indicating that the protein identified by the antiserum is indeed $TR\beta_2$. However, the pI of the protein recognized in extracts of the pituitary gland, was 5.0, suggesting extensive posttranslational modification in this organ, most likely phosphorylation. The strong staining intensity of the $TR\beta_2$-like protein in many organs implied easily detectable mRNA levels. Nevertheless, we were unable to detect the corresponding mRNA with our RT-PCR protocol outside the pituitary in the tissues investigated. The limit of detection of this method is less than 0.1 mRNA molecule per cell. Extremely low cellular levels of $TR\beta_2$ mRNA in association with easily detectable $TR\beta_2$ protein levels were reported before (Lechan et al., 1993; Schwartz et al., 1994) and imply either an extremely high translational efficiency or an extremely stabile protein (Ercan-Fang et al., 1996). Another possibility is a pronounced diurnal mRNA rhythm with our measurement of the mRNAs in the nadir of the daily wave. We did observe a diurnal rhythm for $TR\beta_1$ with a nadir at noon (B.Z. Doulabi et al., submitted), but did not yet investigate $TR\beta_2$. We also tested the antisera by staining the pituitary glands of neonatal and adult rats. The sections revealed that the $TR\beta_2$-like protein concentration was still very low in the ND4 pituitary, but was present in the adult organ as described (Li and Boyages, 1997). Because $TR\beta_2$ is thought to play an important role in the feedback regulation of the hypothalamic-pituitary-thyroid axis (Abel et al., 1999), its absence in the neonatal pituitary may serve to accommodate the perinatal rise in circulating thyroid hormones in rats (Dubois and Dussault, 1977).

The expression patterns that we describe generally fit with the observed effects of targeted inactivation of the respective TR genes [for a recent review, see (Forrest and Vennstrom, 2000)]. Thus, $TR\beta_2$ deficiency was found to lead to central resistance to thyroid hormone (Abel et al., 1999; Forrest et al., 1996; Kaneshige et al., 2000), while we observed a delayed appearance of $TR\beta_2$ in the pituitary, which we hypothesize to be

responsible for the perinatal rise in thyroid hormone production. Similarly, it was shown that $TR\beta_1$ deficiency leads to failure of induction of malic enzyme and spot-14 induction in liver (Weiss et al., 1998), while we showed that this receptor is the predominant TR in the hepatocytes in which these genes are expressed (B.Z. Doulabi et al., submitted). In contrast to TRβ deficiency, TRα deficiency leads to hypothyroidism and delayed tissue maturation (Fraichard et al., 1997), in accordance with the widespread expression of the gene in mesenchymal tissues. However, $TR\alpha^{-/-}$, $TR\beta^{-/-}$ mice survive up to several weeks after birth, even though the $TR\alpha^{-/-}$- and $TR\beta^{-/-}$ phenotype suggests only a limited redundancy of TRα- and TRβ-dependent functions (Gauthier et al., 1999). In view of the pronounced phenotype of hypothyroid neonates, one should perhaps interpret these findings as indicating that the absence of TRs interferes less with normal development than the presence of unoccupied TRs. Alternatively, thyroid hormones become important only after birth, at least in altricial species like most rodents. The latter interpretation seems to be supported by the findings that experimentally induced prenatal hypothyroidism in the mouse (Wallace et al., 1995) does not lead to major problems until after birth (P.J.E. Blommaart et al., in preparation).

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Part III

ABNORMAL MECHANISMS OF PULMONARY DEVELOPMENT

Chapter 7

Dual-hit hypothesis explains pulmonary hypoplasia in the Nitrofen model of congenital diaphragmatic hernia.

Based on:
Keijzer, R., Liu, J., Deimling, J.F., Tibboel, D., and Post, M. (2000) Dual-hit hypothesis explains pulmonary hypoplasia in the Nitrofen model of congenital diaphragmatic hernia. *Am J Pathol,* 156: p.1299-1306.

7.1 Abstract

Pulmonary hypoplasia associated with congenital diaphragmatic hernia (CDH) remains a major therapeutical problem. Moreover, the pathogenesis of pulmonary hypoplasia in case of CDH is controversial. In particular little is known about early lung development in this anomaly. To investigate lung development separate from diaphragm development we used an *in vitro* modification of the 2,4-dichlorophenyl-p-nitrophenyl (Nitrofen) animal model for CDH. This enabled us to investigate the direct effects of Nitrofen on early lung development and branching morphogenesis in an organotypic explant system without the influence of impaired diaphragm development. Epithelial cell differentiation of the lung explants was assessed using surfactant protein-C and Clara cell secretory protein-10 mRNA expression as markers. Furthermore, cell proliferation and apoptosis were investigated. Our results indicate that Nitrofen negatively influences branching morphogenesis of the lung. Initial lung anlage formation is not affected. In addition, epithelial cell differentiation and cell proliferation are attenuated in lungs exposed to Nitrofen. These data indicate that Nitrofen interferes with early lung development prior to and separate from (aberrant) diaphragm development. Therefore, we postulate the dual-hit hypothesis, which explains pulmonary hypoplasia in CDH by two insults, one affecting both lungs before diaphragm development and one affecting the ipsilateral lung after defective diaphragm development.

7.2 Introduction

Congenital diaphragmatic hernia (CDH) has a mean prevalence of approximately 1 in 3000 newborns (Torfs et al., 1992). Even sophisticated management techniques, such as extracorporeal membrane oxygenation (ECMO) and fetal surgery have not significantly influenced the mortality rate of CDH in high-risk patients and consequently, CDH remains a major problem in Pediatric Surgery and Neonatology. A recent review shows that the mortality remains high at 50 to 60% in high-risk patients (Katz et al., 1998). However, other reports suggest a modest reduction in mortality to approximately 30% in comparable series of patients (Reickert et al., 1998; Wung et al., 1995). A combination of morphological features characterizing this anomaly: pulmonary hypoplasia and persistent pulmonary hypertension, is held responsible for the high mortality. In particular the degree of pulmonary hypoplasia is almost impossible to evaluate pre- and immediately

postnatal and in most cases the degree of pulmonary hypoplasia is an important determinant of the outcome (Thebaud et al., 1998).

The pathogenetic events resulting in the diaphragmatic defect and pulmonary hypoplasia are unknown. Classically, the primary defect is believed to be located in the diaphragm. Abdominal organs that herniate through this defect will interfere with normal pulmonary development. This results in a secondary defect: pulmonary hypoplasia and abnormal pulmonary vascular development (Allan and Greer, 1997; Harrison et al., 1986). However, since the introduction of the 2,4-dichlorophenyl-p-nitrophenylether (Nitrofen) animal model for CDH, an alternative hypothesis has been suggested (Cilley et al., 1997; Iritani, 1984). This animal model is based on the teratogenic effects of the herbicide Nitrofen. When administered in the right dosage and at the right time to pregnant rats and mice, Nitrofen interferes with development of the lungs and the diaphragm of the offspring (Cilley et al., 1997; Kluth et al., 1990; Tenbrinck et al., 1990). Studies with this animal model have suggested that CDH might be due to primary disturbance of pulmonary growth into the pleuroperitoneal canal, thereby disturbing the growth of the posthepatic mesenchymal plate, the main origin of the diaphragm (Iritani, 1984). After exposure to Nitrofen, 100% of the litter has a variable amount of lung hypoplasia, whereas depending on animal strain and timing of Nitrofen administration, a smaller percentage, varying from 60 to 90% in rats and 40 to 60% in mice, has a diaphragmatic hernia as well (Cilley et al., 1997; Kluth et al., 1990; Tenbrinck et al., 1990). Observations that both the contralateral and ipsilateral lung are hypoplastic suggest that proper pulmonary development in CDH is already impaired prior to the failed closure of the diaphragm. On the ipsilateral side, growth of the lung is hampered at a later phase by the presence of abdominal organs in the thoracic cavity and eventually interference with fetal breathing movements.

We hypothesized that the lungs are the primary target-organ in the pathogenesis of pulmonary hypoplasia in case of Nitrofen-induced CDH. This implies that the lungs have to be hypoplastic before defective closure of the diaphragm has occurred. In order to test this hypothesis, we investigated early pulmonary development in an *in vitro* modification of the Nitrofen model. We have set up a foregut/lung explant system in which it is possible to study lung formation and branching morphogenesis without the influence of normal or defective closure of the diaphragm. The direct effects of Nitrofen on early pulmonary development *in vitro* were investigated by culturing explants in the presence of Nitrofen. In addition, the effects of removal of physical constraint on branching

morphogenesis after Nitrofen treatment *in vivo* were tested. We show herein that Nitrofen interferes with branching morphogenesis both *in vitro* and *in vivo*. In contrast, initial lung bud outgrowth is not disturbed. Epithelial cell differentiation is attenuated even before effects of a defective closure of the diaphragm could have occurred. Furthermore, the interplay between proliferation and apoptosis is disturbed in lungs exposed to Nitrofen and therefore contributes to pulmonary hypoplasia.

7.3 Materials and methods

Animals

Female (200-250 g) and male (250-300 g) Wistar rats were obtained from Charles River (St. Constant, Quebec, Canada). The animals were kept in a controlled light-dark cycle and food and water were supplied *ad libitum*. Rats were mated overnight and the finding of a sperm-positive vaginal smear was designated day 0 of gestation. At 11 and 13 days of gestation (term = 22 days of gestation), timed-pregnant rats were killed by cervical dislocation after a short exposure to diethyl ether in order to anesthetize them. The fetuses were delivered by Caesarian section using aseptic surgical techniques and kept in Hank's balanced salt solution (Gibco, Burlington, ON). All protocols were evaluated and approved by the Animal Care Committee of the Hospital for Sick Children.

Explant cultures

Foreguts and lungs were cultured according to previously reported studies (Souza et al., 1994). In short, at 11 days of gestation, foreguts and at 13 days of gestation, lungs were harvested from fetal rats under a dissection microscope using microsurgical techniques. The foreguts and lungs were transferred to porous membrane inserts (4 μm pore size) from Millipore (Bedford, MA), and incubated in 4-well cell culture plates from Nunc (Intermed, Denmark). The membrane inserts were pre-soaked in MEM (Gibco, Burlington, ON) for 1 hour before the explants were placed on them. The explants were incubated as floating cultures in 200 μl Dulbecco's modified Eagle medium, nutrient mixture F-12 (Gibco, Burlington, ON) supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, 0.25 mg/ml ascorbic acid and 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Burlington, ON). Heat inactivation of the serum was required to support long-term culture. The explants were cultured at 37°C in 95% air and 5% CO_2.

Nitrofen

The herbicide 2,4-dichloro-phenyl-p-nitrophenylether (Nitrofen) was obtained from Rohm & Haas Co. (Philadelphia, PA). To induce congenital diaphragmatic hernia and pulmonary hypoplasia *in vivo*, 100 mg Nitrofen dissolved in 1 ml olive oil was administered orally on day 9 of gestation. To investigate the effects of Nitrofen *in vitro*, the explants were exposed during the first day of culture to a concentration of Nitrofen, which is similar to that used *in vivo*: 0.25 mg.200 μl^{-1} medium. To mimic the half-life of Nitrofen *in vivo* the explants were exposed to half of this during the second day of culture (Costlow and Manson, 1983; Manson, 1986). During the remaining culture period the explants were cultured in medium alone. Non-treated explants and explants cultured in the presence of vehicle [dimethyl sulphoxide: DMSO] (BDH, Toronto, ON) in the same concentration as was used to dissolve the Nitrofen served as controls (see Table 1). Since Nitrofen is extremely toxic, all handling was done in a fume hood or laminar flow using protective gear to prevent inhalation and contact with the skin. Disposal of all waste products containing Nitrofen was done according to local regulations of The Hospital for Sick Children and all experimental procedures were approved by the department of Occupational Health and Safety of the Hospital for Sick Children.

Quantification of branching morphogenesis

During the entire period of culture, lung formation and branching morphogenesis was monitored daily by phase-contrast microscopy. At approximately the same time point each day, branching morphogenesis was assessed by manually counting the number of terminal buds and in addition, the lung explants were photographed.

Tissue preparation

After 8 (foregut) or 4 and 6 (lung) days of culture, explants were removed from the inserts and fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 16 to 18 hours, dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraplast (Oxford labware, St. Louis, MO). Sections of 5 μm were cut and mounted on Superfrost slides (Fisher Scientific, Unionville, ON) and baked at 42°C for 16 to 18 hours.

In situ hybridization

After dewaxing and rehydrating, tissue sections were post-fixed in 4% (w/v) paraformaldehyde in PBS and permeabilized with proteinase K (20 $\mu g.ml^{-1}$ in 50 mM

Tris/HCl, 5 mM EDTA, pH 8.0) for 15 minutes at room temperature. Following another post-fixation step in 4% (w/v) paraformaldehyde in PBS for 5 minutes, the sections were carbethoxylated by 0.1% (v/v) active diethylpyrocarbonate treatment twice for 15 minutes in order to reduce background by inactivating RNases in the sections. Subsequently, the sections were equilibrated in 5x SSC (NaCl 0.75 mol/L, sodium citrate 0.075 mol/L) for 15 minutes and prehybridized for 2 hours at 58°C in 50% (v/v) formamide, 5x SSC and 40 $\mu g.ml^{-1}$ salmon sperm DNA. The sections were hybridized overnight at 58°C with digoxigenin (DIG)-labeled Surfactant protein-C (SP-C) and Clara cell secretory protein-10 (CC-10) RNA probes in the same hybridization mixture. Rat specific SP-C and CC-10 cDNA fragments (330 and 315 bases, respectively) were DIG-labeled according to a protocol provided by the manufacturer (Boehringer Mannheim, Montreal, Quebec). The next day, sections were washed in 2x SSC for 30 minutes at room temperature, 1 hour at 58°C and in 0.1x SSC for 1 hour at 58°C. The DIG Nucleic Acid Detection Kit from Boehringer Mannheim was used for immunological detection of the hybridized probe. Unspecific labeling was removed in 95% ethanol and the sections were counterstained with methylgreen for 1.5 minutes. After dehydration in a graded series of ethanol and xylene, the sections were mounted with coverslips using Permount (Fisher Scientific, Unionville, ON).

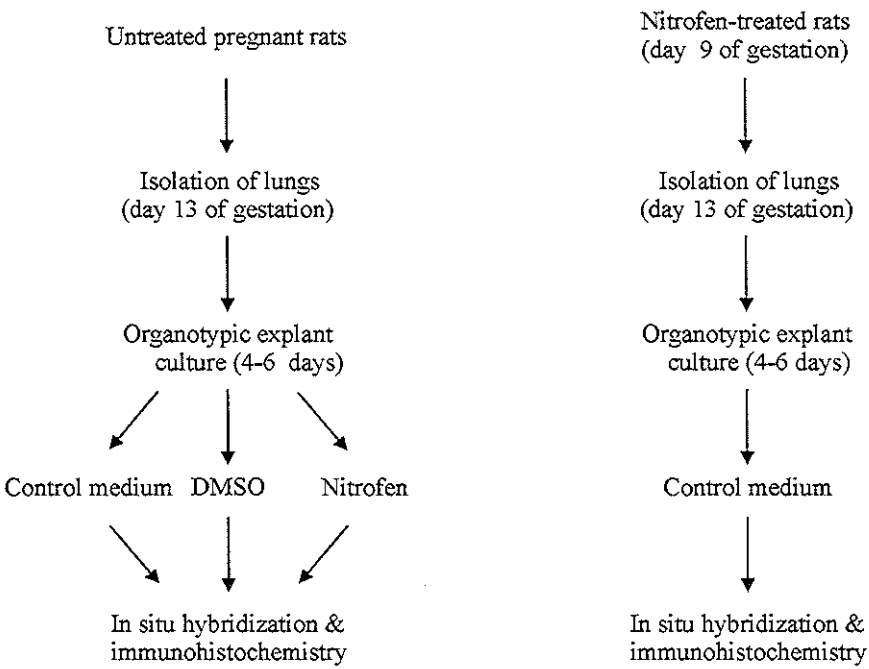
Immunohistochemistry

Sections were dewaxed in xylene after heating them at 60°C for 5 minutes and rehydrated in a graded series of ethanol. Subsequently, antigen retrieval was performed by boiling the sections in a 10 mM sodium citrate solution, pH 6.0 for two periods of 5 minutes in a microwave at medium high. Between the boiling periods, the sections cooled down for 20 minutes. After rinsing the slides in PBS endogenous peroxidase activity was blocked by exposing the slides to a 3% (v/v) hydrogen peroxide in methanol solution for a period of 10 minutes. Following two rinses in PBS, the sections were blocked with 5% (v/v) normal goat serum and 1% (w/v) bovine serum albumin in PBS for the period of 1 hour. The sections were incubated overnight at 4°C with 1:1000 diluted primary monoclonal antibody to proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA). The sections were washed in PBS containing 0.05% (v/v) Tween followed by two washes in PBS alone. The sections were then incubated with a 200-fold dilution of biotinylated anti-mouse IgG for 1 hour at room temperature. After washing, the sections were incubated with avidin-biotin peroxidase complex (Vectastain) kit from Vector

Laboratories (Burlingame, CA) for 2 hours at room temperature. Subsequently, after washes in PBS and Tris- buffered saline (TBS), the sections were developed using 3,3'-diaminobenzidine as substrate. Following washes in TBS and PBS, sections were counterstained with Carazzi's hematoxylin. Subsequently, the sections were dehydrated in a graded series of ethanol and xylene and mounted with coverslips using Permount (Fisher Scientific, Unionville, ON).

Table 1

Organotypic explant cultures



Conditions for the organotypic explant cultures. For details see Materials and Methods section.

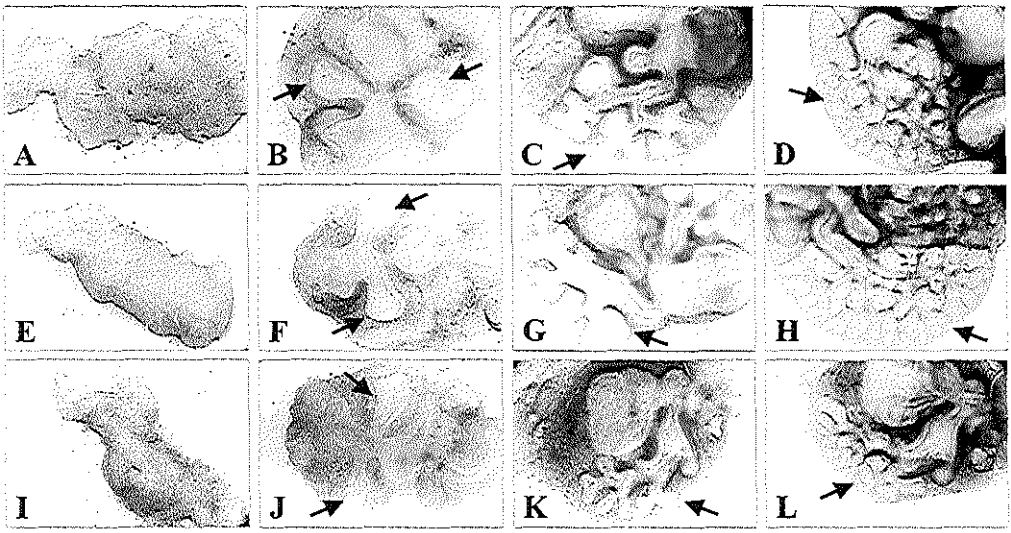


Figure 1
Lung formation and branching morphogenesis of the lung in an explant system of rat foregut. Foreguts of rats, 11 days of gestation, were harvested and cultured in a semi-dry system. Pictures representative of a series of experiments of untreated (A-D), vehicle DMSO-exposed (E-H) and Nitrofen-exposed (I-L) foreguts developing into branching lungs are shown at culture-day 0 (A, E, I), 2 (B, F, J), 6 (C, G, K) and 8 (D, H, L), respectively. The Nitrofen-exposed group was exposed to Nitrofen in a concentration similar to that used *in vivo*: 0.25 mg.200 μl^{-1} medium on the first culture-day, and to half of this on the second culture-day. Lung formation in this group occurred at the same time-point as in the control groups (J), which were cultured in medium with vehicle DMSO (F) or in medium alone (B). Branching morphogenesis was clearly reduced in the explants exposed to Nitrofen during the remaining culture-period (L). (All images at same magnification) (Arrow indicates lung)

Terminal Deoxyribonucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay
After dewaxing and rehydrating, tissue sections were incubated with proteinase K, 20 $\mu g.ml^{-1}$ in 10 mM Tris/HCl, pH 7.6 for 15 minutes at 37°C. Following two washes with PBS, the sections were incubated with 25 µl TUNEL reaction mixture (Boehringer Mannheim, Montreal, Quebec) for 1 hour at 37°C. Subsequently, the sections were washed in PBS and mounted with coverslips using Vectashield mounting medium with DAPI from Vector Laboratories (Burlingame, CA).

Data presentation
All results are expressed as mean ± SE. Statistical significance was determined by one way analysis of variance followed by assessment of differences using a Tukey test for pairwise multiple comparison procedures (Hochberg and Tamhane, 1987). Linear regression was performed after transformation of the curves in straight lines by changing the numerical y-axis scaling into a natural log scale. The rate of branching (slope) was depicted by the coefficient ± SE and differences were assessed using Student's t-test. Significance was defined as $p < 0.05$.

7.4 Results

Lung formation and branching morphogenesis

After 72 hours of culture, lung formation was observed in all foregut explants as a diverticulum arising from the foregut to form two primary lung buds (see Figure 1B, F & J). The timing of lung bud appearance was approximately the same in all explants. The same holds true for initial lung bud outgrowth after Nitrofen treatment *in vivo*, since the endodermal part of the lungs in this group appeared similar at the moment of isolation (see Figure 2D). However, the mesenchymal parts of the lungs treated with Nitrofen *in vivo* appeared much thicker than the mesenchymal parts of the control lungs (see Figure 2D). During the remaining period of culture, dichotomous branching was observed in all lung explants (see Figure 2E-H). In the Nitrofen exposed explants, only lobular branching was observed (see Figure 2G & H), and the rate of branching was reduced (see Figure 3 and Table 2). The explants exposed to Nitrofen *in vitro* showed an arrest in branching during the first 2 days of culture, the period of exposure to Nitrofen. However, the rate of branching was also reduced during the subsequent 2-day culture period (see Figure 3). The number of branches of explants exposed to Nitrofen *in vitro* was significantly

reduced when compared to control explants ($p<0.05$) (Table 2). Exposure to Nitrofen *in vivo* did not result in a statistically significantly reduced number of branches. When only the 75% lowest mean numbers of branches were used for comparison, statistical significance was reached (Table 2). No significant differences were observed between control and DMSO exposed explants. The size of the explants was reduced after exposure to Nitrofen, suggesting lung hypoplasia. Nitrofen exposure *in vitro* resulted in more severe reduction in branching and size than exposure *in vivo*.

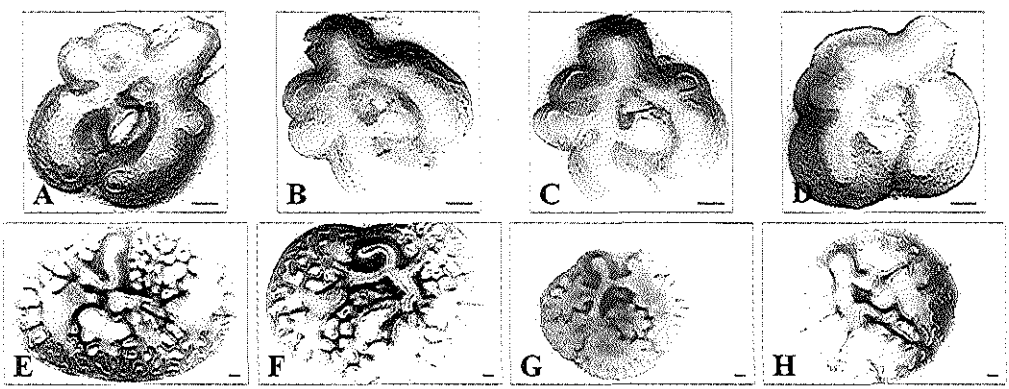


Figure 2
Branching morphogenesis in a rat lung explant system. At 13 days of gestation, lungs were removed from the fetuses and cultured in a semi-dry system. The upper row of pictures are representative for lungs at 13 days of gestation at culture day 0 just after isolation of the lungs from the fetus. The bottom row of pictures represents lungs after a culture period of 4 days. One group was treated with Nitrofen *in vivo* by administration of the Nitrofen to the mother at 9 days of gestation (D, H) Another group was treated with Nitrofen *in vitro* as described before (G). Branching morphogenesis was compared to groups cultured in medium alone (E), and groups cultured in medium plus vehicle DMSO (F). In all Nitrofen-exposed explants a statistically significant ($p < 0.05$) reduced number of branches was observed after 4 days of culture (G, H), whereas no effects of exposure to DMSO were observed (F)[see also fig. 3. and table 2]. In addition, the size of the explants exposed to Nitrofen was clearly reduced (G, H). The effects of Nitrofen exposure *in vitro* were more severe than the effects after Nitrofen exposure *in vivo*. All pictures are representative of a series of experiments. (Scalebar represents magnification level)

Epithelial cell differentiation

In the sections of the control explants and DMSO explants, major airways and a pseudoglandular aspect were distinguished (see Figure 4A, B, E & F). In the sections of Nitrofen exposed explants, large airspaces, due to lobular branching were observed (see

Figure 4C, D, G & H). After 4 days of culture, SP-C mRNA was present in the control and DMSO exposed explants as well as in the explants exposed to Nitrofen *in vivo* (see Figure 4A, B & D). Expression was observed in the epithelial lining of the distal airways. In contrast, no expression was observed in the explants exposed to Nitrofen *in vitro* (see Figure 4C). No differences in the spatial expression pattern of SP-C mRNA were observed between DMSO, control and Nitrofen *in vivo* treated explants. After 6 days of culture, the same expression pattern was observed in the explants exposed to Nitrofen *in vitro* (results not shown). After 4 days of culture, CC-10 mRNA expression was only observed in the DMSO and control explants in the epithelial lining of the major airways (see Figure 4E & F). This time, expression was not observed in any of the explants exposed to Nitrofen (see Figure 4G & H).

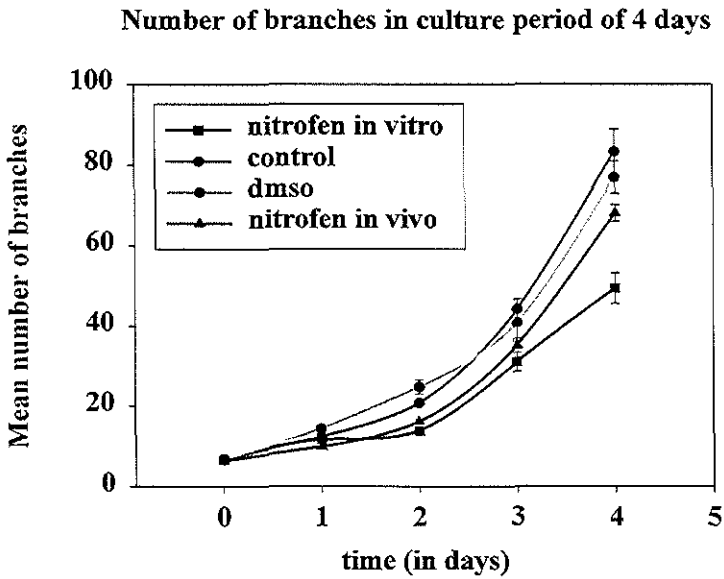


Figure 3

Branching morphogenesis plotted as a graph. Number of branches, mean ± s.e.m., is plotted against days of culture. Explants exposed to Nitrofen *in vitro* showed an arrest in branching during the first 2 days of culture, the period of exposure to Nitrofen. In addition, the rate of branching (slope) was also reduced after the first 2 days of culture during the remaining culture-period. After exposure to Nitrofen *in vivo* explants showed also a reduced number of branches after 3 days of culture and a reduced rate of branching (slope) although to a lesser extent than the ones exposed to Nitrofen *in vitro*.

Proliferation and apoptosis

To investigate whether the interplay between proliferation and apoptosis played a role in the early pathogenesis of Nitrofen-induced pulmonary hypoplasia *in vitro*, we investigated proliferation, using immunohistochemistry with an antibody against PCNA (Takasaki et al., 1981). Apoptotic cells were detected using TUNEL assay (Gavrieli et al., 1992). Proliferating cells (PCNA positive nuclei) were localized mainly in the epithelial lining of the branching terminal lung buds (see Figure 5A & B). A smaller number of proliferating cells was observed in the mesenchyme (see Figure 5A & B). In mesenchyme of the Nitrofen exposed explants, attenuated PCNA immunoreactivity was observed (see Figure 5C). Unexpectedly, in the epithelial lining of the terminal buds, PCNA protein was localized perinuclearly and in the cytoplasm, and not in the nuclei (see Figure 5C).

Table 2

Branching morphogenesis is reduced in Nitrofen exposed rat lung explants, 13 days of gestation after 4 days of culture

Treatment	No. of branches (means ± SE)	*n*	Slope
Control	83.278 (5.576)	16	0.63
DMSO	76.917 (4.008)	12	0.58
Nitrofen *in vitro*	49.385 (3.790)*	13	0.49*
Nitrofen *in vivo*	64.207 (2.099)*	40	0.60

The number of branches after 4 days of culture and the rate of branching (slope) were significantly reduced in the explants exposed to Nitrofen *in vitro*. When only the 75% of animals with lowest number of branches (corresponding to the frequency of diaphragmatic hernias in the animal model) was taken into account for the *in vivo* treated group, statistical significance was also reached for this group. However, the slope in this group remained unchanged. No difference was detected between the control explants and the explants exposed to vehicle DMSO. *n*, number of lungs taken from different fetuses of different litters and in different experiments; *, $P<0.05$

In all explants TUNEL positive cells were observed mainly in the mesenchyme, and occasionally in the epithelium. Although not quantitated, the number of apoptotic cells appeared similar in all explants, including the ones exposed to Nitrofen (see Figure 5D-F). All nuclei of the cells were shown by staining with DAPI (see Figure 5G-I).

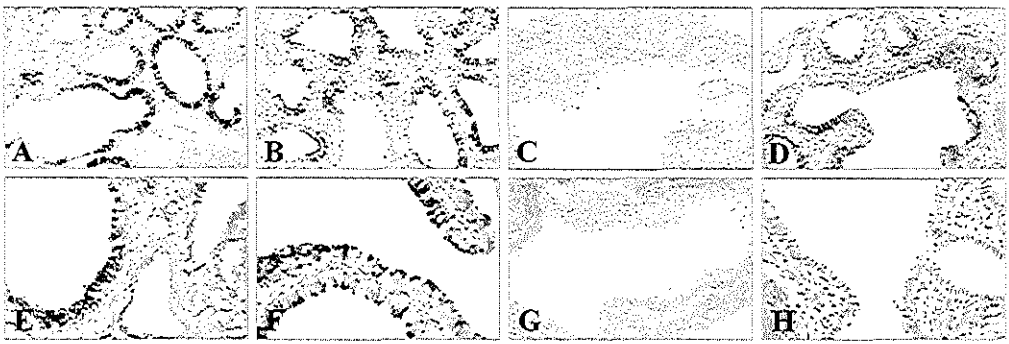


Figure 4
Expression of markers for distal and proximal epithelial cell differentiation, SP-C (upper row) and CC-10 (bottom row) mRNA respectively, in lung explants after 4 days of culture. Expression of SP-C mRNA was observed in control, DMSO and Nitrofen *in vivo* treated explants in the epithelial lining of the terminal lung buds after 4 days of culture (A, B & D). No expression was observed in the explants exposed to Nitrofen *in vitro* (C). Expression of CC-10 mRNA was observed in control and DMSO explants in the epithelial lining of the major proximal airways. (E, F). No expression was observed in the explants exposed to Nitrofen both *in vitro* (G) and *in vivo* (H). All pictures are representative of a series of experiments. [All images at same magnification]

7.5 Discussion

Despite many years of clinical efforts to find alternative treatment modalities, the mortality rate of CDH has not really changed during the past decades. A poor understanding of the pathogenesis of CDH might be partly responsible for this. It is still not clear whether the diaphragmatic defect is the cause or result of the pulmonary hypoplasia. We hypothesized that the lungs are the primary target-organ in Nitrofen-induced CDH, and in order to test this hypothesis, we separated the pathogenesis of CDH in events happening before and after (defective) closure of the diaphragm. In this study we investigated early pulmonary development in an *in vitro* modification of the Nitrofen model 4-6 days before normal closure of the diaphragm (day 17 of gestation) takes place in the rat fetus (Kluth et al., 1996). We studied the direct effects of Nitrofen on lung formation, branching morphogenesis and epithelial cell differentiation in our organ culture system.

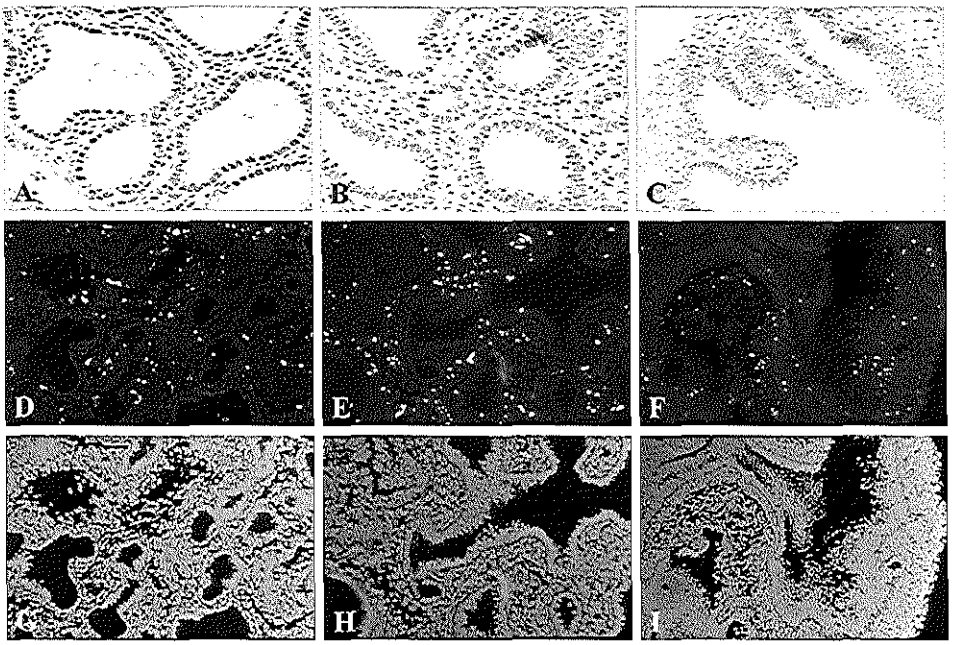


Figure 5

Following a culture period of 4 days, proliferation (A-C) and apoptosis (D-F) were investigated. Immunolocalization of PCNA indicates proliferating cells. Strong PCNA immunoreactivity was observed in the epithelial lining of the terminal buds of the control and DMSO explants, and also, but lower in the mesenchyme (A, B). Surprisingly immunoreactivity was not observed in the mesenchyme and only perinuclear in the explants exposed to Nitrofen (C). Apoptosis in the explants was assessed using TUNEL assay. TUNEL positive cells were observed in all explants in the mesenchyme only, and the number of TUNEL positive cells appeared similar in all explants (D-F). All nuclei were stained using DAPI mounting solution (G-I). All pictures are representative of a series of experiments. (All images at same magnification)

We report here that Nitrofen exposure both *in vitro* and *in vivo* reduces branching morphogenesis before the time that closure of the diaphragm would normally occur in the rat (day 17 of gestation). Only the later phases of branching morphogenesis (i.e. dichotomous branching) are affected, since initial lung bud outgrowth (i.e. primary monopodial branching) and subsequent formation of the lobar bronchi (i.e. secondary branching) were not affected in the Nitrofen-treated explants. The timing and degree of interference with branching morphogenesis by Nitrofen are in accordance with case reports of CDH in which the number of airway generations were assessed by the group of Lynne Reid (Areechon and Reid, 1963; Kitagawa et al., 1971). Both studies report a

reduction in airway generations to about half the normal number. Because development of the bronchial tree is normally complete by the 16th week of intrauterine life, timing of the insult in affected human babies should be early in gestation.

Although *in vivo* exposure to Nitrofen at first did not result in a statistically significant reduction in the number of branches, the statistical significance was observed when the lower 75% of the lungs with reduced branching were considered from among all lungs evaluated. In a previous study, we have shown that in our hands administration of Nitrofen results in a success rate of up to 80% diaphragmatic defects (Ijsselstijn et al., 1997). Therefore, the lower 75% of the lungs with reduced branching might correspond to the most severe cases of pulmonary hypoplasia. All these data indicate that Nitrofen has a direct effect on branching morphogenesis and Nitrofen treatment might thus result in hypoplastic lungs before a diaphragmatic hernia occurs during development. This implies that in case of Nitrofen-induced CDH, the existing hypoplastic lungs might even induce the diaphragmatic defect, which has been suggested before by Iritani (Iritani, 1984) and by Cilley *et al.* (Cilley et al., 1997) However, results of the study of Iritani were based on experiments performed in the presence of Nitrofen during a period from day 5 of gestation until the day of sacrifice. In addition, both studies were done in mice, and the effects of Nitrofen were investigated in an *in vivo* situation, where influences of aberrant diaphragm development cannot be ruled out. To our knowledge, our study is the first to investigate the effects of Nitrofen on lung branching *in vitro* and after a limited period of exposure to Nitrofen, which is similar to the exposure of Nitrofen in the well established *in vivo* animal model.

In addition to the effects of Nitrofen on branching morphogenesis, the epithelial cell differentiation was also disturbed. Again, the effects of Nitrofen exposure *in vitro* were more severe than exposure *in vivo*, in that SP-C mRNA expression was not observed in the explants exposed to Nitrofen *in vitro* after 4 days, but only after 6 days of culture. In the Nitrofen-treated explants no CC-10 mRNA expression could be observed. Thus, *in vitro* treatment specifically resulted in a delay of epithelial cell differentiation. Whereas, apoptotic processes did not change, when evaluated by the number and localization of TUNEL positive cells, PCNA immunoreactivity was clearly reduced in the mesenchymal component of the Nitrofen-treated explants. In addition, PCNA reactivity was only observed perinuclear and in the cytoplasm of epithelial cells of the explants exposed to Nitrofen. This unexpected result clearly indicated that proliferation is disturbed in explants exposed to Nitrofen. Consequently, a disturbed interplay between cell

proliferation and apoptosis may be contributing to the observed hypoplasia in the lung explants exposed to Nitrofen.

Taken together, these data suggest that the primary defect in Nitrofen-induced CDH is located in the lungs and that the lungs are already hypoplastic before the formation of a diaphragmatic defect in the rat model. Our results corroborate with those of Cilley *et al.* (Cilley et al., 1997), who demonstrated in their murine model that Nitrofen-induced pulmonary hypoplasia exists with or without diaphragmatic hernia. The asymmetry of both pulmonary hypoplasia and the diaphragmatic defect can be explained by the fact that there is a difference in timing of closure of the diaphragms. The right diaphragm closes a little earlier than the left (Moore, 1988). It might well be that timing of the insult resulting in hypoplastic lungs and a diaphragmatic defect is crucial and consequently that it occurs more often on the left than on the right side. Strangely, administration of Nitrofen later during gestation results in a higher percentage of diaphragmatic defects occurring on the right side (Kluth et al., 1990; Tenbrinck et al., 1990). In addition, the right lung is much bigger and consists of more lobes (4 in the rat and 3 in the human) than the left lung (1 in the rat and 2 in the human). This might result in a right lung providing a more sufficient basis for communication between the lungs and the diaphragm when the lungs grow into the pleuroperitoneal canal, which is disturbed in CDH as suggested by Iritani (Iritani, 1984). Another possibility is that CDH should be considered as the result of a shared or a separate mechanism that affects, for instance, the mesenchyme of the lungs and diaphragm in a similar way. Although these problems are not clarified yet, it is clear that the occurrence of a diaphragmatic defect and pulmonary hypoplasia requires a sensitive and crucial interplay between several factors not yet identified.

Besides isolated or familial case reports describing chromosomal aberrations, until now a genetic origin or an etiological-environmental factor for CDH has been lacking (Bos et al., 1994). Therefore, the pathogenesis of pulmonary hypoplasia in the Nitrofen model of CDH should be extrapolated to the human situation with a fair amount of reserve. We postulate that the crucial event resulting in human CDH takes place during early branching morphogenesis in a susceptible genetic environment. There have been reports of families with a higher incidence of CDH, suggesting that a genetic defect is in part responsible for the etiology of the CDH cases (Enns et al., 1998; Tibboel and Gaag, 1996). Therefore, more research efforts to unravel the genetic defects in this anomaly are justified and will lead to a better understanding of the pathogenesis of CDH.

Finally, we would like to postulate a new hypothesis describing the pathogenesis of pulmonary hypoplasia in case of CDH: the dual-hit hypothesis. In this hypothesis the pathogenesis of pulmonary hypoplasia in case of diaphragmatic hernia is explained by two developmental insults. The first insult occurs early on in development, before diaphragm development, given a still unidentified background of genetic and environmental factors.

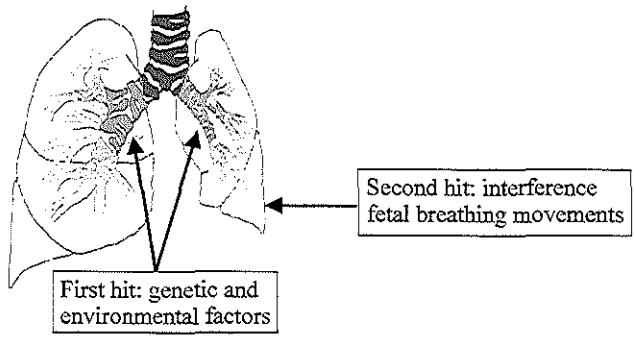


Figure 6 The Dual-hit hypothesis: pulmonary hypoplasia in case of CDH is explained by two developmental insults. The first hit affects both lungs prior to and separate from diaphragm development in a background (unidentified until now) of genetic and environmental factors. The second hit affects only the ipsilateral lung after defective development of the diaphragm because of interference of the herniated abdominal organs with fetal breathing movements of this lung.

This insult 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. In this scenario herniated abdominal organs will interfere with fetal breathing movements of the ipsilateral lung resulting in greater impairment of the development of the ipsilateral lung than of the contralateral lung (see Figure 6).

In our view, this hypothesis best describes the pathogenesis of pulmonary hypoplasia in case of CDH based on current available data, which were obtained mainly from studies in the Nitrofen model. However, more research will be necessary both in humans and in the Nitrofen model to solve the chicken-or-egg question that is still associated with this anomaly. In conclusion, in the Nitrofen model of CDH, pulmonary hypoplasia precedes the diaphragmatic defect and might even be the cause of the diaphragmatic defect, instead of the result. This insight in the pathogenesis of pulmonary hypoplasia in case of CDH leads to a different perspective on the hypoplastic lungs that are characteristic of this anomaly.

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Chapter 8

Steroid hormone receptor superfamily expression is not altered in pulmonary hypoplasia in case of Nitrofen-induced congenital diaphragmatic hernia in rats.

Based on:
Keijzer, R., Blommaart, P.J.E., Lamers, W.H., and Tibboel, D. Steroid hormone receptor superfamily expression is not altered in pulmonary hypoplasia in case of Nitrofen-induced congenital diaphragmatic hernia in rats. (submitted)

8.1 Abstract

Antenatal administration of glucocorticoids will soon be evaluated in a multicenter clinical trial as a new treatment modality for pulmonary hypoplasia associated with congenital diaphragmatic hernia (CDH). However, not much information is available about the sensitivity of hypoplastic lungs for glucocorticosteroids. We and others have suggested that changes in thyroid hormone metabolism are involved in the pathogenesis of Nitrofen-induced CDH associated with pulmonary hypoplasia. Hence, the aim of this study was to determine the expression patterns of the relevant nuclear receptors, that is, the glucocorticoid receptor (GR), the TRs (TR) alpha and beta, and their preferred partners of dimerization, the retinoid-X receptor (RXR) alpha and beta in normal and Nitrofen-induced hypoplastic lungs by *in situ* hybridization. TR alpha was specifically expressed in the developing mesenchyme and TR beta in the developing epithelium. A comparable expression pattern was observed for both the RXRs, whereas the GR was ubiquitously expressed in both germ layers. No differences in timing or localization were observed between normal and hypoplastic lungs. Consequently, clinical trials using antenatal hormonal modulation of pulmonary growth are justified in the light of steroid hormone receptor expression patterns in the hypoplastic lung. In addition, modulation of timing of steroid drugs in prenatally diagnosed CDH is not warranted based on the expression pattern of the steroid hormone receptors in the developing lung.

8.2 Introduction

During the past decades congenital diaphragmatic hernia (CDH) and its underlying pathology, pulmonary hypoplasia and persistent pulmonary hypertension, have been investigated with great effort in order to acquire new treatment modalities for this anomaly (Beresford and Shaw, 2000; Desfrere et al., 2000; IJsselstijn and Tibboel, 1998; Kays et al., 1999). Although a number of recent publications suggest significant changes in survival rate, a great variety in survival rate is still reported. One of the reasons for this lack of progress may well be that the pathogenesis of CDH is still not completely understood (Katz et al., 1998; Skari et al., 2000). Recently, two new strategies have been put forward to supplement the already broad spectrum of sophisticated treatment modalities, which include extracorporeal membrane oxygenation (ECMO) and fetal surgery. The first is prenatal fetoscopic modulation of pulmonary growth by tracheal

clipping. This approach can only be used in selected cases and is nowadays subject to an NIH sponsored trial (Harrison et al., 1998). The second is prenatal hormonal modulation of pulmonary growth using preferentially glucocorticoids (Keijzer et al., 2000). The first strategy can only be implemented in specialized centers and, moreover, has not proven to be superior to conventional treatment modalities in cases without liver up (Harrison et al., 1998). In contrast, the second strategy was adapted from a widely and successfully used therapy for the treatment of Respiratory Distress Syndrome (RDS) in Pediatrics and Obstetrics [for review see (Crowley, 2000)]. In case of imminent premature delivery the mother is treated with glucocorticoids, which dramatically reduces the risk of RDS. More recently, a debate has started about the risks and benefits of repeated dosages and the long term effects on neurodevelopmental outcome (Matthews, 2000; Smith et al., 2000). We and others have demonstrated beneficial effects of prenatal administration of glucocorticoids (with or without thyrotropin-releasing hormone) on pulmonary development in both the 2,4-dichlorophenyl-p-nitrophenyl ether (Nitrofen) rat model and the surgical sheep model for CDH (Hedrick et al., 1997; IJsselstijn et al., 1997; Losty et al., 1996; Losty et al., 1995; Schnitzer et al., 1996; Suen et al., 1994a; Suen et al., 1994b). Based on these observations, an international multicenter clinical trial is forthcoming to evaluate the effects of antenatal corticosteroids on CDH-associated pulmonary hypoplasia in humans. However, up till now no published data are available on the ontogeny of the sensitivity to hormonal modulation in developing (hypoplastic) lungs. Since corticosteroids exert their effects via specific nuclear receptors [for review see (Evans, 1988)], a study of the spatio-temporal distribution of these receptors in (hypoplastic) lungs was warranted.

In addition to this, we investigated a hypothetical explanation for the teratological effects of Nitrofen on pulmonary and diaphragm development. Nitrofen is widely used in a rodent model for CDH, in which administration of Nitrofen to a pregnant dam results in a high incidence of CDH in the progeny (Kluth et al., 1990; Tenbrinck et al., 1990). Since the original toxicological studies, the connection between Nitrofen and thyroid hormones has been made. First, the chemical structure of Nitrofen resembles that of thyroid hormones. Second, when thyroid hormones are administered in addition to the exposure to Nitrofen, the percentage of anomalies in the litter decreases (Manson et al., 1984). Recently, our group showed that the same holds true for vitamin A and Nitrofen, because antenatal treatment with vitamin A significantly reduces the incidence and severity of CDH and pulmonary hypoplasia (Thebaud et al., 1999). In addition to this, we showed

before that Nitrofen decreases binding of thyroid hormone to the alpha 1 and beta 1 form of the thyroid hormone receptor (TR) in a non-competitive way (Brandsma et al., 1994). Therefore, the purpose of this study was to investigate the mRNA expression pattern of the TR alpha and beta in normally developed lungs and compare this to the distribution of these receptors in hypoplastic lungs in case of Nitrofen-induced CDH. Others have shown that upon binding of thyroid hormone to the TR, this hormone/receptor complex binds to DNA as dimers composed of two different TRs or in combination with other receptors of the steroid hormone receptor superfamily, preferentially the 9-*cis* retinoic-acid receptors (RXRs) (Rosen et al., 1993; Wahlstrom et al., 1992; Yen et al., 1992). Therefore, the expression patterns of the RXR alpha and beta were included in our studies.

We demonstrate a specific heterogeneous spatio-temporal mRNA expression pattern for the TRs alpha and beta, the RXRs alpha and beta and the glucocorticoid receptor (GR). TR alpha mRNA was observed in developing pulmonary mesenchyme, whereas TR beta mRNA was observed in developing pulmonary epithelium. A comparable distribution was observed for the RXR alpha mRNA and beta mRNA, respectively, but for the RXR beta mRNA expression was also observed in the mesenchymal part of the lung. Finally, GR mRNA expression was ubiquitously observed in the lung, but was pronounced in the developing pulmonary epithelium. When we investigated expression of these receptor mRNAs in (hypoplastic) lungs of fetuses exposed to Nitrofen, we did not observe any changes in either localization or timing of expression of these mRNAs, indicating that Nitrofen-induced CDH is not resulting from a change in the expression neither in time or tissue localization of the investigated receptors. In addition, investigating prenatal hormonal modulation with glucocorticoids of hypoplastic lungs in CDH is justified.

8.3 Materials and methods

Animals

Adult Wistar rats were obtained from the HSD animal farm in Zeist, The Netherlands. The animals were kept in a controlled light/dark cycle and food and water were supplied ad libitum. Rats were mated at the end of the morning. The day of finding a sperm-positive vaginal smear was designated day 1 of gestation. Fetuses were delivered by Caesarian section at 15, 18, and 20 days of gestation. To induce congenital diaphragmatic hernia and pulmonary hypoplasia, 100 mg of 2,4-dichloro-phenyl-p-nitrophenylether (Nitrofen) dissolved in 1 ml olive oil, was administered to the mother rats by gavage on day 10 of

gestation (Kluth et al., 1990; Tenbrinck et al., 1990). This regime is known to result in 60 to 90 % diaphragmatic hernias and 100 % pulmonary hypoplasia in the offspring.

Tissue isolation and preparation

Fetuses of 15 (pseudoglandular stage of lung development) and 18 (canalicular stage of lung development) days of gestation were fixed in toto. Fetuses of 20 days of gestation (saccular stage of lung development) were decapitated. After removal of limbs and pelvis, their skin was removed and their body cavities were opened for optimal penetration of the fixative. All tissues were fixed in 4% phosphate-buffered formaldehyde (wt/vol) at 4°C for 16-18 hours, dehydrated in a graded series of ethanol, cleared with iso-butanol and embedded in Paraplast Plus (Monoject, Kildare, Ireland). 7 μm serial frontal sections were cut, and mounted onto RNAse-free 3-aminopropyltriethoxysilane (Sigma A3648) coated slides. From the group treated with Nitrofen, only fetuses with a diaphragmatic hernia were selected and included in this study.

Specification and preparation of RNA probes

GR cDNA (2.4 kb) was isolated from rat liver and recloned in pBluescript SK+ in the BamHI restriction site of the multiple cloning site (kind gift of Dr. Paul Godowski). The antisense probe was generated with T3 RNA polymerase after linearization with PstI. As probes for the TR alpha and beta mRNA detection, EcoRI-HindIII cDNA fragments of the clones rc-erbA-α (nucleotide -29 to nucleotide +1821) and rc-erbA-β (nucleotide -37 to nucleotide +2165) were used, respectively. The fragments contain the coding region of the gene and recognize both the TR alpha1 and TR alpha2 and TR beta1 and TR beta2 isoforms. Both cDNA fragments were obtained from H.C. Towle (Bradley et al., 1992; Murray et al., 1988). The [α-^{35}S]UTP labeled anti-sense probes for both TR alpha and beta were generated with Sp6 RNA polymerase, after linearization of the plasmids with HindIII. RXR alpha and RXR beta mRNAs were detected using an EcoRI-EcoRI cDNA fragment (1850 bp) and a EcoRI-HindIII cDNA fragment (1360 bp), respectively, containing the coding sequences obtained by anchored PCR from a total cDNA library of mouse embryos of 11 and 14 days of gestation (Leid et al., 1992). The RXR alpha cDNA fragment was subcloned from the expression vector pSG5 into the EcoRI site of pBluescript SK+ and the RXR beta cDNA fragment from the expression vector pTL1 into EcoRI and HindIII sites of pBluescript SK+. The [α-^{35}S]dUTP labeled anti-sense probes of RXR alpha and RXR beta were generated

with T7 RNA polymerase after linearization of the plasmids with PstI and EcoRI, respectively.

All plasmids were linearized by enzyme digestion using enzymes of Gibco/BRL (Life Technologies B.V., The Netherlands). Radioactively labeled RNA-transcripts were obtained using either T3, T7 (Gibco/BRL, Life Technologies, The Netherlands) or Sp6 (HT Biotechnology ltd., Cambridge, England) RNA polymerases in the presence of 50 μCi [α-^{35}S]UTP (Amersham, United Kingdom). After degradation of the full-length transcripts by alkaline hydrolysis, the fragments were purified by phenol-chloroform extraction and ethanol precipitation.

In situ hybridization

In situ hybridization was carried out as described (Moorman et al., 2000). In short, after deparaffination in xylene and rehydration in a graded series of ethanol, sections were treated with 0.2 M HCl for 20 minutes at room temperature (RT). Subsequently, the sections were washed in bidistilled water and incubated in 2x standard saline citrate (SSC; 1x SSC contains 0.15 M NaCl and 0.015 M Na citrate, pH 7.2) for 10 minutes at 70°C. This step improves pepsin digestion, unfolding of the target RNA, and RNA accessibility in the tissues in the next steps. The proteins in the sections of rat fetuses were digested with pepsin (Sigma P7000); 0.1% (wt/vol) in 0.01 M HCl for 7 minutes (15 days of gestation), for 10 minutes (18 days of gestation) and for 15 minutes (20 days of gestation) at 37°C. Digestion was stopped with 0.2% (vol/vol) glycine in phosphate-buffered saline (PBS), 30 seconds at RT. The sections were postfixed in 4% phosphate-buffered formaldehyde (wt/vol) for 20 minutes at RT, treated with 10 mM EDTA for 5 minutes at RT to dissociate the ribosomes from the RNA and incubated in 10 mM dithiothreitol (DTT) for 5 minutes at 4°C to reduce the tissue and therefore lower the background. Subsequently, the sections were air-dried and hybridized with the [^{35}S]-labeled anti-sense RNA probes for 16-18 hours at 54°C in a mixture containing 50% (vol/vol) deionized formamide, 10% (wt/vol) dextran sulphate, 2x SSC, 2x Denhardt's, 0.1% (vol/vol) Triton X-100, 10 mM DTT and 200 ng.μl^{-1} herring sperm DNA. The probe concentrations were approximately 50 pg.μl^{-1} hybridization mixture and 6-12 μl of hybridization mixture was applied per section. The specific activity of the anti-sense RNA probe was approximately 500 dpm.pg^{-1}. After hybridization the sections were washed and treated with RNAse A (10 μg/ml in 10 mM Tris-HCl (pH 8.0), 5 mM EDTA and 500 mM NaCl) for 30 minutes at 37°C to reduce nonspecific binding. The sections were dehydrated in a graded series of ethanol containing 0.3 M ammonium acetate

and air-dried. Subsequently, the sections were exposed to photographic emulsion (Ilford Nuclear Research Emulsion G-5). After developing, the sections were counterstained with nuclear fast red, dehydrated in a graded series of ethanol and xylol and mounted in Malinol (Chroma-Gesellschaft, Schmidt Gmbh+Co, Köngen, Germany).

8.4 Results

Expression of glucocorticoid receptor mRNA during normal pulmonary development

The lung primordium, an outgrowth of foregut endoderm, appears in rat embryos at 10 to 11 days of gestation. Subsequently, the primordium develops into two lung buds. During the next two days, dichotomous branching forms conducting airways, while the pulmonary epithelium is growing into a conspicuous mass of mesenchyme. In control rats, expression of GR mRNA was first observed in the endodermal part of the developing conducting airways at 15 days of gestation (Figure 1A). From 18 days of gestation, GR mRNA expression was observed more ubiquitously in both epithelium and mesenchyme, albeit more pronounced in the epithelium (Figure 2A). A similar, but more faint distribution was observed at 20 days of gestation (Figure 3A).

Expression of retinoid-X receptor alpha and retinoid-X receptor beta mRNAs during normal pulmonary development

At 15 days of gestation, mRNA expression of both RXRs was observed in both mesenchyme and epithelium. However, RXR alpha mRNA expression was more pronounced in the mesenchyme (Figure 1C), whereas RXR beta mRNA expression was more pronounced in the epithelium (Figure 1E). Three days later in gestation, RXR alpha mRNA was observed predominantly in the mesenchyme (Figure 2C) and RXR beta mRNA in the epithelium (Figure 2E). At 20 days of gestation, distinguishing between mesenchymal and epithelial distribution of RXR mRNAs was more difficult, because of thinning of mesenchymal tissue. However, both RXR mRNAs appeared to be distributed over both germlayers, but again, RXR alpha was more pronounced in the mesenchyme (Figure 3C), and RXR beta in the epithelium (Figure 3E).

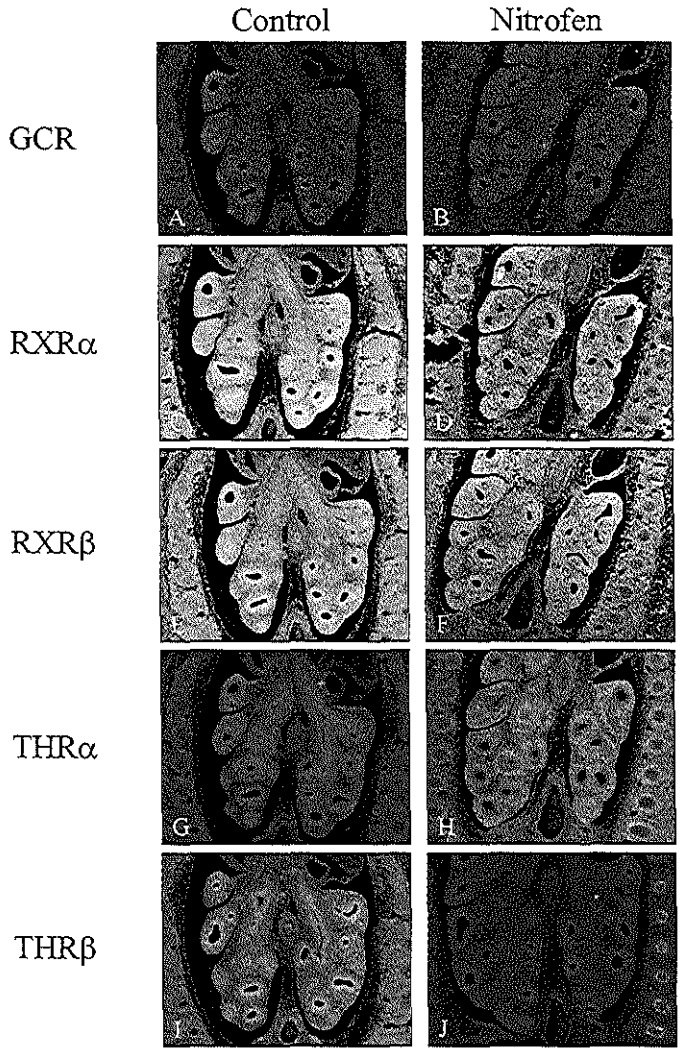

Figure 1 Steroid hormone receptor expression in normal and Nitrofen-induced hypoplastic lungs at 15 days of gestation. GR (A,B), RXR alpha (C,D), RXR beta (E,F), TR alpha (G,H) and TR beta (I,J) expression in normal (A,C,E,G,I) and hypoplastic (B,D,F,H,J) lungs at 15 days of gestation. GR mRNA is observed in the endoderm lining the branching airways (A,B). RXR alpha mRNA (C,D) and RXR beta mRNA (E,F) were observed in both germ layers, but the former was more pronounced in the mesenchyme, whereas the latter was more pronounced in the epithelium. TR alpha mRNA (G,H) was exclusively expressed in the mesenchyme. In contrast, TR beta mRNA (I,J) was exclusively expressed in the epithelium. No differences in steroid hormone receptor expression were observed between normal and hypoplastic lungs. All pictures are representative of a series of experiments and were taken at the same magnification.

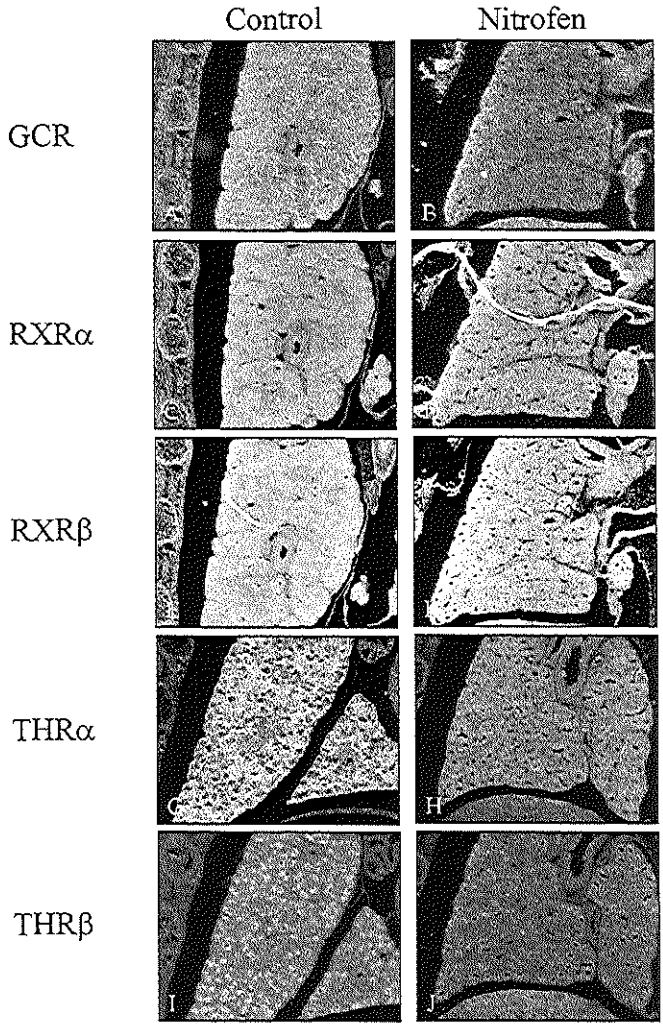

Figure 2 Steroid hormone receptor expression in normal and Nitrofen-induced hypoplastic lungs at 18 days of gestation. GR (A,B), RXR alpha (C,D), RXR beta (E,F), TR alpha (G,H) and TR beta (I,J) expression in normal (A,C,E,G,I) and hypoplastic (B,D,F,H,J) lungs at 18 days of gestation. At this stage, GR mRNA was faintly expressed in the entire lung (A,B). RXR alpha mRNA expression (C,D) appeared higher in the mesenchyme, whereas RXR beta mRNA (E,F) was predominantly observed in the epithelium. Again, TR alpha mRNA (G,H) was exclusively expressed in the mesenchyme at this stage. In contrast, TR beta mRNA (I,J) was exclusively expressed in the epithelium. No differences in steroid hormone receptor expression were observed between normal and hypoplastic lungs. All pictures are representative of a series of experiments and were taken at the same magnification.

Expression of TR alpha and TR beta mRNAs during normal pulmonary development

From 15 to 18 days of gestation, during the canalicular stage of pulmonary development, expression of TR beta mRNA became detectable, predominantly in the pulmonary epithelium of the distal conducting airways (Figures 1I & 2I). Expression of TR alpha mRNA was confined to the pulmonary mesenchyme surrounding the conducting airways (Figures 1G & 2G). At 20 days of gestation the prominence of the interstitial tissue is decreased (Figure 3). At this time-point expression of TR beta mRNA was more difficult to observe in the pulmonary epithelium (Figure 3I), whereas expression of the TR alpha mRNA remained present in the developing pulmonary mesenchyme (Figure 3G). An accurate description of the expression patterns became more difficult near term because the walls of the airways become very thin due to expansion of the gas-exchange surface. Expression of TR beta mRNA was observed at all time points in pulmonary arteries (Figures 1I, 2I & 3I), while expression of TR alpha mRNA was continuously observed in pulmonary veins (Figures 1G, 2G & 3G).

Expression of thyroid hormone receptor alpha, thyroid hormone receptor beta, retinoid-X receptor alpha, retinoid-X receptor beta and glucocorticoid receptor mRNAs during abnormal pulmonary development in case of congenital diaphragmatic hernia

In approximately 60-90% of the fetuses a left-sided diaphragmatic hernia with pulmonary hypoplasia and herniated abdominal organs (in particular the liver) developed upon treatment of the dams with Nitrofen at day 10 of pregnancy. For all investigated receptors, an expression pattern comparable to that observed in normally developed lungs was observed (Figures 1B, D, F, H, J; Figures 2B, D, F, H, J and Figures 3B, D, F, H, J). Both timing of expression and localization of the mRNAs were similar in the normal and hypoplastic lungs. In addition, no differences were observed in expression patterns of the receptors between the ipsilateral and contralateral lung in case of congenital diaphragmatic hernia.

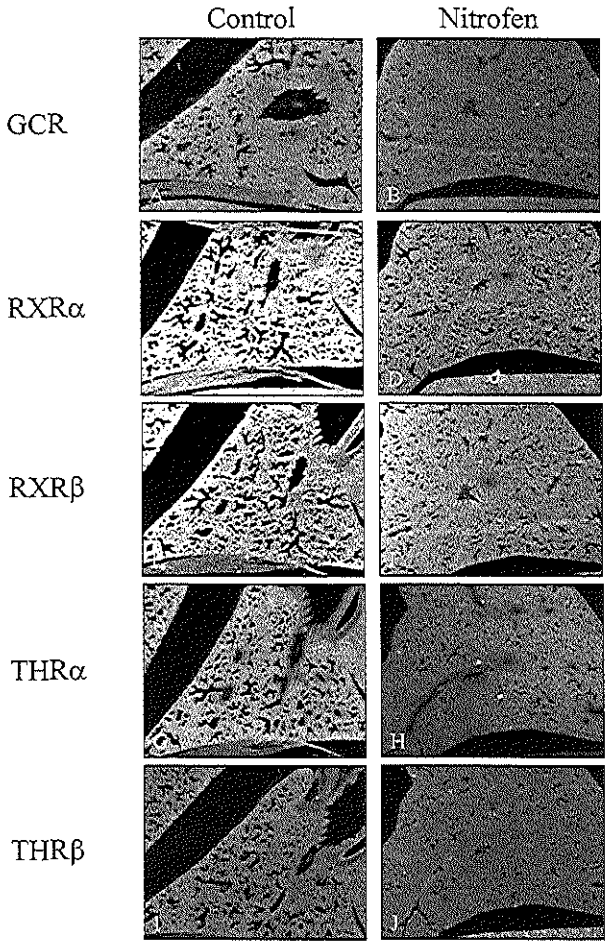

Figure 3 Steroid hormone receptor expression in normal and Nitrofen-induced hypoplastic lungs at 20 days of gestation. GR (A,B), RXR alpha (C,D), RXR beta (E,F), TR alpha (G,H) and TR beta (I,J) expression in normal (A,C,E,G,I) and hypoplastic (B,D,F,H,J) lungs at 20 days of gestation. Because of thinning of mesenchymal tissue, differences between expression in the mesenchyme and epithelium were more difficult to distinguish at this stage. GR mRNA was faintly expressed in the entire lung (A,B). RXR alpha mRNA expression (C,D) and RXR beta mRNA (E,F) were appeared more evenly distributed at this stage. Again, TR alpha mRNA (G,H) was exclusively expressed in the mesenchyme at this stage. In contrast, TR beta mRNA (I,J) was exclusively expressed in the epithelium. No differences in steroid hormone receptor expression were observed between normal and hypoplastic lungs. All pictures are representative of a series of experiments and were taken at the same magnification.

8.5 Discussion

A multicenter clinical trial incorporating 300 prenatally diagnosed CDH-fetuses to evaluate the therapeutical benefits of antenatal hormonal modulation on pulmonary growth in case of CDH-associated hypoplastic lungs is underway. The study was inspired by the promising results obtained with prenatal glucocorticoids in both the Nitrofen rat and surgical sheep model for CDH. However, the presence of the GR in hypoplastic lungs has never been shown despite speculations about it.

In this study, we demonstrate that the GR is indeed expressed in a similar fashion in Nitrofen-induced hypoplastic lungs as in normally developed lungs. This finding justifies the start of a multicenter trial to evaluate the beneficial effects of prenatal glucocorticoids on pulmonary growth. Awaiting the results of the trial we speculate that this form of antenatal lung "surgery" may offer a good contribution to the array of treatment modalities for this poorly treatable anomaly.

Since the first teratological studies with Nitrofen, a relation between Nitrofen and thyroid hormone metabolism has been suggested (Manson et al., 1984). In order to gain more insight in the pathogenesis of the Nitrofen model for CDH, we performed *in situ* hybridization studies to localize transcripts of the TRs. TRs belong to the steroid hormone receptor superfamily and bind preferably to DNA as dimers composed of two different TRs or in combination with other receptors of the superfamily, the 9-*cis* retinoic-acid receptors (RXRs) (Rosen et al., 1993; Wahlstrom et al., 1992; Yen et al., 1992). Interestingly, a link between vitamin A and CDH has been made as early as 1953 when Wilson et al. reported a high incidence of CDH in offspring from rats on a vitamin A-deficient diet (Wilson et al., 1953). Recently, vitamin A was again related to CDH in a report from Thébaud et al. Their studies showed that antenatal treatment with vitamin A has a positive effect on the incidence and severity of CDH in the Nitrofen rat model (Thebaud et al., 1999).

In this manuscript, we report specific heterogeneous spatio-temporal expression patterns for both the TR alpha and beta and the RXR alpha and beta. The TR alpha is expressed in the mesenchyme throughout development of the lung and the TR beta is expressed in the epithelium during the late pseudoglandular and canalicular phase of lung development. The expression patterns were not changed in hypoplastic lungs of fetuses with Nitrofen-induced CDH. This clearly indicates that induction of pulmonary hypoplasia in Nitrofen-induced CDH is not the result of an alteration in expression of the investigated receptors. This is in contrast to a study from Causak et al., who reported

reduced expression of a part of the TR gene, which was found by differential display (Causak et al., 1998). Another report by Tovar et al. showed decreased plasma T3 and T4 levels without changes in thyroid stimulating hormone (TSH). However, the levels of T3, T4 and TSH were not changed in tissues (Tovar et al., 1997). Hence, the conclusion of their study was that Nitrofen-induced pulmonary hypoplasia is not related to a decrease in tissue thyroid hormone levels. Since we found no changes in TR expression, our study complements these results.

An explanation for this could be that redundant factors mask the interference of Nitrofen with thyroid hormone metabolism. The TRs belong to the steroid/TR superfamily of ligand-dependent transcription factors, which share highly conserved DNA-binding domains (Evans, 1988). It is therefore possible that one receptor of the superfamily replaces another receptor of which the function is hampered by for instance Nitrofen. This would explain the normal tissue levels of thyroid hormone and unaltered TR expression in Nitrofen-induced hypoplastic lungs.

Taken together, our data suggest that hypoplastic lungs are as sensitive to corticosteroids as normally developed lungs as for the expression of the glucocorticoid receptor is concerned. This observation justifies the initiated multicenter clinical trial in which the effects of prenatal hormonal modulation of pulmonary growth in CDH are evaluated. Furthermore, we demonstrate that an alteration of TR expression is not causing pulmonary hypoplasia in case of Nitrofen-induced congenital diaphragmatic hernia.

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Part IV

GENERAL DISCUSSION AND SUMMARY

Chapter 9

General discussion

9.1 Introduction

The description of the DNA double helix structure by Watson and Crick in the fifties (Watson and Crick, 1953) began the era of molecular biology. Since the seventies when it became possible to manipulate pieces of DNA and study animal models for human genetic diseases (and congenital anomalies). This completely new research line in the life sciences has resulted in a better comprehension of many biological processes. In particular neuroscience and developmental biology have benefited from these approaches. For example, we are increasingly able to understand the morphogenesis of complex organs and structures such as the lungs and limbs [for review see (Hogan, 1999)]. In sharp contrast with this is the relatively minimal progress that has been made in understanding abnormal developmental processes: congenital anomalies of organs which result in a high mortality and increasing long term morbidity in childhood and sometimes even into adulthood. One of the major congenital anomalies, congenital diaphragmatic hernia and the associated pulmonary hypoplasia forms the basis of this thesis. With an average prevalence of approximately 1 in 3000 newborns (Torfs et al., 1992), CDH is a common problem in children's hospitals. Despite the large arsenal of sophisticated and available treatment modalities, such as inhalated Nitric Oxide (NO) with or without High Frequency Oscillation (HFO), ECMO and liquid ventilation, children with early onset of respiratory insufficiency in CDH still experience a high mortality. The clinical problems in case of CDH: pulmonary hypoplasia, persistent pulmonary hypertension and the sequelae of artificial ventilation are responsible for this. Hence, the studies described in this thesis were designed to gain more insight into developmental regulation of normal and abnormal pulmonary development in CDH. Led by the philosophy that one first needs to understand normal development in order to better understand abnormal development, a large portion of the studies were designed to increase our understanding of developmental processes of normal pulmonary formation. The aims of the described studies were as follows:

1. To specify the role of the transcription factor GATA6 during fetal pulmonary development;
2. To investigate the relative role of and the balanced interplay between proliferation and apoptosis during normal lung morphogenesis and abnormal pulmonary development;

3. To evaluate the role of the thyroid hormone receptors during fetal organogenesis and pulmonary development;
4. To examine the direct effects of Nitrofen on pulmonary development, and the relation to the diaphragmatic defect in CDH;
5. To investigate the expression of members of the steroid hormone receptor superfamily in Nitrofen-induced hypoplastic lungs and compare this with normal lungs.

9.2 Interpretations and implications of the studies

The transcription factor GATA6 has been implicated in pulmonary endoderm specification (Morrisey et al., 1998; Whitsett and Tichelaar, 1999) (see also Figure 1). This assumption was based on data in the literature describing the expression pattern of GATA6 in mice, one chimeric experiment in mice and extrapolation of results for GATA homologues obtained in lower organisms, such as *C. Elegans* (Kalb et al., 1998; Morrisey et al., 1996; Morrisey et al., 1998). Using a chimeric approach again, we found that GATA6 is not involved in pulmonary endoderm specification, but plays a more extensive role in branching morphogenesis and epithelial cell differentiation of the lung. The discrepancy between our results and the results of the other group could be explained by the different approach we took to generate the chimeric mice. As opposed to the usual chimeric experiment (Hogan et al., 1994), we used up to double the amount of mutant embryonic stem cells in order to generate highly chimeric embryos. This enabled us to investigate the role of GATA6, because absence of GATA6 resulted in abnormally branched and differentiated endoderm or epithelium.

In our study we did not address some important remaining questions concerning GATA6 and pulmonary development. For instance, we could not conclude whether the observed phenotype was due to direct effects of missing GATA6 in the endoderm, or was the result of secondary effects through defective epithelial/mesenchymal signaling. To investigate this aspect, tissue recombination studies would be informative. For example, epithelium derived from GATA6-/- lungs can be recombined with wild-type mesenchyme, and vice versa. Another important issue which was not addressed in our studies is the question which genes in the developing lungs are the target for transcriptional regulation by GATA6. Are only the known "master" genes for pulmonary development, such as FGF-10, BMP-4 and SHH, involved or do other, not yet identified

genes, play an important role? Of interest is the fact that, GATA6 is the only GATA factor expressed in pulmonary endoderm, whereas another GATA factor, namely GATA5 is expressed in pulmonary mesenchyme (Morrisey et al., 1997). To date, no relationship between these two GATA factors have been demonstrated in relation to pulmonary development.

Following another approach, we used a lung specific promoter to overexpress GATA6 in distal pulmonary epithelium during pulmonary development. We found that overexpression of GATA6 results in abnormal lung development characterized by disturbed branching and epithelial cell differentiation. These results suggest that overexpression of GATA6 in pulmonary endoderm leads to a block of differentiation in this endoderm and are in accordance with results from studies in which other GATA factors have been overexpressed (Briegel et al., 1993; Hendriks et al., 1999; Pevny et al., 1991; Whyatt et al., 1997; Zheng and Flavell, 1997). However, this study was not conclusive in determining the exact mechanism by which GATA6 acts during pulmonary development, nor in identifying the target genes that are transcriptionally regulated by GATA6 during pulmonary development. Hence, the two studies on GATA6 and pulmonary development described in this thesis have partly revealed the role of GATA6 in fetal pulmonary development and more studies on this subject are currently planned in our laboratory.

In another study we investigated the interplay between proliferation and apoptosis during morphogenesis of the lung. Employing a cell recombinant model for fetal pulmonary development, we demonstrated that both proliferation and apoptosis are developmentally regulated, and in addition, that mesenchymal apoptosis during late pulmonary development occurs as the result of a loss of inhibition by the neighbouring epithelium. Although this study was in principle descriptive, the results clearly indicate that for normal lung morphogenesis to occur, a tight regulation of the ratio between proliferating and apoptotic cells is required. Our main finding is that the massive apoptosis that normally occurs in prenatal mesenchymal cells is due to intrinsic properties, but can be inhibited by immature epithelial cells. We did not identify any molecular factors involved in either proliferation or apoptosis, and therefore, more studies aimed at unraveling the molecular mechanisms involved in these processes are warranted.

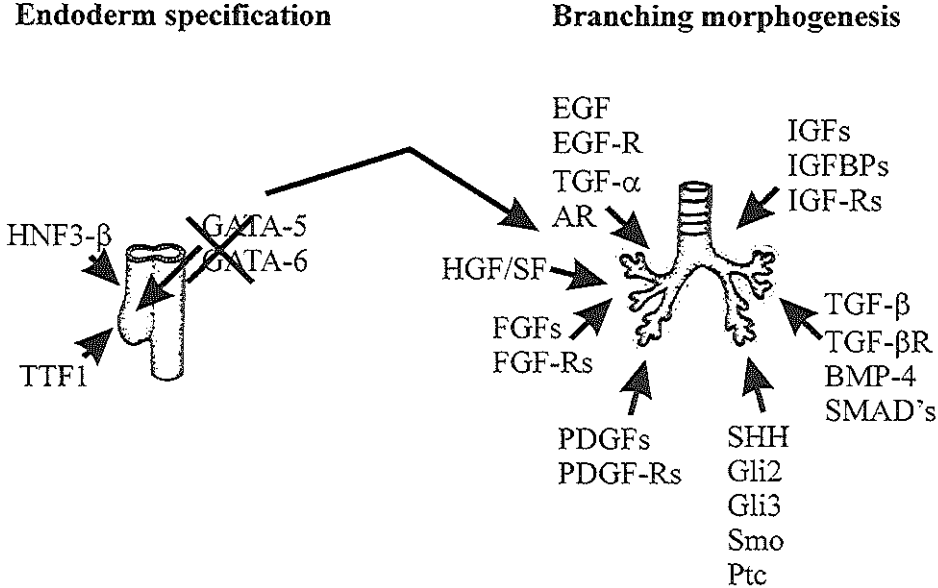

Figure 1

Based on chimeric experiments using GATA-6-/- ES cells, we demonstrate that GATA-6 is not involved in pulmonary endoderm specification, but branching morphogenesis and epithelial cell differentiation of the lung.

In the final study on aspects of normal pulmonary development, we described the specific heterogeneous expression of thyroid hormone receptors both at the mRNA and protein level during (pulmonary) organogenesis. We found that thyroid hormone receptor α mRNA was expressed in developing mesenchyme, whereas thyroid hormone receptor β mRNA was predominantly expressed in developing epithelia of the investigated organs. This result suggests that thyroid hormone receptors are very important for epithelial-mesenchymal interactions. Up until now it was clear that thyroid hormones play an important role during organogenesis, but whether the effects of thyroid hormones were direct or indirect was not established. Our study indicates that the dynamic expression of the different isoforms of the thyroid hormone receptors might be the underlying mechanism. All different isoforms (plus the combinations) of the thyroid hormone receptors have been knocked out in transgenic mice (Fraichard et al., 1997; Gauthier et al., 1999; Weiss et al., 1997; Wikstrom et al., 1998). Surprisingly, these mutant mice exhibit very mild phenotypes. These results were not anticipated because the absence of thyroid hormone results in severe disturbance of normal development in particular of the central nervous system after birth.

The toxicity of unoccupied thyroid hormone receptors that are normally expressed in the absence of thyroid hormone may have negative influences on normal organogenesis. However, when the thyroid hormone receptors are mutated, the effects are relatively mild.

Another very interesting aspect of our study was the large discrepancy between the mRNA expression patterns and the protein expression patterns. In some organs high levels of mRNA were measured, whereas no protein could be detected, and in other organs the opposite situation was observed. This observation suggests that besides the dynamic expression of thyroid hormone receptors, the effects of thyroid hormones on organogenesis are regulated at the translational and posttranslational level. Although another study had already demonstrated that the mRNA content and thyroid hormone binding capacity are different (Strait et al., 1990), we demonstrated for the first time that the distribution of thyroid hormone receptor mRNA can be very different from the distribution of its protein. Despite its descriptive character, this study enhances our knowledge on how thyroid hormones and their receptors influence normal organogenesis. More detailed studies on translational and posttranslational regulation of thyroid hormone receptor expression during fetal development are warranted, however, they are beyond the scope of this thesis.

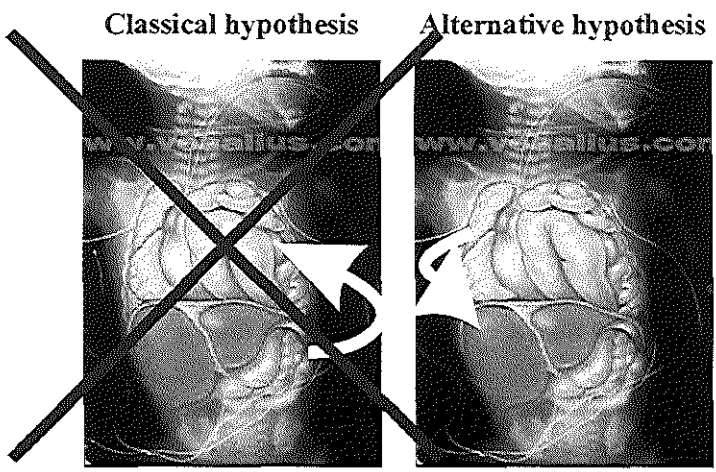

Figure 2 Based on our experiments, pulmonary hypoplasia in case of Nitrofen-induced CDH can only be explained by the alternative hypothesis.

In the third part of this thesis some aspects of abnormal pulmonary development are described. In the first study we utilized the Nitrofen rat model for CDH to investigate abnormal pulmonary development and the direct effects of Nitrofen on pulmonary development. With the first description of the Nitrofen model in mice by Iritani in 1984, it was suggested that the lungs are the primary target organ in CDH, and that abnormal development of the lungs might be the cause of abnormal diaphragm development (Iritani, 1984). This formed the basis for the alternative view on pulmonary hypoplasia associated with CDH. However, because of the sustained administration of Nitrofen in the experiments of Iritani, his alternative view was not generally accepted. Our results are conclusive in identifying pulmonary hypoplasia as the primary defect at least in the Nitrofen model of CDH (see Figure 2). Using an organ explant system we investigated Nitrofen induced pulmonary hypoplasia separate from abnormal diaphragm development. We were able to demonstrate that Nitrofen directly influences pulmonary development in a negative manner. The results are consistent and supported by the results of other studies that were published around the same time using the same animal model (Guilbert et al., 2000; Jesudason et al., 2000).

As a consequence of our *in vitro* organotypic experiments, pulmonary hypoplasia in case of Nitrofen-induced CDH can be explained by the dual-hit hypothesis (see Figure 3). The first hit occurs prior to and separate from abnormal diaphragm development in an, up to this moment, unidentified environment of genetic and environmental factors. This hit affects both lung anlagen in a similar way and results in diminished branching morphogenesis. The second hit takes place after occurrence of the diaphragmatic defect and affects only the ipsilateral lung with resulting higher severity of pulmonary hypoplasia. It is the result of interference of the herniated abdominal organs with fetal breathing movements, which are required for normal pulmonary development. In our opinion based on the currently available data, this hypothesis best explains pulmonary hypoplasia associated with CDH. However, the group of Greer advocates a different opinion. Based on their studies on embryological development of the diaphragm in the Nitrofen-model, they state that the early diaphragm, in particular the pleuroperitoneal fold (PPF), is the primary affected structure in Nitrofen-induced CDH (Allan and Greer, 1997). The PPF is a pyramid-shaped tissue that extends medially from the lateral cervical wall to the esophageal mesentery and fuses ventrally with the septum transversum. Three-dimensional reconstruction studies demonstrated that the malformations of the PPF were limited to the dorsolateral part of the caudal regions of the PPF, the region where the diaphragmatic defect is localized in CDH (Greer, et al., 2000). It

is tempting to suggest that all these results can be explained and combined by considering pulmonary hypoplasia and the diaphragmatic defect as part of a syndrome called CDH.

Extrapolation of results obtained in the Nitrofen model to the human situation should be done with a certain amount of reserve, since they are based on the teratogenic effects of Nitrofen as a CDH model. At this moment no direct correlation between Nitrofen as a teratogen and the etiology of human CDH can be made. Another point of criticism of our and all other studies using Nitrofen to induce CDH is that the exact working mechanism of Nitrofen remains obscure. Alterations in thyroid hormone metabolism have been held responsible for the teratogenic effect, however, there is still no direct evidence available for this. To come to an explanation of the mechanism of action of Nitrofen it will be very helpful when the target genes of Nitrofen are known. In addition, since there is a different sensitivity for Nitrofen between different rat and mice strains it should be relatively easy to employ techniques such as quantitative trait loci in order to clone the responsible gene(s). At the present time, such a laborious project is probably too big for the different small research groups working on this subject worldwide.

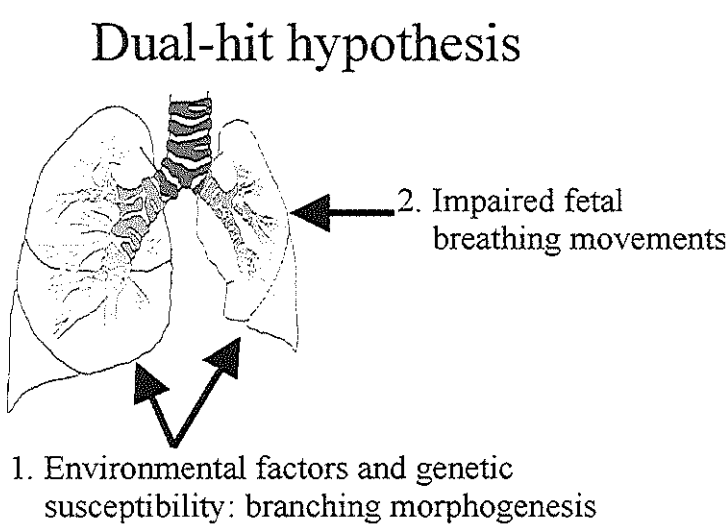

Figure 3
Dual-hit hypothesis explains pulmonary hypoplasia in case of CDH with two insults. The first affects both lungs before (abnormal) diaphragm development occurs. The second affects only the ipsilateral lung and occurs after the diaphragmatic defect has occurred.

In the second study we touched on the mechanism of action of Nitrofen. As stated before, our group has previously demonstrated that Nitrofen decreases the binding of thyroid hormone to its receptor *in vitro* (Brandsma et al., 1994). Combining Nitrofen administration with thyroid hormone results in a decrease in observed abnormalities (Manson et al., 1984) which led us to hypothesize that Nitrofen influences expression of the thyroid hormone receptors. Using in situ hybridization, we could not demonstrate a change in distribution or expression of the thyroid hormone receptors and their preferred partners of dimerization, the retinoid-X receptors. In our studies we included another member of the steroid hormone receptor superfamily, namely the glucocorticoid receptor. The implementation of antenatal hormonal modulation with corticosteroids as a suggested new treatment modality for CDH-associated pulmonary hypoplasia was another argument. From the fact that we did not observe any major changes in localization or expression of all investigated receptors between control and Nitrofen lungs, we concluded that Nitrofen does not function through alteration of the expression of these members of the steroid hormone receptor superfamily. Furthermore, it is not necessary to alter the proposed protocol for antenatal hormonal modulation in case of hypoplastic lungs, since there is no change in the expression of the glucocorticoid receptor in these lungs.

9.3 Future research

As stated in the introduction, molecular approaches and animal models have increased our understanding of many basic biological processes, such as organogenesis of the lung. Recently, new milestones in biology have been accomplished with the completion of the human genome sequence. The knowledge gained from the human genome will have an enormous impact on how research will be done in the near future. And although the function of all the genes is not known yet, the cloning of genes responsible for certain processes such as organogenesis, but also for diseases and congenital anomalies will be done behind the personal computer, instead of the laboratory bench. This will certainly alleviate the research aimed at understanding multifactorial diseases or anomalies such as CDH.

Another promising new technique is the use of micro arrays or "gene chips", which is currently establishing a stable position in the laboratory (Schena et al., 1995). This will make it easily possible to compare sets of genes from affected (for instance CDH) and unaffected (control) tissues, thereby identifying the genes responsible for the observed

phenotypes. Preliminary experiments have been undertaken with this new technique in the CDH model (Malah Chinoy, personal communication). However, the biggest problem of this technique at present, is the difficulty in analyzing and interpreting the huge amount of information that is generated. Bioinformatics will probably bring solutions for this in the near future.

The final promising recent discovery, which is in my opinion of importance for future research in the field of lung development is the use of human or mouse (embryonic) stem cells. It is becoming increasingly clear that (embryonic) stem cells can be forced to take on the identity of almost every cell of the human body. Although there is no data available yet on the forced differentiation of stem cells into pulmonary cells, I believe that this will be possible in the near future. It is tempting to speculate whether it would come to the point that we understand the process of lung development and stem cell differentiation in such a way that we are able to grow lungs in culture starting with embryonic, but preferably somatic stem cells. Although I am aware that we are at present not even able to mimic three-dimensional growth in culture, let alone the growth of complete organs, I believe that it will be possible in the not so far away future.

Finally I would like to put these "science fiction" ideas into a bit more down-to-earth perspective. One should not forget that at present we still do not know which genes are essential for the transition of a group of endoderm foregut cells into primary endodermal lung buds. Some of the lung "master" genes appear to have been identified. However, FGF-10 which is thought to be one of the pivotal genes is not necessary for development of the trachea, indicating that the earliest onset of lung development occurs normally in these mice (Min et al., 1998; Sekine et al., 1999). Moreover, we do not even know which particular group of cells from the foregut are responsible for the formation of the future lung. Therefore, a start has to be made in the process of identifying the cells of the foregut that are responsible for the formation of the future lung. This will provide new insights into very early pulmonary development. As a result we will be able to better understand the processes involved in lung organogenesis, and I believe that such an understanding will eventually lead to the prevention and improved treatment of congenital lung anomalies.

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Chapter 10

Summaries

10.1 Summary

Pulmonary hypoplasia, underdevelopment of the lung, remains an important cause of mortality and morbidity in the Neonatal Intensive Care Unit. It is either an isolated entity based on for example oligohydramnios, or part of a larger problem, such as Congenital Diaphragmatic Hernia (CDH). The minimal improvement of outcome following intensive treatment of pulmonary hypoplasia associated with CDH might be partly due to poor understanding of the processes involved in normal pulmonary development. Consequently, the mechanisms by which disturbances in normal pulmonary development result in pulmonary hypoplasia are not understood at all.

This thesis consists of four parts: *Part I* (Chapter 1 and 2) forms the introduction, *Part II* (Chapter 3 to 6) describes the studies on mechanisms of normal pulmonary development, *Part III* (Chapter 7 and 8) describes the studies on mechanisms of abnormal pulmonary development associated with CDH, and *Part IV* (Chapter 9 and 10) contains the discussion and summaries.

Chapter 1 reviews the literature on various aspects of normal pulmonary development. First, the respiratory structures of several organisms are described and compared. Next follows a description of the developmental anatomy and embryology of normal mammalian pulmonary development, starting from pulmonary endoderm specification. Finally, the most important genes, transcription and growth factors, extracellular matrix proteins and hormones involved in normal pulmonary development are discussed.

Chapter 2 introduces aspects of abnormal pulmonary development, and in particular pulmonary hypoplasia associated with CDH. The pathogenetic and clinical background of CDH and pulmonary hypoplasia is discussed, and the animal models used for investigation of this congenital anomaly are reviewed. The chapter ends with the aims of the studies and the questions addressed in this thesis.

Chapter 3 and Chapter 4 describe studies on the role of the transcription factor GATA6 during normal pulmonary development. The expression pattern of GATA6 mRNA, predominantly in the pulmonary endoderm and epithelium, suggested involvement of GATA6 in branching morphogenesis of the lung. Antisense

oligonucleotides against GATA6 abrogated branching morphogenesis of organotypic lung explants. This role of GATA6 in branching morphogenesis was more extensively investigated with loss-of-function experiments using GATA6-/- chimeric embryos. The lungs of these embryos showed diminished branching morphogenesis, both *in vitro* and *in vivo*. In addition, the lungs displayed disturbed epithelial cell differentiation as indicated by diminished SP-C and CC-10 expression, markers for proximal and distal pulmonary epithelial cell differentiation, respectively. In contrast, TTF-1 expression (a marker for pulmonary endoderm specification) was not disturbed. We concluded therefore, that pulmonary endoderm specification occurred normally in the chimeric embryos. This phenomenon had already been demonstrated by the fact that the chimeric embryos had lungs, albeit abnormal lungs. Chimeras generated in a ROSA26 background showed that endodermal cells in the abnormally branched areas were derived from GATA6 mutant ES cells, indicating that the defect is intrinsic to the endoderm. From these studies we concluded that GATA6 is essential for branching morphogenesis and epithelial cell differentiation during fetal pulmonary development.

In *Chapter 4* studies are described in which the opposite approach, "gain-of-function" is used to investigate the role of GATA6 in pulmonary development. Following on the studies described in *Chapter 3*, a well-characterized lung-specific promoter was used to maintain high levels of GATA6 protein during fetal pulmonary development. This resulted in a phenotype comparable to that seen in the GATA6 -/- chimeras. The transgenic lungs displayed diminished branching morphogenesis as well as a lack of distal epithelial differentiation (absent or abnormal SP-C expression), whereas proximal epithelial cell differentiation was unaffected (normal CC-10 expression). This was confirmed at the ultrastructural level and with glycogen staining, which both indicated that transgenic lungs lacked differentiation into mature type II cells. Taken together, GATA6 might function as an anti-differentiation factor for pulmonary epithelial cell development, in analogy to the other GATA factors effecting the differentiation of blood cells.

Chapter 5 describes studies on the balanced interplay between proliferation and apoptosis during epithelial-mesenchymal interactions of the developing lung. An *in vitro* cell model served to recombine various combinations of mesenchymal cells, fibroblasts, endodermal cells and epithelial cells. During culture of the cell clumps, we observed

alveolization-like morphogenesis. Most proliferating cells were observed in homotypic recombinants of early cells and this appeared to be intrinsic to, in particular, the epithelial cells. In the homotypic recombinants of late cells, most apoptotic cells were seen between fibroblasts, but this was not observed when these fibroblasts were cultured together with early endodermal cells. This led us to conclude that apoptosis of late fibroblasts was due to loss of inhibition by late epithelial cells, which does not occur with early endodermal cells.

In *Chapter 6* studies on the role of thyroid hormone receptors during (pulmonary) organogenesis are presented. Using different visualization and quantitation techniques, RNA and protein expression combined with RNA and protein levels are described during organogenesis of liver, lung, bone and kidney. These organs are dependent upon epithelial-mesenchymal interactions for their development. We found that the TRα isoform was expressed in mesenchymal tissues, and TRβ in epithelial tissues. The expression patterns were more pronounced at the mRNA than at the protein level, which we contributed to extensive posttranscriptional control. TRβ protein levels reached adult levels before $TR\alpha_1$, suggesting an initiating, TRβ-mediated signalling from the epithelium, followed by a TRα-mediated response from the mesenchyme. TRα and TRβ were co-expressed in the developing brain and intestine. In addition, these organs had a very low translational efficiency of TR mRNA or higher degradation of TR protein, which was indicated by a very low TRα and TRβ protein/mRNA ratio. We concluded from these studies that there must be a TR-dependent regulation which functions differently in organs with complementary or co-expression of TRα and TRβ genes.

Both *Chapter 7* and *Chapter 8* describe studies on mechanisms of abnormal pulmonary development. In both chapters the Nitrofen rat model for CDH is used to investigate pulmonary hypoplasia associated with CDH. In *Chapter 7* an *in vitro* modification of the Nitrofen model was used to investigate the direct effects of Nitrofen on early lung development before and separate from abnormal diaphragm development. This was done using an organotypic lung explant system. We found that Nitrofen negatively influences branching morphogenesis, epithelial cell differentiation (SP-C and CC-10 expression) and proliferation of the lung, whereas apoptosis did not appear to be affected. Therefore, we postulate the dual-hit hypothesis to explain pulmonary hypoplasia in CDH with two insults. The first hit affects both lungs before (abnormal) diaphragm

development and the second affects only the ipsilateral lung after defective diaphragm development.

In *Chapter 8* two aspects of CDH and pulmonary hypoplasia were investigated. In the first part of the study the expression of the glucocorticoid receptor in hypoplastic lungs was compared with that in control lungs. In the second part a possible mechanism of action of Nitrofen in producing CDH and pulmonary hypoplasia was investigated. Radioactive *in situ* hybridization was used in order to investigate the expression patterns of the glucocorticoid receptor, thyroid hormone receptors, together with their preferred partner of dimerization, the retinoid-X receptors in the Nitrofen model. TRα was specifically expressed in the developing pulmonary mesenchyme, and TRβ in the developing pulmonary epithelium. A comparable expression pattern was observed for the retinoid-X receptors, whereas the glucocorticoid receptor was ubiquitously expressed in both germ layers. No differences in timing or localization were observed between normal and hypoplastic lungs. This suggests that hypoplastic lungs are as sensitive to glucocorticoids as normally developed lungs. In addition, an alteration of thyroid hormone receptor expression is not the underlying cause of pulmonary hypoplasia in case of Nitrofen-induced congenital diaphragmatic hernia.

In conclusion, the studies presented in this thesis have supplied the following new insights into some aspects of normal and abnormal pulmonary development:

- GATA6 is not involved in endoderm specification, but plays a role in branching morphogenesis and epithelial cell differentiation of the lung;
- a developmentally regulated interplay between proliferation and apoptosis is essential for normal pulmonary morphogenesis; the former is cell or tissue intrinsic, whereas the latter is dependent on epithelial-mesenchymal interations;
- normal organogenesis requires developmental changes in the expression of the thyroid hormone receptors alpha and beta in rat tissues; this might function via extensive post-transcriptional regulation;
- Nitrofen interferes with early pulmonary development before and separate from (aberrant) diaphragm development;
- steroid hormone receptor superfamily expression is not altered in pulmonary hypoplasia in case of Nitrofen-induced CDH in rats.

10.2 Samenvatting

Pulmonale hypoplasie, onvolkomen ontwikkeling van de long, is nog steeds een belangrijke oorzaak van morbiditeit en mortaliteit op de Pediatrische Intensive Care. De aandoening kan op zichzelf staan, zoals in het geval van oligohydramnios, of deel uitmaken van een syndroom zoals congenitale hernia diafragmatica (CHD). De minimaal verbeterde uitkomst na intensieve behandeling van pulmonale hypoplasie (in combinatie met CHD) is waarschijnlijk te wijten aan onvoldoende kennis van de normale longontwikkeling. De mechanismen die betrokken zijn bij afwijkende longontwikkeling en die leiden tot pulmonale hypoplasie zijn niet bekend.

Dit proefschrift bestaat uit vier delen: Deel I (hoofdstuk 1 en 2) vormt de introductie, Deel II (hoofdstuk 3 tot en met 6) beschrijft de onderzoeken naar mechanismen van normale longontwikkeling, Deel III (hoofdstuk 7 en 8) beschrijft de studies naar mechanismen van abnormale longontwikkeling in combinatie met CHD, en Deel IV (hoofdstuk 9 en 10) bevat de discussie en samenvattingen.

Hoofdstuk 1 geeft een overzicht van de literatuur over verschillende aspecten van de normale longontwikkeling. Allereerst worden de ademhalingsstructuren van diverse organismen beschreven en met elkaar vergeleken. Dit wordt gevolgd door een beschrijving van de anatomie en de embryologie van de normale longontwikkeling bij zoogdieren te beginnen bij hoe het primitieve voordarmendoderm zich specificeert tot longendoderm. Tenslotte worden de belangrijkste genen, transcriptie- en groeifactoren, extracellulaire matrix eiwitten en hormonen die een rol spelen bij normale longontwikkeling besproken.

Hoofdstuk 2 geeft een inleiding op aspecten van de abnormale longontwikkeling, met name pulmonale hypoplasie bij CHD. De achtergronden bij CHD en pulmonale hypoplasie worden beschreven en de diermodellen die gebruikt worden om deze aangeboren afwijking te bestuderen worden besproken. Dit hoofdstuk eindigt met een opsomming van de doelstellingen van de diverse onderzoeken, alsmede de onderzoeksvragen die behandeld worden in dit proefschrift.

Hoofdstuk 3 en *Hoofdstuk 4* beschrijven het onderzoek naar de rol van de transcriptiefactor GATA6 tijdens de normale longontwikkeling. Gezien het

expressiepatroon van GATA6 mRNA in longendoderm en –epitheel, werd aangenomen dat GATA6 een rol speelt bij het vroege vertakken van de luchtwegen in de long. Het bleek mogelijk om dit vertakken in long kweken te blokkeren met behulp van antisense oligonucleotiden tegen GATA6. Deze functie van GATA6 werd verder onderzocht in "loss-of-function" onderzoeken met GATA6-/- chimere embryos. De longen van deze embryos vertoonden minder vertakkingen van de luchtwegen dan normale longen, zowel *in vitro* als *in vivo*. Daarnaast was de differentiatie van deze longen afwijkend, zoals bleek uit de afwijkende SP-C en CC-10 expressie, wat betekent dat de proximale en distale differentiatie van longepitheel anders verloopt. Daarentegen was de expressie van TTF-1 niet afwijkend, en dit betekent dat de specificatie van longendoderm normaal verloopt. Dit werd al vermoed, aangezien de chimere embryos longen hadden, zij het abnormale. In chimere embryos, gemaakt met behulp van een ROSA26 genetische achtergrond, bleek dat de endodermale cellen uit de gebieden met abnormale vertakkingspatronen afkomstig waren van gemuteerde GATA6 embryonale stamcellen. Derhalve is het probleem gerelateerd aan het endoderm. We concludeerden dat GATA6 essentieel is bij het vroege vertakken van de luchtwegen en de differentiatie van luchtwegepitheel tijdens de foetale longontwikkeling.

In *Hoofdstuk 4* wordt onderzoek beschreven waarin een tegenovergestelde aanpak wordt gebruikt om de rol van GATA6 tijdens long ontwikkeling te onderzoeken, namelijk "gain-of-function" onderzoeken. Naar aanleiding van de resultaten van het onderzoek uit *Hoofdstuk 3* werd nu een longspecifieke promoter gebruikt om het GATA6-eiwit gehalte kunstmatig hoog te houden tijdens de foetale longontwikkeling. Dit resulteerde in een fenotype dat vergelijkbaar was met dat van de GATA6-/- chimere embryos. Transgene longen vertoonden minder vertakkingen van de vroege luchtwegen en geen distale epitheeldifferentiatie (afwezige of abnormale SP-C expressie), terwijl de proximale epitheeldifferentiatie normaal verliep (normale CC-10 expressie). Dit werd bevestigd met behulp van elektronenmicroscopie en een kleuring voor glycogeen. Beide toonden aan dat er in de transgene longen gebrekkige differentiatie naar volwaardige type II cellen plaatsvindt. Samenvattend, het zou kunnen zijn dat GATA6 functioneert als een anti-differentiatie factor voor de ontwikkeling van longepitheel-cellen, net zoals andere GATA factoren in relatie tot bloedcellen.

Hoofdstuk 5 beschrijft onderzoek naar de relatie tussen proliferatie en apoptosis gedurende epitheliale-mesenchymale interacties van de zich ontwikkelende long. Er werd een *in vitro* recombinatiemodel van mesenchymale cellen, fibroblasten, endodermale cellen en epitheelcellen gebruikt om verschillende combinaties van de genoemde cellen te maken. Tijdens het kweken van de groepjes cellen werd morphogenese waargenomen die leek op normale alveolarisatie. De meeste prolifererende cellen werden gezien in de homotypische recombinanten van langer ontwikkelde cellen, de meeste apoptotische tussen de langer ontwikkelde fibroblasten. Dit laatste werd echter niet gezien als de fibroblasten gecombineerd werden met vroege endodermale cellen. Daarom concludeerden wij dat apoptosis van langer ontwikkelde fibroblasten het gevolg was van een verlies van inhibitie door langer ontwikkelde epitheel cellen, en dit gebeurt dus niet als de fibroblasten gecombineerd worden met vroege endodermale cellen.

In *hoofdstuk 6* wordt onderzoek naar de rol van schildklierhormoon-receptoren tijdens de (long) organogenese beschreven. Met behulp van verschillende visualiserings- en kwantificeringstechnieken werden RNA- en eiwitexpressie samen met de RNA- en eiwitgehaltes vastgesteld gedurende de organogenese van de lever, longen, bot en de nieren, allen organen, die afhankelijk zijn van epitheel-mesenchym interacties voor hun ontwikkeling. TRα bleek met name tot expressie te komen in mesenchymale weefsels, en TRβ met name in epitheliale weefsels. De expressiepatronen waren op RNA-niveau duidelijker dan op eiwit-niveau, en dit werd toegeschreven aan uitgebreide posttranscriptionele regulatie. TRβ eiwit gehaltes bereikten volwassen waardes voordat TRα gehaltes dit deden, wat doet vermoeden dat er een initieel TRβ geleid signaal vanuit het epitheel gevolgd wordt door een TRα-geleid antwoord van het mesenchym. TRα en TRβ kwamen samen tot expressie in de zich ontwikkelende hersenen en darmen. Daarnaast hadden deze organen een zeer lage translatie-efficiëntie voor TR-mRNA of een hoge degradatie van TR-eiwit, vanwege de zeer lage TRα en TRβ eiwit/RNA ratio. We concludeerden dat er een TR-afhankelijke regulatie moet bestaan die anders functioneert in organen met tegenovergestelde expressie van TRα en TRβ genen dan in organen met co-expressie.

Zowel *hoofdstuk 7* als *hoofdstuk 8* beschrijven onderzoeken naar de mechanismen van abnormale longontwikkeling. In beide hoofdstukken wordt het Nitrofen-rattenmodel gebruikt om pulmonale hypoplasie bij CHD te bestuderen. In *Hoofdstuk 7* wordt een *in*

vitro modificatie van het Nitrofenmodel gebruikt om de effecten van Nitrofen op vroege longontwikkeling voorafgaande aan en los van de abnormale ontwikkeling van het middenrif te onderzoeken. Hierbij werd gebruik gemaakt van een longkweek-systeem. Het bleek dat Nitrofen het vroege vertakken van de luchtwegen, de differentiatie van epitheel cellen (SP-C en CC-10 expressie) en de proliferatie van de long negatief beïnvloedde, terwijl het op apoptosis geen effect had. Naar aanleiding hiervan postuleerden wij de dual-hit hypothese om pulmonale hypoplasie bij CHD tijdens de ontwikkeling te verklaren met twee problemen. De eerste hit treft beide longen en treedt op voorafgaande aan (abnormale) ontwikkeling van het middenrif. De tweede hit treft alleen de long aan de aangedane zijde en treedt op nadat er een gat in het middenrif is ontstaan.

In *Hoofdstuk 8* werden twee verschillende aspecten van CHD en pulmonale hypoplasie onderzocht. Ten eerste werd onderzocht of hypoplastische longen de glucocorticoidreceptor net zo tot expressie brengen als controlelongen. Ten tweede werd een mogelijk werkingsmechanisme van Nitrofen voor het veroorzaken van CHD en pulmonale hypoplasie onderzocht. Er werd gebruik gemaakt van radioactieve *in situ* hybridisatie om de expressiepatronen van de glucocorticoid receptor, de schildklierhormoon-receptoren en hun voorkeurspartner voor dimerizatie, de retinoid-X-receptoren in het Nitrofen model te onderzoeken. TRα kwam met name tot expressie in het longmesenchym, en TRβ met name in het longepitheel. Een vergelijkbaar expressiepatroon werd gezien voor de retinoid-X-receptoren, terwijl de glucocorticoid receptor meer in beide kiemlagen tot expressie kwam. Tijdstippen en plaats van expressie verschilden niet tussen normale en hypoplastische longen. Dit suggereert dat hypoplastische longen net zo gevoelig zijn voor glucocorticoïden als normaal ontwikkelde longen. Daarnaast bleek dat een veranderde expressie van de schildklierhormoon receptoren niet de onderliggende oorzaak is van pulmonale hypoplasie als gevolg van CHD geïnduceerd door Nitrofen.

Samengevat heeft dit proefschrift de volgende nieuwe inzichten verschaft in bepaalde aspecten van normale en abnormale ontwikkeling van de long:

- GATA6 is niet betrokken bij de specificatie van endoderm, maar speelt een rol in het vroege vertakken van de luchtwegen en de differentiatie van epitheelcellen van de long;

- een ontwikkelingsgebonden relatie tussen proliferatie en apoptosis is van belang voor de normale morphogenese van de long; het eerste is wezenlijk voor het weefsel of de cel, en het laatste is afhankelijk van epitheel-mesenchym interacties;
- voor normale organogenese is het van belang dat er ontwikkelingsgebonden veranderingen in de expressie van de schildklierhormoon receptoren alpha en beta optreden in weefsels van de rat; dit zou kunnen plaatsvinden door middel van uitgebreide post-transcriptionele regulatie;
- Nitrofen verstoort de vroege longontwikkeling voorafgaande aan en los van de (abnormale) ontwikkeling van het middenrif;
- de expressie van de steroidhormoon-receptor superfamilie is onveranderd bij ratten met pulmonale hypoplasie en CHD geïnduceerd door Nitrofen.

Part V

APPENDIX

Dankwoord

Het onderzoek beschreven in dit proefschrift werd verricht op vier afdelingen, te weten de afdeling Kinderheelkunde van het Sophia Kinderziekenhuis in Rotterdam, de afdeling Anatomie en Embryologie van het Academisch Medisch Centrum in Amsterdam, de afdeling Lung Biology Research van het Hospital for Sick Children in Toronto (Canada) en de afdeling Celbiologie en Genetica van de Erasmus Universiteit in Rotterdam. Veel mensen van deze afdelingen hebben geholpen bij het tot stand komen van dit proefschrift. Een aantal van hen wil ik speciaal bedanken.

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Curriculum Vitae

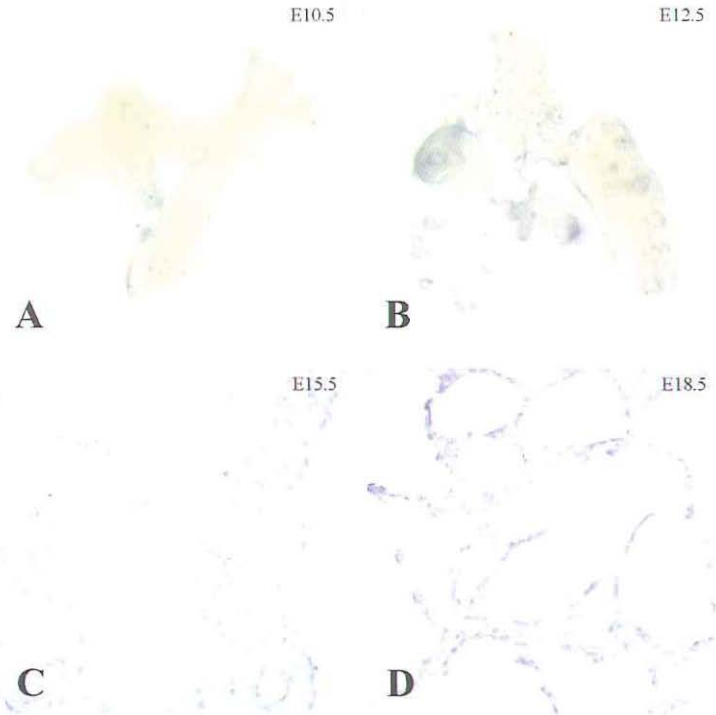
Richard Keijzer was born on November 1 1971 in Voorburg, the Netherlands. In 1990 he obtained his V.W.O. "diploma" from O.S.G. Professor Casimir in Vlaardingen. In the same year he started his medical training at the Erasmus University Rotterdam. Since 1994 he worked on a research project at the Department of Pediatric Surgery of the Sophia Children's Hospital in Rotterdam under guidance of Prof.dr. D. Tibboel. As a part of this research project he worked in the Department of Anatomy and Embryology of the University of Amsterdam (Prof.dr. W.H. Lamers) in 1994 and 1995. In 1997 he obtained E.C.F.M.G. certification after taking Step I (1995) and Step II (1997) of the United States Medical Licensing Education for foreign medical graduates. In December 1997 he obtained his medical degree cum laude.

During 1998 he lived in Toronto, Canada, where he worked in the Lung Biology Research laboratory of Prof.dr. M. Post in the Hospital for Sick Children and the University of Toronto. After returning to the Netherlands he worked from 1999 to 2000 in the laboratory of the Department of Pediatric Surgery at the Department of Cell Biology and Genetics of the Erasmus University Rotterdam (Prof.dr. F. Grosveld).

In January 2001 he started his residency in general surgery at the "Reinier de Graaf Gasthuis", Delft (dr. L.P.S. Stassen) as part of his specialty training in general surgery (Prof.dr. H.J. Bonjer).

On May 5 he married Els de Gussem.

Color figures



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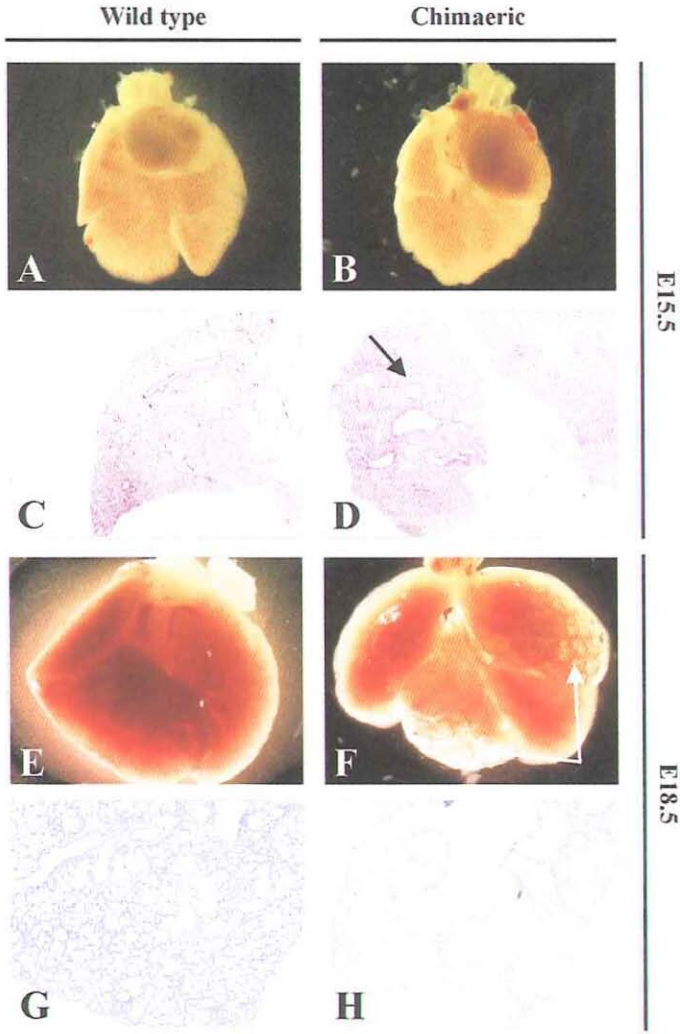

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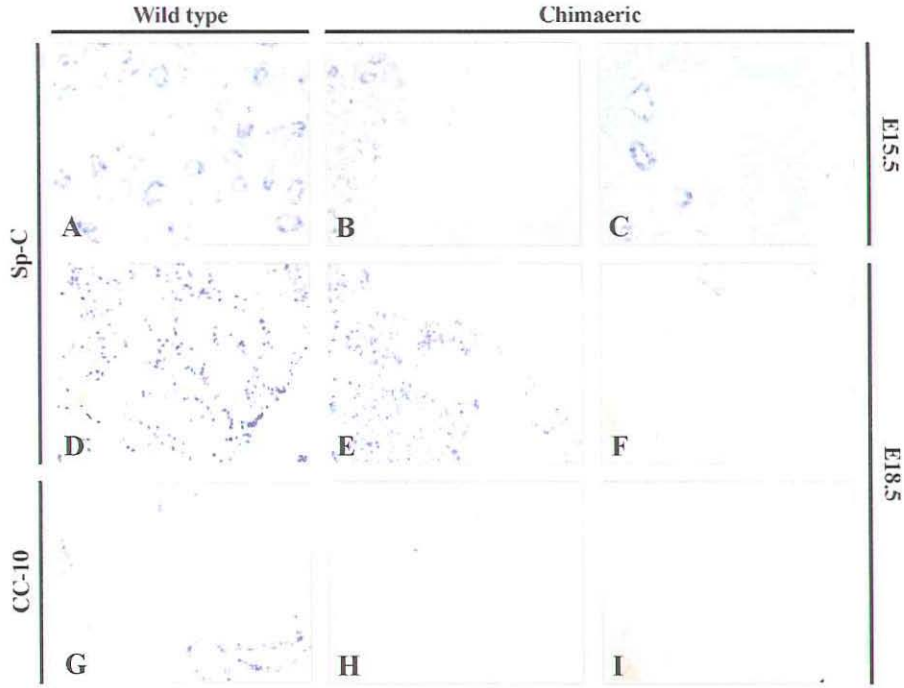

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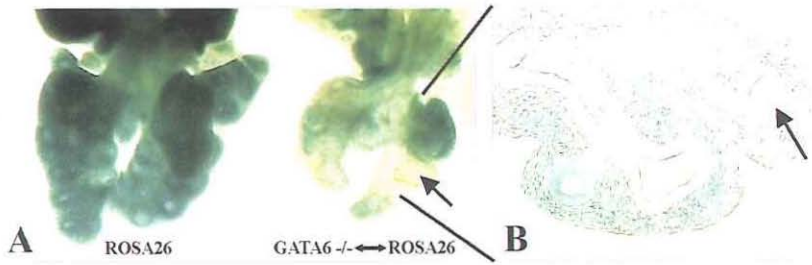

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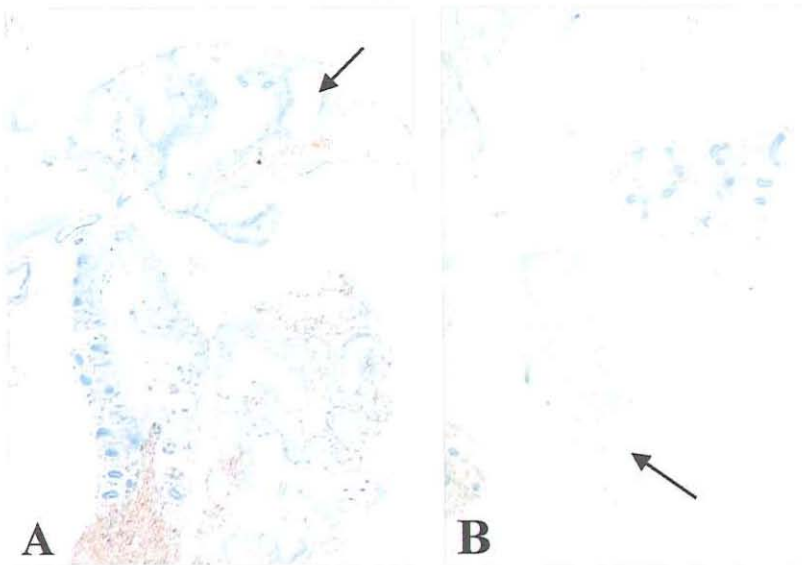

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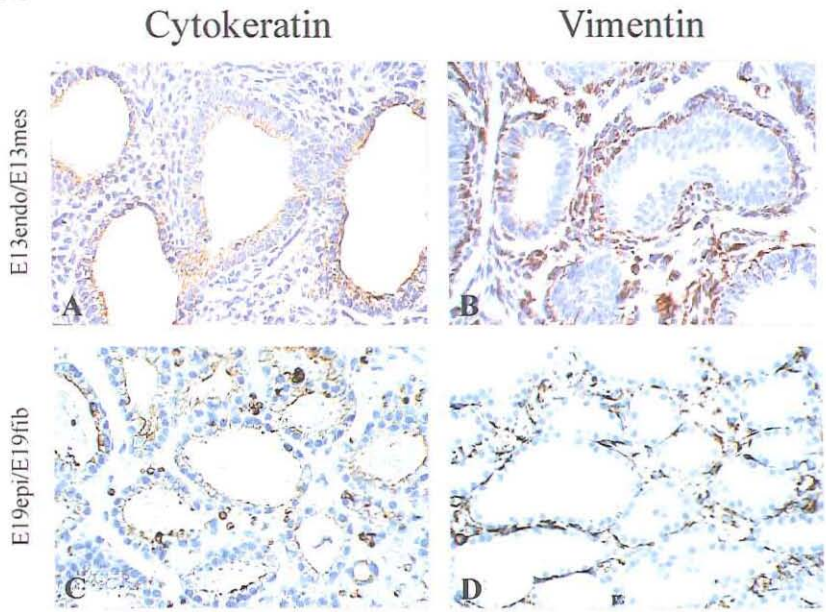

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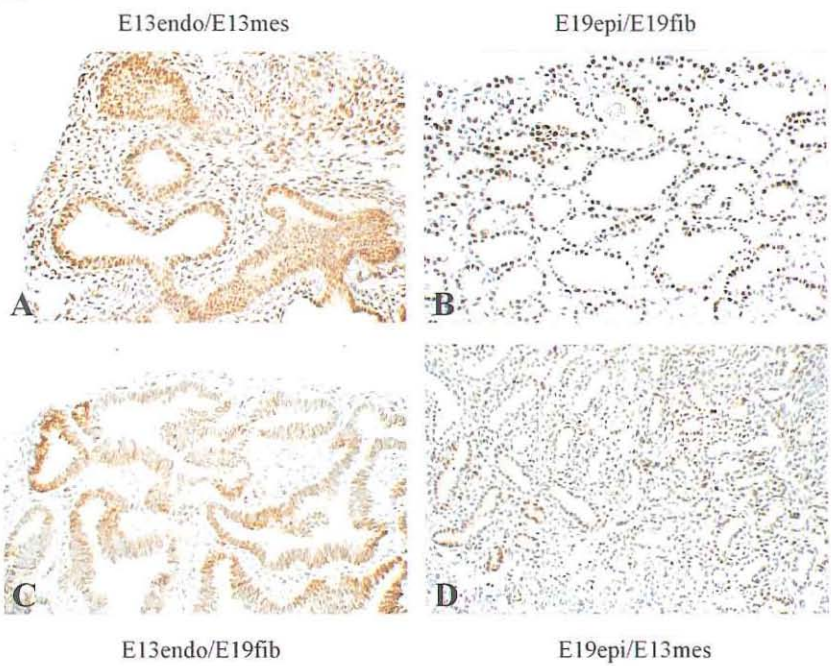

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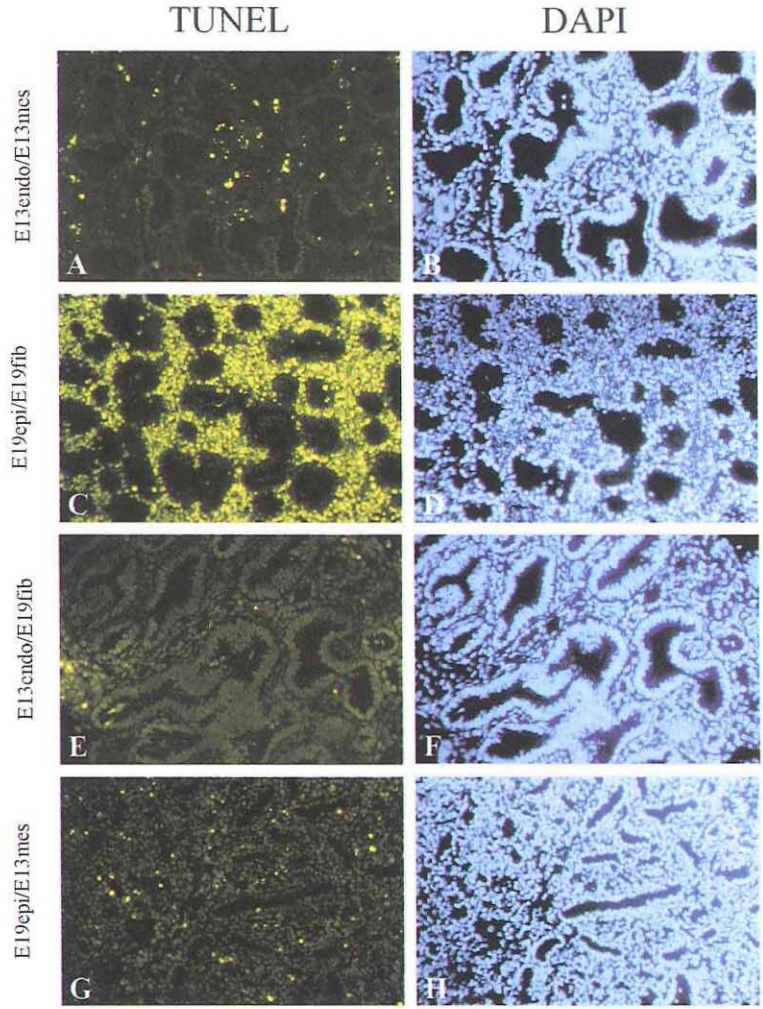

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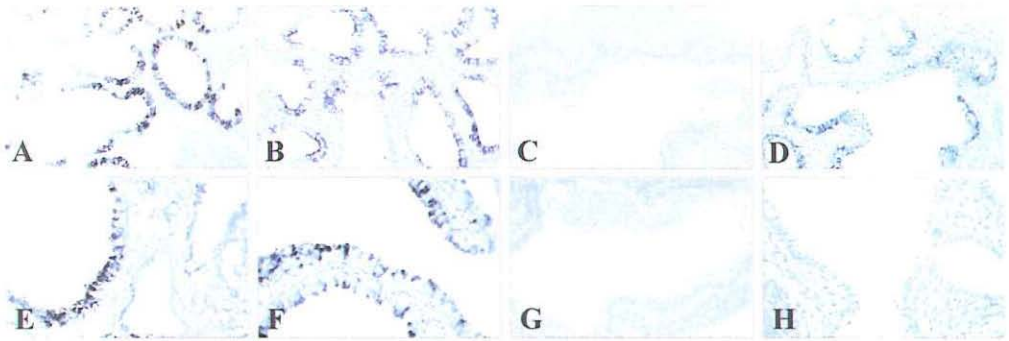

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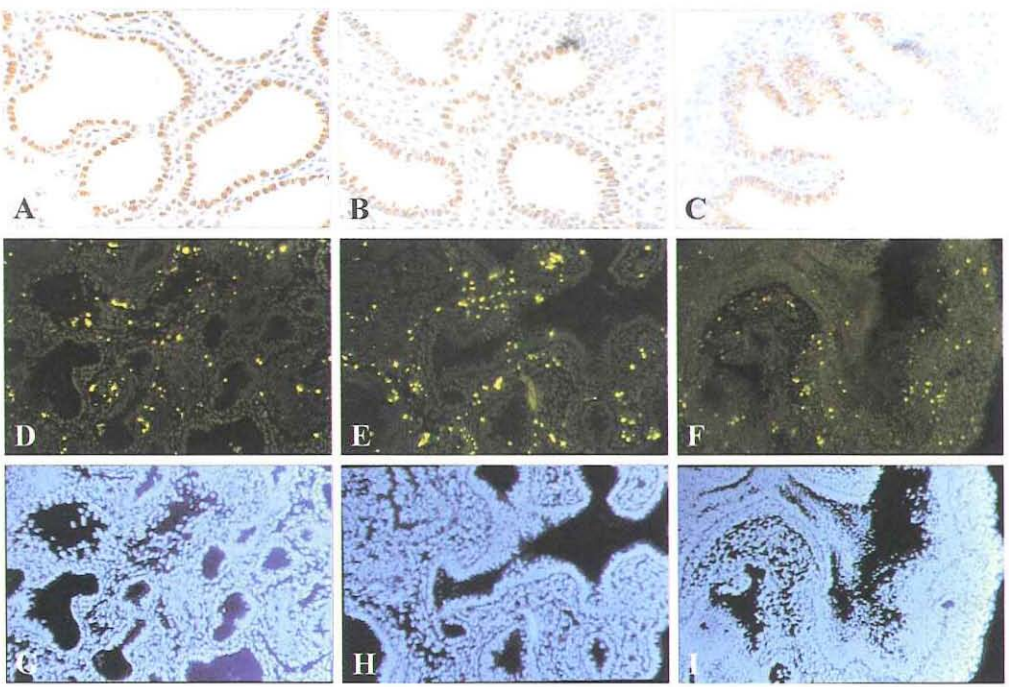

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