Cristina Isabel Nogueira da Silva

**Looking for Novel Physiological
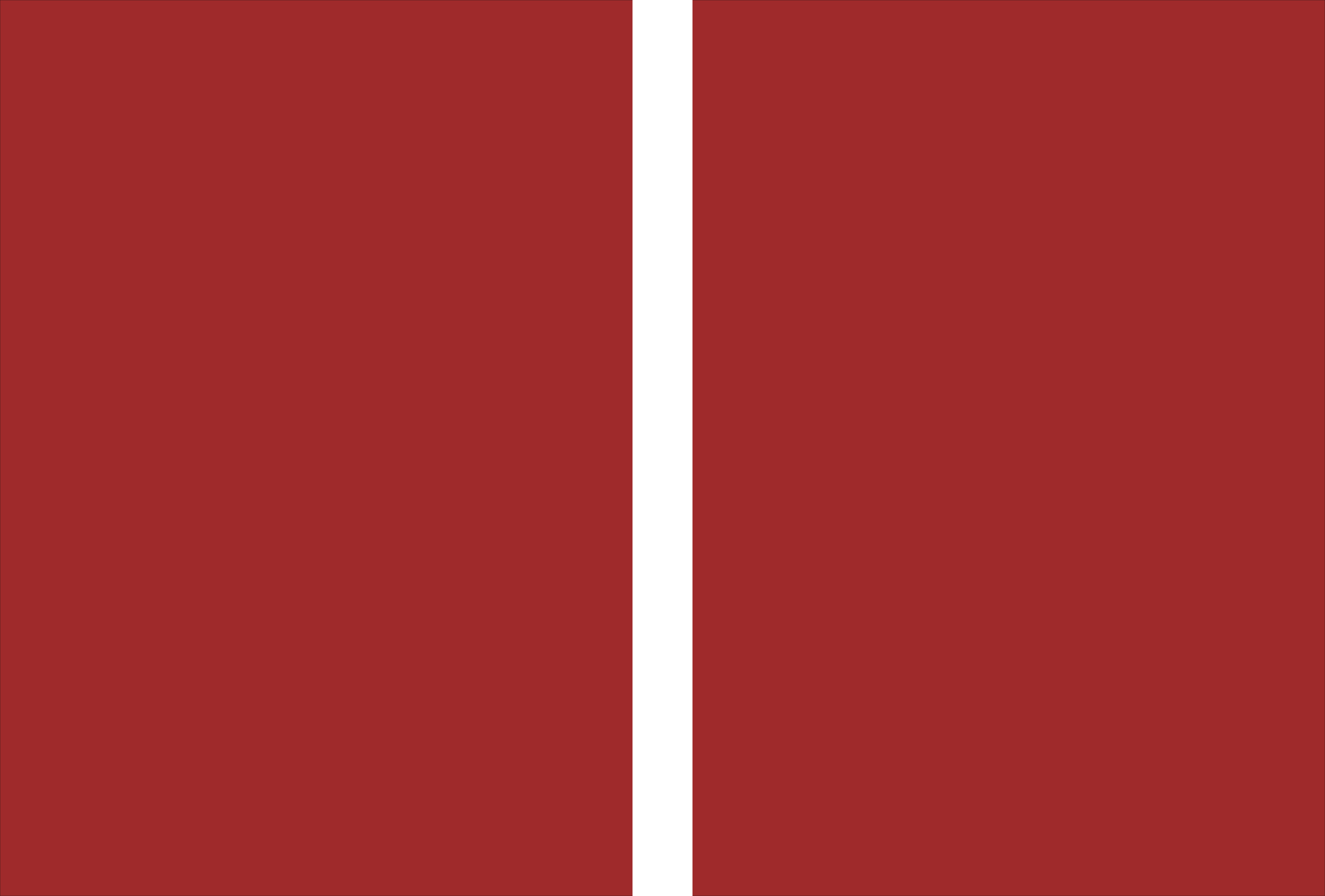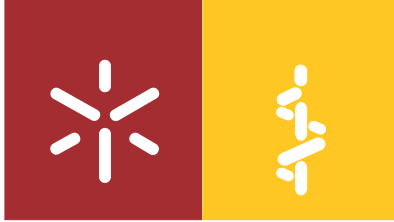Regulators of Lung Development**

Universidade do Minho
Escola de Ciências da Saúde

Cristina Isabel Nogueira da Silva

Looking for Novel Physiological Regulators of Lung Development

Tese de Doutoramento em Medicina

Trabalho realizado sob a orientação do:

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Declaração

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Looking for Novel Physiological Regulators of Lung Development

Novos Reguladores Fisiológicos do Desenvolvimento Pulmonar

Orientadores:

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Doutora Isabel Maria Mestre Marques Palmeirim Alfarra Esteves

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Medicina

**É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA
TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO,
MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO
QUE A TAL SE COMPROMETE.**

Universidade do Minho,   /   /2012

Assinatura:

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Agradecimentos

O momento em que atingimos uma meta, alcançamos um propósito, concretizamos um sonho, concluímos uma etapa, é inevitavelmente um momento de reflexão sobre o caminho percorrido. E dessa reflexão surge de imediato o ímpeto de agradecimento a todos quantos nos acompanharam nesse longo percurso. A concretização de uma Tese de Doutoramento é precisamente uma meta, um propósito, um sonho, uma etapa e um longo caminho que só pode ser percorrido de uma forma: andando (*"Caminhante, não há caminho, o caminho faz-se andando"*, António Machado, Campos de Castilla, 1912). E andando lado a lado com a sabedoria, o apoio, a motivação e a presença de muitos. É a estes, que constituíram em uníssono a pedra angular neste trilho repleto de desafios, que dedico as linhas que se seguem e expresso o meu agradecimento sincero.

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Abstract

Pediatric and adult diseases characterized by lung hypoplasia or dysplasia are an epidemiological relevant issue, involving important morbidity and mortality rates. The understanding of the complex process of lung development, regulated by several genetic, chemical and physical determinants, has clinical relevance since it can open new perspectives in the treatment of these lung diseases as well as modulation of lung repair. Regardless of the advanced knowledge of normal lung growth, there are several possible regulators and determinants that need to be investigated. Therefore, the main aim of the present dissertation was to discover novel physiological regulators of normal lung morphogenesis, expecting to contribute for the development of new strategies for lung diseases, namely for fetal lung hypoplasia, in context of congenital diaphragmatic hernia (CDH).

Using different laboratorial approaches, namely histological, molecular, and functional studies, the role of glycoprotein 130 (gp130) family of cytokines and renin-angiotensin system (RAS) was evaluated throughout fetal lung development, in rat model of either normal and hypoplastic (the nitrofen-induced CDH model) lung development..

In this thesis, it was demonstrated that interleukin 6 (IL-6) and leukemia-inhibitory factor (LIF) are constitutively expressed during fetal lung development and they have a physiological role on pulmonary branching mechanisms. Indeed, cytokines signaling through gp130 homodimers (IL-6 and IL-11) stimulate lung branching, whereas cytokines acting in a gp130 heterodimer receptor (LIF, oncostatin M) inhibit lung growth. It was also established that there is a local and physiologically active RAS during lung morphogenesis. In fact, all RAS components are constitutively expressed in the lung throughout gestation and angiotensin II (ANG II), the physiologically active peptide of RAS, induces a stimulatory effect on lung branching, mediated by type 1 (AT_1) receptor of ANG II, through p44/42 and Akt phosphorylation.

After the description of these two new physiological regulators/modulators of fetal lung growth, gp130 family of cytokines and RAS, their role in pulmonary hypoplasia, in the CDH context, were assessed. First of all, it was showed that hypoplastic fetal lung has the intrinsic ability to regulate its growth and to recover from growth retardation through a way that resembles the *catch-up growth* phenomenon. Moreover, IL-6 might be involved in the mechanisms underlying this phenomenon. Finally, the discovery of RAS as a new regulator of fetal lung growth allowed the establishment of an antagonist of type 2 (AT_2) receptor of ANG II (PD-123319) as a putative antenatal therapy for pathologies characterized by fetal lung hypoplasia, such as CDH. *In vivo*, in nitrofen-induced CDH model, antenatal PD-123319 treatment improved the key determinants of mortality associated with CDH, namely lung hypoplasia and pulmonary hypertension, and also improved lung function and survival, without maternal or fetal deleterious effects.

In summary, in this thesis, according to the proposed aims, two novel physiological regulators of fetal lung development were identified: gp130 family of cytokines and local lung RAS. Moreover, we demonstrated that fetal lung has the intrinsic ability to regulate its growth and proposed a model for regulation of correct lung size and growth. Nonetheless, future studies are necessary to clarify this regulatory loop and the mediators (afferent arms, sensors, efferent arms) involved on this hypothetic feedback mechanism of compensatory growth. Finally, we established that selective inhibition of AT_2 receptor is a putative antenatal therapy for pathologies characterized by lung hypoplasia, such as CDH, which open perspectives for future pre-clinical studies.

Resumo

As doenças pulmonares pediátricas ou do adulto, que cursam com hipoplasia ou displasia pulmonar, associam-se a elevadas taxas de morbilidade e mortalidade. A compreensão dos mecanismos complexos do desenvolvimento pulmonar, regulados por determinantes genéticos, químicos e mecânicos, apresenta relevância clínica ao permitir o desenho de novas abordagens terapêuticas que mimetizem o normal crescimento e maturação pulmonares, bem como a regeneração pulmonar. Nos últimos anos, o conhecimento sobre desenvolvimento pulmonar normal muito ter evoluído. Contudo, são vários os fatores de crescimento e reguladores que permanecem por esclarecer. Assim, nesta dissertação investigaram-se novos reguladores fisiológicos da morfogénese pulmonar, procurando contribuir para o desenvolvimento de novas estratégias terapêuticas para as doenças pulmonares, nomeadamente a hipoplasia pulmonar fetal, no contexto da hérnia diafragmática congénita (HDC).

No modelo animal do rato, quer em animais controlo quer no modelo de HDC induzida pelo nitrofeno, a função da família das citocinas da glicoproteína 130 (gp130) e do sistema renina-angiotensina (SRA) foi avaliada ao longo do desenvolvimento pulmonar fetal. Diferentes técnicas laboratoriais foram utilizadas, nomeadamente estudos histológicos, moleculares e funcionais.

Os nossos resultados demonstraram que a interleucina 6 (IL-6) e o fator inibidor da leucemia (LIF) são constitutivamente expressos ao longo do desenvolvimento pulmonar fetal e desempenham um papel fisiológico nos mecanismos de ramificação pulmonar. Assim, a IL-6 e a IL-11, cujos recetores são homodímeros da gp130, estimulam a ramificação pulmonar. Por sua vez, o LIF, a oncostatina M, que sinalizam via heterodímeros de gp130, inibem o crescimento pulmonar. Para além disso, foi também descrita a presença de um SRA local e fisiologicamente ativo durante a morfogénese pulmonar. Na verdade, todos os componentes do SRA são expressos no pulmão em todas as idades gestacionais estudadas e a angiotensina II (ANG II), o peptídeo fisiologicamente ativo deste sis-

tema, estimula a ramificação pulmonar, via recetor de tipo 1 (AT_1) e a fosforilação da p44/42 e Akt. Uma vez descritos estes dois novos reguladores/moduladores do crescimento pulmonar fetal, a família das citocinas da gp130 e o SRA, o seu papel na hipoplasia pulmonar fetal, no contexto da HDC, foi avaliado. Foi possível demonstrar que o pulmão fetal hipoplásico apresenta capacidade intrínseca para regular o seu crescimento e recuperar do atraso de crescimento por um mecanis-mo similar ao clássico fenómeno de *catch-up growth*. A IL-6 revelou-se como um dos mediado-res envolvidos nos mecanismos subjacentes a este fenómeno. Finalmente, a descoberta do SRA como um novo regulador do crescimento pulmonar fetal permitiu apresentar o PD-123319, um antagonista específico do recetor de tipo 2 da ANG II (AT_2), como um potencial alvo terapêutico para patologias caraterizadas por hipoplasia pulmonar fetal, das quais a HDC é um exemplo. No modelo de HDC induzida pelo nitrofeno, *in vivo*, a administração materna antenatal de PD-123319 melhorou os determinantes chave associados a esta patologia, nomeadamente a hipoplasia e hipertensão pulmonares, e melhorou a função pulmonar e a taxa de sobrevida, sem aparentes efeitos deletérios maternos ou fetais.

Em resumo, nesta tese, de acordo com os objetivos inicialmente estabelecidos, foram identi-ficados dois novos reguladores fisiológicos do desenvolvimento pulmonar fetal: a família das citocinas da gp130 e o SRA pulmonar. Foi, também, demonstrado que o pulmão fetal apresenta capacidade intrínseca para regular o seu crescimento e foi proposta a existência de um modelo de regulação do crescimento pulmonar. Contudo, estudos futuros são necessários a fim de se clarificar este hipotético mecanismo de regulação do crescimento e os seus mediadores (vias aferentes, sensor e vias eferentes). Por fim, a apresentação do recetor AT_2 como um potencial alvo terapêutico antenatal para patologias caraterizadas por hipoplasia pulmonar, tais como a HDC, promove o desenvolvimento de estudos pré-clínicos futuros.

Contents

Chapter I - General Introduction

Abbreviations list

ACE	Angiotensin-converting enzyme
AECI	Type I alveolar epithelial cells
AECII	Type II alveolar epithelial cells
ANG II	Angiotensin II
AT1	Type 1 receptor of angiotensin II
AT2	Type 2 receptor of angiotensin II
BMP-4	Bone morphogenetic protein 4
CaR	Calcium-sensing receptor
CCSP	Clara cell-secretory protein
CDH	Congenital diaphragmatic hernia
CGRP	Calcitonin gene-related peptide
CLC	Cardiotrophin-like cytokine
CNTF	Ciliary neurotrophic factor
COUP-TFII	Chicken ovalbumin upstream promoter-transcription factor II
CRABP1	Cellular retinoic acid binding protein 1
CT-1	Cardiotrophin-1
DKK	Dickkopf
DNA	Deoxyribonucleic acid
Dpc	Days post-conception
ECM	Extracellular matrix
ECMO	Extra corporeal membrane oxygenation
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinases-1 and -2

ERα	Estrogen receptor α
ERβ	Estrogen receptor β
Eya1	Eyes absent 1
FBLN4	Fibulin-4
FBN1	Fibrillin-1
FETO	Fetoscopic endoluminal tracheal occlusion
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Flk-1	Fetal liver kinase 1
Flt-1	FMS-like tyrosine kinase 1
FOG	Friend of GATA
FOX	Forkhead box proteins
FZ	Frizzled
Gab1	Grb2-associated binding 1
GATA	A/TGATA/G -binding transcription factor
GLI	Glioblastoma transcription factor
Gp130	Glycoprotein 130
GPC3	Glypican-3
GRP	Gastrin-releasing peptide
GSK3β	Glycogensynthase kinase-3β
HFH-4	Hepatocyte nuclear factor-3/forkhead homologue 4
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HIP	Hedgehog interacting protein
HNF-3β	Hepatocyte nuclear factor 3β
IGF	Insulin-like growth factor
IGFR	Insulin-like growth factor receptor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
JAK	Janus kinase
JNK	c-Jun NH(2)-terminal kinase
KO	Knockout

LHR	Lung area to head circumference ratio
LIF	Leukemia-inhibitory factor
Lox	Lysyl oxidase
LRP	Low-density-lipoprotein receptor-related proteins
MAPK	Mitogen-activated protein kinase
MRI	Magnetic resonance imaging
MyoD	Myogenic differentiation
NF-$\kappa\beta$	Nuclear factor $\kappa\beta$
NNT-1/BSF- 3	Novel neurotophin-1/B cell-stimulating factor-3
NP	Neuropoietin
OSM	Oncostatin M
PCP	Planar-Cell-Polarity
PDGF	Platelet-derived growth factor
PH	Pulmonary hypertension
PI3K	Phosphatidylinositol-3 kinase
PKC	Protein Kinase C
PLs	Phospholipids
PNEC	Pulmonary neuroendocrine cells
PTC	Patched receptor
RA	Retinoic acid
RALDH	Retinal dehydrogenase
RAR	Retinoic acid receptor
RAS	Renin-angiotensin system
RDS	Respiratory distress syndrome
ROBO1	Roundabout
RXR	Retinoic acid receptor X receptor
Sema	Semaphorins
sFRP	Secreted Frizzled-related Proteins
SHH	Sonic hedgehog
Sim2	Single-minded homolog 2
Six1	Drosophila sine oculis gene
Smads	Drosophila mothers against decapentaplegic

SOX	Sex determining region Y-box
SP	Surfactant proteins
SPARC	vascular endothelial growth factor
SPRY	Sprouty
STAT	Signal transducers and activators of transcription
STRA6	Stimulated by retinoic acid gene 6 homolog
T3	Triidothyronine
T4	Thyroxine
TCF/LEF	T Cell Factor/Lymphoid Enhancer Factor
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TTF-1	Thyroid-specific transcription factor 1
VEGF	Vascular endothelial growth factor
WNT	Wingless
Wt1	Wilm's Tumor 1

CHAPTER I
GENERAL INTRODUCTION
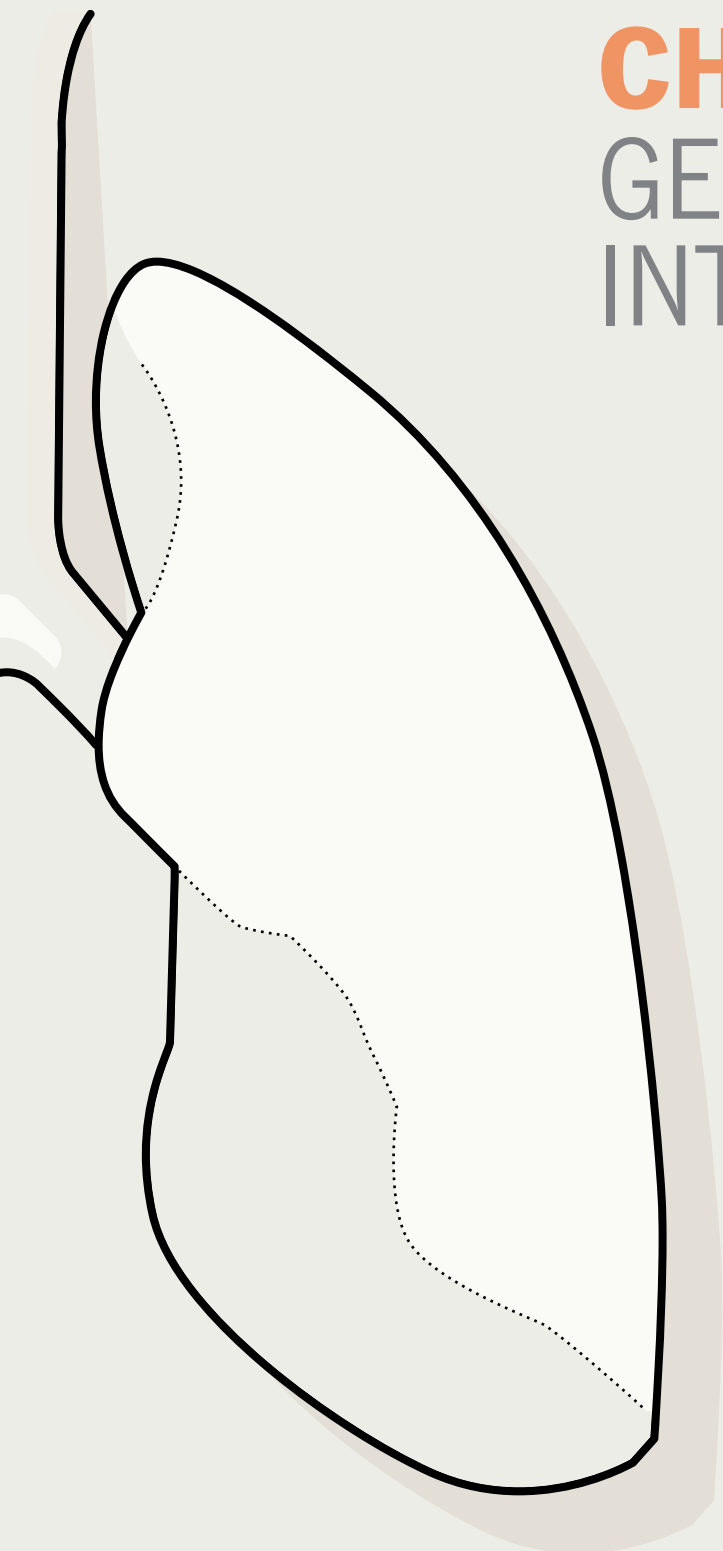
General Introduction

1. Normal Lung Development

The human lung is a vital organ, which is able to achieve a final gas diffusion surface of 70 m^2 in area by 0.1 mm in thickness in young adulthood. In this way, it is capable of supporting systemic oxygen consumption ranging between 250 and 5500 mL/min, and it can accommodate pulmonary blood flow rising from 4 to 40 L/min, at rest and during maximal exercise, respectively [Warburton *et al.*, 2000].

Histological and anatomically, lung is a very complex organ. The lung consists of two extensively branched, tree-like systems - the airways and the vasculature - that develop in a coordinated way in order to generate millions of alveolar gas exchange units [Morrisey & Hogan, 2009; Shi *et al.*, 2009]. The complexity of lung morphogenesis is immediately evident from the multiple cell types that comprise the lung, all present in appropriate numbers and sites to support respiration [Maeda *et al.*, 2007]. Regarding cell types, different authors point to more than 40 specific types of cells are differentiating during lung development, either in airway or vascular structures, derived of the same epithelial or mesenchymal common precursors. The lung epithelium composition changes significantly along the proximal-distal axis and the differentiation of airway epithelium follows a centrifugal pattern. In proximal pseudostratified airway epithelium, there are three major cell components, ciliated columnar cells, basal and mucous (goblet) cells. Regarding distal columnar epithelium, there are pulmonary neuroendocrine cells (PNEC), Clara and alveolar cells. Regarding alveolar surface, its majority is covered by flattened type I alveolar epithelial cells (AECI) or pneumocytes. Indeed, type I pneumocytes covered 95% of the surface area of the alveolar wall. The other 3% of the alveolar surface is covered by rounded cells, type II alveolar epithelial cells (AECII) or pneumocytes. Regarding the pulmonary mesenchyme, it is composed by several specialized cell types, including fibroblasts, myofibroblasts, parabronchial
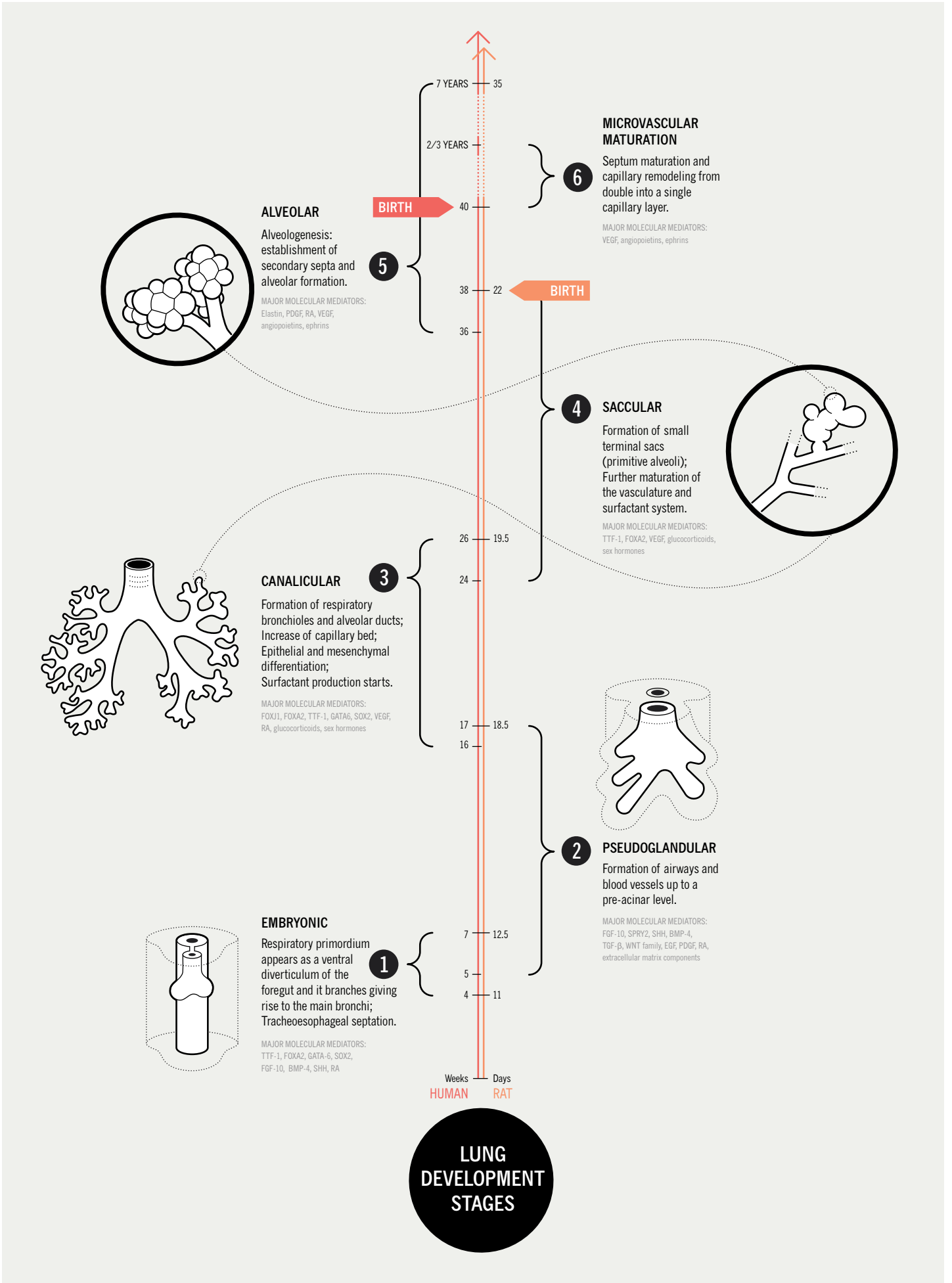
and vascular smooth muscle cells, endothelial cells, vascular pericytes, and chondrocytes in large airways [Morrisey & Hogan, 2009; Shi *et al.*, 2009; Cardoso & Lü, 2006].

Anatomically, in humans the right lobe is trilobed and the left is bilobed, with 23 generations of airway branching (in opposition to mouse and rat lung that consist of right four lobes and one left lobe). Thus, the bronchial tree of the human lung has more than 10^5 conducting and 10^7 respiratory airways, with a matching capillary network developing in close apposition to the alveolar surface [Correia-Pinto *et al.*, 2010; Maeda *et al.*, 2007].

Classically, although a continuous process, lung development has been divided in six different stages: embryonic, pseudoglandular, canalicular, saccular, alveolar and microvascular stages (Figure 1).

The **embryonic** stage is characterized by formation of the respiratory primordium that giving rise to the <u>main bronchi</u> (left and right), and also by the tracheoesophageal septation. In human, this process occurs about day 26 after conception (4th week of gestation), whereas in mice and rats begins at 9.5 and 11 days post-conception (dpc), respectively [Cardoso & Lü, 2006; Warburton *et al.*, 2005; Copland & Post, 2004]. During embryo development, following gastrulation, the definitive endoderm undergoes complex morphogenetic movements that ultimately lead to the formation of the primitive gut tube [Chuang & McMahon, 2003]. The foregut is the most anterior (cranial) region of this tube, while the midgut and hindgut are located at progressively more posterior (caudal) regions of the embryo. The mammalian lung formation initiates with the emergence of a small diverticulum, the respiratory diverticulum or laryngotracheal groove, from the ventral-lateral aspect of the foregut endoderm, which then invade the surrounding splanchnic mesoderm [Chuang & McMahon, 2003]. The proximal portion of the laryngotracheal groove gives rise to the larynx and trachea, whereas the distal portion gives rise to the left and right mainstem bronchial buds, which in turn give rise to the left and right lobar branches of the bronchial tree. Interestingly, the trachea and lungs originate from the same endoderm layer, like the thyroid, esophagus, stomach, liver, and pancreas. Thus, early on gestation, several factors needs to regulate the commitment of specific regions of the foregut into development of specific organs. Expression of transcription factors or markers characteristic of specific organs are observed along the anterior-posterior axis of the foregut tube before formation of each organ [Maeda *et al.,* 2007].

Figure 1. Overview of lung development: stages, structure, characteristic events and major molecular mediators. Human lung development has been divided in six different stages: embryonic (4 to 7 weeks), pseudoglandular (5 to 17 weeks), canalicular (16 to 26 weeks), saccular (24 to 38 weeks), alveolar (36 weeks – 7 years) and microvascular stages (birth to 2-3 years). The braces are representing the duration of each stage of human lung development. The duration of rat lung stages are also depicted [11 to 12.5 days post-conception (dpc); 12.5 to 18.5 dpc; 18.5 to 19.5 dpc; 19.5 to 22 dpc; and 7 to 35 postnatal days, respectively]. In opposition to human, in rat, the alveolar stage is all a postnatal phenomenon.

7 YEARS — 35

2/3 YEARS —

6 MICROVASCULAR MATURATION

Septum maturation and capillary remodeling from double into a single capillary layer.

MAJOR MOLECULAR MEDIATORS: VEGF, angiopoietins, ephrins

ALVEOLAR

BIRTH 40

Alveologenesis: establishment of secondary septa and alveolar formation.

5

MAJOR MOLECULAR MEDIATORS: Elastin, PDGF, RA, VEGF, angiopoietins, ephrins

38 — 22 **BIRTH**

36 —

4 SACCULAR

Formation of small terminal sacs (primitive alveoli); Further maturation of the vasculature and surfactant system.

MAJOR MOLECULAR MEDIATORS: TTF-1, FOXA2, VEGF, glucocorticoids, sex hormones

26 — 19.5

CANALICULAR **3**

24 —

Formation of respiratory bronchioles and alveolar ducts; Increase of capillary bed; Epithelial and mesenchymal differentiation; Surfactant production starts.

MAJOR MOLECULAR MEDIATORS: FOXJ1, FOXA2, TTF-1, GATA6, SOX2, VEGF, RA, glucocorticoids, sex hormones

17 — 18.5

16 —

2 PSEUDOGLANDULAR

Formation of airways and blood vessels up to a pre-acinar level.

MAJOR MOLECULAR MEDIATORS: FGF-10, SPRY2, SHH, BMP-4, TGF-β, WNT family, EGF, PDGF, RA, extracellular matrix components

EMBRYONIC

7 — 12.5

Respiratory primordium appears as a ventral diverticulum of the foregut and it branches giving rise to the main bronchi; Tracheoesophageal septation.

1

5 —

4 — 11

MAJOR MOLECULAR MEDIATORS: TTF-1, FOXA2, GATA-6, SOX2, FGF-10, BMP-4, SHH, RA

Weeks — Days

HUMAN   RAT

LUNG DEVELOPMENT STAGES

The **pseudoglandular** stage occurs, in human, between 5 to 17 weeks of gestation (12.5 to 18.5 dpc in rat) and it is characterized by development of the hierarchical pattern of all preacinar airways (until terminal bronchioles) and blood vessels [Morrisey & Hogan, 2009]. During the pseudoglandular stage occurs the first sixteen generations of branching that are stereotypically reproducible (genetically predetermined). The remaining seven generations, the last of which lead into the alveoli, occurring in other stages, appears to follow a more random-appearing distribution [Metzger *et al.*, 2008; Maeda *et al.*, 2007; Shi *et al.*, 2007; Warburton *et al.*, 2000]. Further step in lung development is production of cartilage, which can already be found around the main bronchi before 10 weeks after conception [Correia-Pinto *et al.,* 2010].

The **canalicular** stage comprise the period between 16 and 26 weeks in humans (18.5 to 19.5 dpc in rat). The cranial part of the lung develops relatively faster than the caudal part, resulting in partial overlap between this stage and the previous stage. This stage is characterized by further expansion of respiratory tree in diameter and length, division of terminal bronchioles into respiratory bronchioles and alveolar ducts, and an increase of capillaries number leaning against the epithelium, marking the beginning of the prospective gas-exchange region. Moreover, the pulmonary epithelium differentiates into AECI (cells of thinned air-blood barrier) and AECII (surfactant producers) and surfactant production starts at the end of this stage [Correia-Pinto *et al.,* 2010; Shi *et al.,* 2009]. Pulmonary surfactant is a complex mixture of about 90% phospholipids (PLs) and 10% proteins that function to decrease the surface tension at the alveolar air-liquid interface, therefore stabilizing and maintaining the alveoli in an open position that prevents alveolar collapse. The PLs, mainly phosphotidylcholine, are assembled in the endoplasmic reticulum and the Golgi apparatus of AECII and are packaged in lamellar bodies until exocytosis [Gower & Nogee, 2011; Osanai *et al.*, 2006]. Regarding surfactant proteins (SP), there are four types synthesized by AECII (SP-A, B, C and D). SP-A and SP-D are hydrophilic proteins that regulate surfactant secretion and support immune responses in the lung. On the other hand, SP-B and SP-C are hydrophobic proteins that allow proper functioning of surfactant, with SP-B interacting with calcium ions and SP-C enhancing the movements of PLs molecules that keeps the surfactant properties [Gower & Nogee, 2011; Whitsett & Weaver, 2002]. The importance of surfactant for perinatal survival is further supported by the findings that inherited disorders of surfactant metabolism cause lethal respiratory distress in mature infants after birth [Gower & Nogee, 2011; Maeda *et al.*, 2007].

The canalicular stage is also characterized by mesenchymal cells differentiation and interstitial tissue decrease. The major source for these lung mesenchymal cells is lateral splanchnic me-

soderm. However, about 30% of the vascular smooth muscle cells as well as some mesenchymal cells outside the blood vessel walls are derived from mesothelial cells [Shi *et al.*, 2009; Que *et al.*, 2008]. Lung mesenchyme development is crucially influenced by signals from the epithelium and the pleura that, in concert, appear to maintain a balance of differentiated and proliferating multipotent progenitors while the lung grows [Weaver *et al.*, 2003].

The **saccular** (terminal sac) stage begins at about 24 weeks in human pregnancy (19.5 to 22 dpc in rat). There is again a partial temporal overlap between this stage and the previous stage, since in first 2 weeks of this stage, proximally are occurring saccular specific phenomena, whereas distally are taking place yet canalicular phenomena. In this stage, the terminal or acinar tubes narrow and give rise to small terminal sacs or primitive alveoli. There is substantial thinning of the interstitium that it results from apoptosis as well as ongoing differentiation of mesenchymal cells. The capillaries also grow rapidly in the mesenchyme surrounding the primary alveoli to form a complex double network. Indeed, the airspaces are smooth-walled transitory ducts and saccules with primary septa, which are thick and are tightly associated with a double capillary network. Furthermore, during this period, preparation for the real alveolarization starts by deposition of elastic fibers at the localizations where future secondary septa will form. Additionally, at this stage, the alveolar epithelial cells are more clearly differentiated into mature squamous AECI, providing close apposition between pulmonary blood vessels and the respiratory epithelium, and secretory surfactant AECII, with further maturation of the surfactant system occurring. These factors are determinant to support relatively inefficient gas exchange, but sufficient to maintain the life of prematurely born neonates [Correia-Pinto *et al.*, 2010; Morrisey & Hogan, 2009; Copland & Post, 2004].

The **alveolar** stage begins around 36 weeks of pregnancy in human, and continues postnatally at least up to 7 years of age, giving rise to about 300 million alveoli. In opposition, in rodents (mouse and rat), perhaps the most extensively studied laboratory species for lung development, the alveolar stage is all a postnatal phenomenon (7 to 35 postnatal days) [Shi *et al.*, 2009; Zoetis & Hurtt, 2003]. During alveolarization the sacs are subdivided by the ingrowth of ridges or crests known as secondary septae. In fact, the establishment of secondary septa within terminal sacs is the key step for differentiation of the saccules into alveoli, and the increase of airway surface area. The alveologenesis involves a complex interaction between airway epithelial cells, vascular endothelial cells, and myofibroblasts that migrate to the proper position within nascent alveolar septa, and synthesize and deposit of elastin [Correia-Pinto *et al.*, 2010; Morrisey

& Hogan, 2009]. The trans-section of these newly formed interalveolar walls (secondary septa) demonstrates a doubled capillary layer separated by a sheet of connective tissue. This structure does not yet correspond to the adult morphology characterized by thin interalveolar septa, in which a capillary monolayer occupies almost the whole space of the septum. Therefore, these structures will undergo more restructuring, the microvascular maturation [Correia-Pinto *et al.*, 2010; Morrisey & Hogan, 2009; Copland & Post, 2004].

The **microvascular** maturation stage is a postnatal stage characterized by septum maturation and formation of the mature interalveolar wall, a functional respiratory membrane that consists of endothelial cells tightly associated with the flattened AECI, which favors optimal gas exchange [Correia-Pinto *et al.*, 2010; Roth-Kleiner & Post, 2003]. Thus, two additional processes occur: the thinning out of the secondary septa; and the maturation of the capillary bed. Thinning of the interstitial tissue involves cells apoptosis in the postnatal lung mesenchyme. Simultaneously, occur capillary remodeling from double capillary layer into a single capillary layer [Schittny *et al.*, 1998].

Lung development is a highly orchestrated process directed by mesenchymal (vessels)-epithelial interactions which control and coordinate the temporal and spatial expression of multiple regulatory factors required for proper lung formation [Correia-Pinto *et al.*, 2010]. In the next sections current knowledge about airway branching morphogenesis, vasculature development and regulation of lung development will be detailed.

1.1 Branching morphogenesis

Once the primary lung buds have formed, they extend into the surrounding mesenchyme and begin the process of branching morphogenesis, generating a complex tree-like structure [Morrisey & Hogan, 2009]. The branching morphogenesis results from three distinct local processes that are repeatedly in different combinations: domain branching, planar bifurcation, and orthogonal bifurcation [Metzger *et al.*, 2008; Warburton *et al.*, 2008]. Domain branching is responsible for the formation of the main secondary branches and it involves the orderly sprouting of new buds at specific distances from the tip of a stalk and at positions around the circumference that are either dorsal/ventral or medial/lateral relative to the axis of the parent stalk [Morrisey & Hogan, 2009]. The other two processes are related to the bifurcation of the tip. They are called planar and orthogonal bifurcation, depending on the axis along which the two new buds are formed.

Planar bifurcation subsequently generates the tertiary and later-generation branches, whereas orthogonal bifurcation results in the formation of three-dimensional branching of the airways.

The importance of mesenchymal-epithelial tissue interactions for lung development has been known for several decades. Each tissue compartment produces unique sets of growth factors and other signaling molecules, which signal in a paracrine manner between the epithelium and the mesenchyme. Their expression must be well coordinated as at some sites they need to enhance cell proliferation (at the edge of the growing lung buds), while at other sites they have to inhibit cell division (at branching points). The exact mechanisms responsible for regulation of lung bud branching are not completely understood. However, the current model suggests that lung buds undergo repetitive cycles of four processes: lung bud position and elongation, cessation of outgrowth, expansion of the tip, and bifurcation [Morrisey & Hogan, 2009; Metzger *et al.*, 2008]. At the molecular level, these processes result from a dynamic change in relative activity of fibroblast growth factor 10 (FGF-10), fibroblast growth factor receptor 2IIIb (FGFR2IIIb), sprouty 2 (SPRY2), sonic hedgehog (SHH), and bone morphogenetic protein 4 (BMP-4) [Correia-Pinto *et al.*, 2010; Morrisey & Hogan, 2009; Metzger *et al.*, 2008]. It has been proposed that high SHH signaling and low FGF-10 signaling occurs where branching is not supposed to proceed. In opposition, where a branch emerges, SHH is locally suppressed by patched receptor (PTC) and hedgehog interacting protein (HIP), which re-establish FGF-10 signal. FGF-10 induces the dynamic expression of its inhibitor SPRY2 as branches extend. As the bud elongates towards the mesenchymal source of FGF-10, SPRY2 begins to be synthesized at the most distal tip until it reaches maximal expression and almost abrogates FGF-10 expression, thus determining interbranch length. Proximally, bud outgrowth is regulated by SHH signaling. BMP-4 is also an important factor that appears to enhance lung branch tip outgrowth according together with FGF-10. FGF-10 is shown stimulating BMP-4 expression (Figure 2) [Morrisey & Hogan, 2009; Cardoso & Lü, 2006; Warburton *et al.,* 2005].
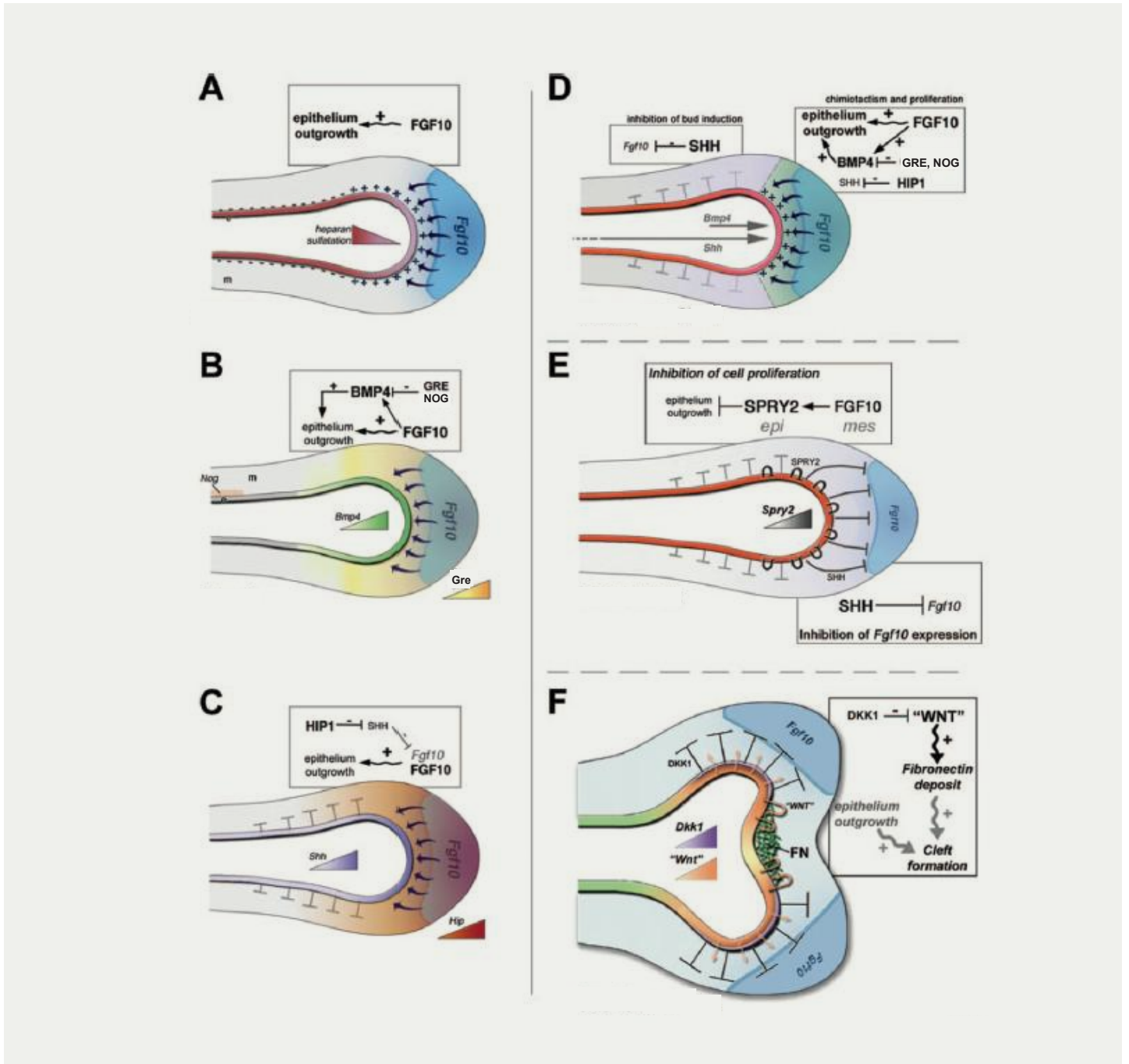
Figure 2. Current model of molecular events involved in lung bud position and elongation, cessation of outgrowth, expansion of the tip and bifurcation [from Warburton et al., Pediatr Res, 2005]. A. FGF-10 distal mesenchyme expression induces chemotaxis of the bud tip toward the FGF-10 source. Heparan sulfate is important for FGF-10 function. B. BMP-4 enhances bud tip branching according together with FGF-10. FGF-10 induces BMP-4 epithelial expression, whereas the BMPs binding proteins gremlin (GRE) and noggin (NOG) repress BMP-4 expression. C. SHH inhibits FGF-10 expression at the branch tip and, simultaneously, HIP inhibits SHH which re-establish FGF-10 signal. D. Lung bud position and elongation: FGF-10, BMP-4 and SHH promote the balance between chemotaxis and proliferation resulting in bud induction versus inhibition of bud outgrowth. E. Cessation of outgrowth and expansion of the tip: interbranch length may be determined by arrest of bud outgrowth by concerted action of SPRY2 and SHH. FGF-10 mesenchymal gradient induces SPRY2 epithelial gradient as branches extend, thus inhibiting epithelial outgrowth. In proximal regions of lung bud, SHH mediates branching suppression by inhibiting FGF-10 expression in the peripheral mesenchyme. F. Lung bud bifurcation: Wingless (WNT) signaling propels fibronectin (FN) deposition between the branch tips, leading to epithelial cleft formation. Dickkopf 1 (DKK1) inhibits WNT signaling away from the cleft, with consequent lower deposition of FN where the cleft does not have to occur.

1.2 Development of Pulmonary Vascular System

Pulmonary vascular system is divided into pulmonary and bronchial circulations. The pulmonary arteries supply the intrapulmonary structures and regulate gas exchange. The bronchial system is the nutrient and oxygen supplier of the lung. All intrapulmonary structures are drained into the pulmonary veins, whereas the hilar structures drain into the bronchial veins and then to the azygos system [Hislop, 2005; Hislop, 2002].

The vascular development takes place during all stages of lung development and important alterations of the three-dimensional structure of the capillary network lay the foundation for alveolarization [van Tuyl *et al.*, 2005]. Even in the early stages of lung development vascular connections are well established, with the development of the central bronchial arteries during the embryonic period. By the end of the pseudoglandular stage, preacinar bronchi are formed and are accompanied by the pulmonary and bronchial arteries. During the canalicular stage of lung development, the distal circulation develops and there is a marked growth in capillary network which gets closer to the pulmonary epithelium. Thus, the canalicular stage is also known as the vascular stage [Copland & Post, 2004; Warburton *et al.*, 2000]. In the saccular phase capillaries are evident around the saccules. In the alveolar phase the secondary septa contain a capillary bilayer. During the microvascular maturation the vasculature development is completed with the formation of single capillary networks [Copland & Post, 2004].

The pulmonary vasculature develops within the splanchnic mesoderm via two simultaneous processes, vasculogenesis and angiogenesis [Fisher & Summer, 2006; Hislop, 2002; Gebb & Shannon, 2000]. **Vasculogenesis** is characterized by *in situ* new vessels formation from precursors (angioblasts). The angioblasts or endothelial cell precursors are present in mesoderm since early stages of lung development. Thus, these mesodermal cells trans-differentiates into endothelial cells, proliferate, and organize into vessels [Warburton *et al.*, 2000]. **Angiogenesis** is the process of new vessels formation from extension of pre-existing ones. In this process, differentiated endothelial cells proliferate, sprout from previously formed vessels, and form new vascular structures [van Tuyl *et al.*, 2005].

Several studies about pulmonary vasculature development suggest that the large central pulmonary arteries are formed by angiogenesis from the aortic sac, whereas the capillary plexus around the distal endoderm (lung periphery) is generated by vasculogenesis [Morrisey & Hogan, 2009; Anderson-Berry *et al.*, 2005; Hislop, 2002]. Moreover, a fusion process is necessary to connect angiogenic and vasculogenic vessels, the main pulmonary trunks with the peripheral

vasculature [Morrisey & Hogan, 2009; deMello & Reid, 2002]. Communication between the two respective vascular networks rarely occurs in the mid-pseudoglandular stage, but a gradual increase in communication progresses until a complete vascular circuit is achieved by the beginning of the terminal sac stage [Schwarz *et al.*, 2009; deMello *et al.*, 2002]. Nonetheless, questions regarding the process of pulmonary vascular morphogenesis remain. For instance, Parera *et al* suggested distal angiogenesis as the mechanisms for lung vasculature development [Parera *et al.*, 2005]. Recently, Schwarz *et al* also defended that initial pulmonary vessel formation occurring within the mesenchyme is predominately angiogenic [Schwarz *et al.*, 2009].

Regarding molecular regulation of pulmonary vasculature development, it has only recently begun to be elucidated. It has been demonstrated that cell-extracellular matrix and cell-cell interactions as well as growth factors and transcription factors are involved in vascular development [Copland & Post, 2004]. Specifically, members of the vascular endothelial growth factor (VEGF) [Yamamoto *et al.*, 2007; Healy *et al.*, 2000], the angiopoietin [Hato *et al.*, 2009] and the ephrin [Vadivel *et al.*, 2012] families appear to be key players in the control of pulmonary vascularization. Moreover, it is now consensual that lung vascular development requires epithelial-mesenchymal interactions [Crivellato, 2011; Gebb & Shannon, 2000]. For instance, VEGF is expressed in the developing epithelium, whereas their cognate receptors (Flk-1, fetal liver kinase 1; Flt-1, FMS-like tyrosine kinase 1) are expressed in and direct the emergence of developing vascular capillary networks within the mesenchyme [Correia-Pinto *et al.*, 2010; White *et al.*, 2007; Warburton *et al.*, 2005].

Furthermore, there is growing evidence for reciprocal interactions between airways and blood vessels that are critical for normal lung development [Crivellato, 2011]. For instance, hepatocyte growth factor (HGF) produced by the endothelial cells positively regulates the proliferation of the endoderm [Yamamoto *et al.*, 2007]. Ephrin B_2 expressed by the microvasculature is critical for lung alveolization and secondary septae formation [Vadivel *et al.*, 2012; Wilkinson *et al.*, 2008]. On the other hand, it was demonstrated that ablation of lung epithelium impairs lung vascular cells development [Sarah *et al.*, 2000]. Moreover, VEGF inhibition in neonatal rats leads to arrested alveolar development, suggesting that inhibition of vascular growth itself may directly impair lung development [Thébaud *et al.*, 2005; van Tuyl *et al.*, 2005; Compernolle *et al.*, 2002; Healy *et al.*, 2000; Jakkula *et al.*, 2000]. In contrast, addition of VEGF to early mouse embryonic lung explants markedly stimulates epithelial as well as vascular morphogenesis, playing an important role in matching the epithelial-capillary interface during lung morphogenesis [Del Moral *et al.*, 2006; Compernolle *et al.*, 2002]. Interestingly, recently Lazarus *et al* uncovered that vasculature also determines stereotypy

of epithelial branching morphogenesis. Indeed, vascular ablation perturbed branching stereotypy, inducing overall flat lung morphology, and also led to a high frequency of ectopic branching. This role of the vasculature was independent of perfusion, flow or blood-borne substances [Lazarus *et al.*, 2011]. Thus, airway and vascular development are intimately linked processes and the disruption of one system has severe consequences on the development of the other.

1.3 Regulation of Lung Development

The lung development is a highly predetermined program, orchestrated by finely integrated and mutually regulated networks of several controlling factors, namely: i) genetic factors (transcription factors, growth factors, hormones, their receptors and intracellular signaling components, extracellular matrix (ECM) components and intercellular adhesion molecules); ii) mechanical stimuli, including
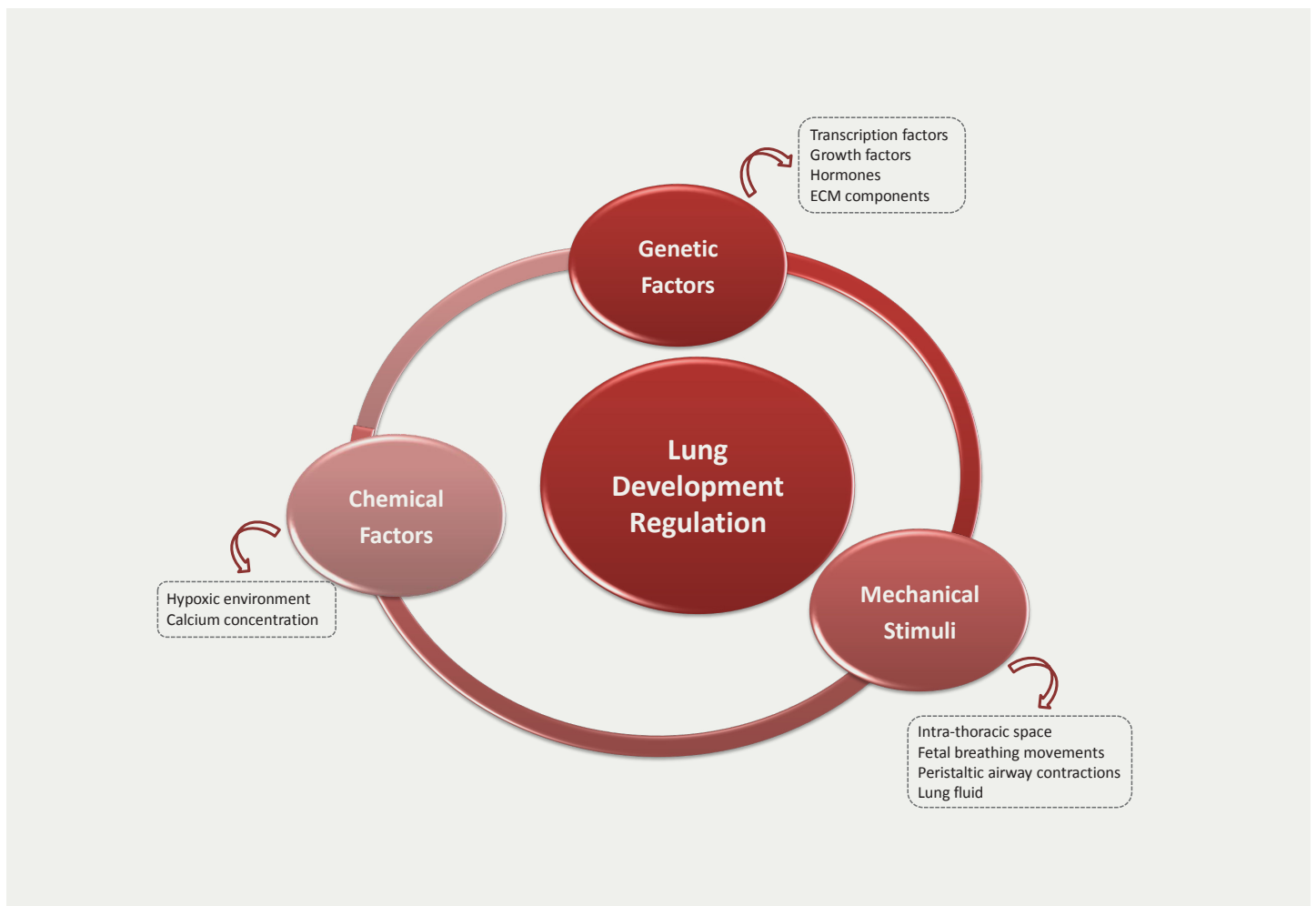
Figure 3. Overview of lung development regulation. The lung development is a complex morphogenetic process, involving genetic factors, mechanical stimuli and chemical factors.

intra-thoracic space, fetal breathing movements, peristaltic airway contractions and lung fluid; and iii) chemical factors such as relative hypoxia and calcium concentration (Figure 3) [Correia-Pinto *et al.*, 2010; Shi *et al.*, 2009; Warburton *et al.*, 2005].

The role and the interplay between major molecular mediators of lung morphogenesis will be reviewed in following sections.

1.3.1 Genetic Factors

Several transcription factors, growth factors, hormones, their receptors and intracellular signaling components have been described as crucial players involved in normal lung development. These genetic factors are programmed to be expressed at the correct time, place and dose to induce and control orderly airway and vasculature development.

1.3.1.1 Transcription Factors

Numerous transcription factors and their binding sites have been characterized and associated with the regulation of lung specific genes. For instance, several transcription factors play a specific part in differentiation of the epithelium and mesenchyme into specific cell types. Nonetheless, the principles that govern the design and evolution of transcriptional networks operating during lung formation and function have just begun to be understood [Maeda *et al.*, 2007]. In the following sections the main transcription factors involved on lung morphogenesis will be briefly reviewed.

Thyroid-specific Transcription Factor 1

The thyroid-specific transcription factor 1 (TTF-1) also known as Nkx2 marks the region from which lung buds arise and is one of most important genes in lung development. Indeed, the earliest known step in the development of the respiratory system (the trachea and lungs) is the establishment of a localized domain of TTF-1 expression in the ventral wall of the anterior foregut. Thus, TTF-1 is the earliest known marker associated with commitment of endodermal cells to pulmonary and thyroid cell lineages, appearing before formation of the definitive lung [Maeda *et al.*, 2007]. Abrogation of *Ttf1* in mice results in full absence of distal airway branches, while two main bronchial stems are still formed, suggesting lung development is arrested at a

very early stage [Cardoso & Lü, 2006]. Null mutation of *Ttf1* is also associated with defects in tracheoesophageal septum development [Morrisey & Hogan, 2009]. Furthermore, TTF-1 also influences perinatal lung maturation, regulating the expression of genes critical for respiratory adaptation at birth [Maeda *et al.*, 2007].

Forkhead Box Proteins

Forkhead box proteins (FOX) are a family of more than 50 transcription factors sharing a winged helix deoxyribonucleic acid (DNA) binding domain, and play important roles in the regulation of the expression of genes involved in cell growth, proliferation, differentiation. Members of FOX proteins have differential expression patterns in lung during development [Maeda *et al.*, 2007].

FOXA2 or hepatocyte nuclear factor 3β (HNF-3β) is expressed in cells believed to be progenitors of respiratory epithelial cells and has a major role in the regional specification of foregut endoderm. *Foxa2* knockout (KO) presents a severe early embryonic lethal phenotype with complete failure of the primitive foregut to close into a tube and, therefore, absence of the lungs [Sasaki & Hogan, 1993]. Later in lung development, FOXA2-binding sites have been found in the promoter regions of several lung-specific genes, such as SP-A, B, C, D and Clara cell-secretory protein (CCSP) [Warburton *et al.*, 2000; Bohinski *et al.*, 1994]. Thus, the FOXA2 is expressed in an overlapping pattern with TTF-1 in respiratory epithelial cells and appears to cooperate with the TTF-1 to determine pulmonary epithelial cell lineage fates [Maeda *et al.*, 2007; Warburton *et al.*, 2000].

FOXF1 has been involved in the regulation of mesenchymal-epithelial interactions. Indeed, it is expressed in the splanchnic mesenchyme in close apposition to endoderm, suggesting its role in mesenchymal-epithelial interaction during lung and gastrointestinal tract morphogenesis. *Foxf1* KO is lethal before the gastrulation whereas *Foxf1* heterozygote presents severe lung malformations and is associated with tracheoesophageal septum anomalies [Morrisey & Hogan, 2009; Maeda *et al.*, 2007; Mahlapuu *et al.*, 2001].

Regarding **FOXJ1**, also known as hepatocyte nuclear factor-3/forkhead homologue 4 (HFH-4), this transcriptional factor is critical in the establishment of right-left asymmetry and in the development of the ciliated cells. In fact, defects of FOXJ1 results in failure of cilia formation and left-right asymmetry that is integral part of the body plan and necessary for normal formation and localization of intrathoracic and intra-abdominal organs [Alten *et al.*, 2012; Maeda *et al.*, 2007; Zhang *et al.*, 2004].

A/TGATA/G -Binding Transcription Factor

GATA-6, a member of the GATA family of zinc finger transcriptional factors, which bind DNA consensus sequence A/TGATA/G, is expressed in respiratory epithelial cells throughout lung morphogenesis [Maeda *et al.*, 2007]. GATA-6 has been implicated in pulmonary endoderm specification. Later in development, GATA-6 also influences epithelial cells differentiation, sacculation and alveolarization of the lung [Zhang et al., 2008]. Interestingly, expression of a dominant negative *Gata6* in mouse respiratory epithelial cells inhibited lung differentiation in late gestation and decreased surfactant proteins. On the other hand, increased activity of GATA-6 inhibited alveolarization and perturbed pulmonary function [Maeda *et al.*, 2007; Yang *et al.*, 2002; Koutsourakis *et al.,* 2001].

Sex determining region Y- Box

A number of sex determining region Y-box (SOX) genes are expressed in the developing lung, including SOX2, -4, -9, -11, and -17 [Maeda *et al.*, 2007].

Regarding **SOX2**, it is involved in tracheoesophageal septation. As respiratory diverticulum caudally grows, the single foregut tube begins to separate into two - a dorsal esophagus that leads into the stomach and a ventral trachea that connects to the lung buds, by the tracheoesophageal septum formation [Morrisey & Hogan, 2009]. A greatest SOX2 expression is observed dorsally in the future esophagus while TTF-1 expression marking the future trachea is greatest ventrally. This patterning also depends on signals from the surrounding mesenchyme. Null mutations of *Sox2* lead to defects in tracheoesophageal septum development including tracheoesophageal fistula and esophageal atresia [Morrisey & Hogan, 2009; Que *et al.*, 2006]. Recently, it was also demonstrated that SOX2 is required for maintenance and differentiation of PNEC, Clara, ciliated, and goblet cells [Tompkins *et al.*, 2009; Tsao *et al.*, 2009;].

Semaphorins, Eyes absent and Six Transcription Factors

Recently, other transcription factors have been described to be important for lung development.

To date, more than 20 types of semaphorins (Sema) have been identified and they have diverse functions in many physiological processes [Arese *et al.*, 2011]. **Sema3A** is a neural guidance cue that also mediates cell migration, proliferation and apoptosis, and inhibits branching morphogenesis. It was demonstrated that Sema3A modulates distal pulmonary epithelial cell development and lung morphogenesis. Indeed, genetic deletion of *Sema3A* induces septae thickness, alveolarization reduction and significant perinatal lethality [Becker *et al.*, 2011].

The evolutionarily conserved homeodomain transcription factor **Six1** (homologous to *Drosophila sine oculis* gene) and its canonical coactivator Eyes absent 1 (**Eya1**) synergistically control organ-specific progenitor cell proliferation and survival and are critical regulators of embryonic development, namely of kidney, muscle and inner ear development [Farabaugh *et al.,* 2012; Li *et al.,* 2003]. Recently, it was demonstrated that Six1 and Eya1 are important coordinators of pulmonary epithelial, mesenchymal and vascular morphogenesis. These transcription factors are expressed at the distal epithelial tips of branching tubules as well as in the surrounding distal mesenchyme. Six1 and Eya1 ensure the proper level of proliferation and differentiation along the proximodistal axis of epithelial, mesenchymal and endothelial cells, and they are critical upstream coordinator of SHH-FGF-10 signaling during embryonic lung development. Indeed, *Six1* and *Eya1* deficient lungs are severely hypoplastic, with reduced epithelial branching, increased mesenchymal cellularity and severe defects in the smooth muscle component of the bronchi and major pulmonary vessels [El-Hashash *et al.,* 2011a; El-Hashash *et al.,* 2011b].

1.3.1.2 Growth Factors

Growth factors and their signal pathways play important regulatory roles in lung morphogenesis. Many of them also play important roles in lung pathologies and lung repair mechanisms [Warburton *et al.,* 2000]. Some authors defense that early formation of the airways is mainly controlled by transcription factors, whereas later division of airways is influenced by growth factors [Hislop, 2002].

Fibroblast Growth Factor Family

In vertebrates, the FGF family is composed by 22 members that have functions on cell proliferation, differentiation and migration during organogenesis [Itoh & Ornitz, 2011; Ornitz & Itoh, 2001]. The developing lung expresses FGF-1, -2, -7, -9, -10, and -18, and FGF family have been shown to be important for the formation of secondary septae [Correia-Pinto *et al.,* 2010; Weinstein *et al.,* 1998].

FGF-10 has proven to be absolutely necessary for lung development. Indeed, mice deficient for *Fgf10* present lung agenesis [Ohuchi *et al.,* 2000]. On the other hand, FGF10 overexpression perturbs pulmonary morphogenesis and induced cystic lung malformations [Hashimoto *et al.,* 2012a; Gonzaga *et al.,* 2008]. FGF-10 is expressed in the distal mesenchyme at sites where epithelial buds will emerge and it was shown to induce chemotaxis of the distal lung epithelium. FGF-10 and its high affinity receptor FGFR2IIIb (present on epithelial cells) are mediators of fundamental

epithelial-mesenchymal interations during lung development [Weaver *et al.*, 2000]. Furthermore, FGF-10 plays a pivotal role in maintaining epithelial progenitor cell proliferation and regulates the formation of multiple mesenchymal lineages during lung development, namely alveolar smooth muscle cell formation and vascular development [Ramasamy *et al.*, 2007].

Regarding **FGF-9**, during early pulmonary development, it is expressed in epithelium and mesothelium, becoming later restricted to the mesothelium. Mesothelial- and epithelial-derived FGF-9 have distinct functions in the regulation of lung development. Mesothelial FGF-9 is primarily responsible for regulating mesenchymal proliferation and differentiation, and it has been suggested that maintains a FGF-10 progenitor population. On the other hand, epithelial FGF-9 primarily affects epithelial branching [Yin *et al.*, 2011]. Thus, embryos lacking *Fgf9* present mesenchymal hypoplasia, epithelial branching decrease and, by the end of gestation, hypoplastic lungs [Weaver *et al.*, 2003; Colvin *et al.*, 2001].

Concerning to **FGF-7** (or keratinocyte growth factor), it is expressed by mesenchymal pulmonary cells and mediates epithelial-mesenchymal interactions by binding to FGFR2IIIb. FGF-7 overexpression in pulmonary epithelium has been related with abnormal morphogenesis similar to cytadenomatoid malformation [Simonet *et al.*, 1995].

At the moment there are four subtypes of FGFR described, which bind members of the FGF family with varying affinity. FGFR1, 2, and 3 encode two receptor isoforms that are generated by alternative splicing, and each binds a specific repertoire of FGF ligands. **FGFR2IIIb** is mainly expressed in epithelium and binds four known ligands (FGF-1, FGF-3, FGF-7, and FGF10), which are primarily expressed in mesenchymal cells [Ornitz & Itoh, 2001]. This receptor has a key role during lung development. In fact, mice deficient for *Fgfr2IIIb* present agenesis and dysgenesis of multiple organs, including the lungs [Peters *et al.*, 1994].

Sprouty Family

In vertebrates have been identified 4 SPRY homologs and all share a cysteine-rich region. SPRY family plays an important role in regulation of lung branching morphogenesis. Indeed, this family is a FGF-inducible signaling antagonist and also an inhibitor of other tyrosine kinase signaling pathways such as epidermal growth factor (EGF) [Hashimoto *et al.*, 2012b; Tefft *et al.*, 2002].

SPRY2 has a critical role in branching morphogenesis. It is expressed at the distal tips of the embryonic lung epithelial branches and is down-regulated at sites of prospective bud formation. SPRY2 high expression at the bud tip induces bud outgrowth arrest and tip splitting event must

occur [Sutherland *et al.*, 1996]. Recently, it was demonstrated that in embryonic day 14 and 16 lungs, SPRY2 was expressed both in epithelial and peripheral mesenchymal cells [Hashimoto *et al.*, 2012b]. Mouse *Spry2* KO presents increase of lung branching morphogenesis and enhanced expression of specific lung epithelial differentiation markers [Tefft *et al.*, 2002]. On the other hand, **SPRY4** is predominantly expressed throughout the distal lung mesenchyme. SPRY4 inhibits endo-thelial cells proliferation and negatively regulates angiogenesis [Tefft *et al.*, 2002].

Sonic Hedgehog Pathway

SHH signaling is crucial for lung branching morphogenesis regulation, since the temporos-patial restriction of FGF-10 expression by SHH appears to be essential to initiate and maintain lung branching [Jia & Jiang, 2006]. Indeed, *Shh* null mutant presents lung hypoplasia [Pepicelli *et al.*, 1998]. Epithelial SHH expression is also a mesenchymal differentiation signal. Moreover, SHH expression is locally suppressed at branch tips by the induction of its **PTC** receptor and **HIP**. These proteins assist local FGF signaling where branching is stereotypically programmed to take place [Zhang *et al.*, 2010; Chuang & McMahon, 2003]. The activation of PTC by SHH induces the glioblastoma (**GLI**) transcription factors (GLI1, GLI2 and GLI3). There are expressed in distinct but overlapping domains in lung mesenchyme and the highest expression registered at the distal tips. Experiments with *Gli* KO are indicating that these transcription factors may be effectors of other signaling pathways besides SHH [Maeda *et al.*, 2007; Motoyama *et al.*, 1998].

SHH pathway has also been implicated in tracheoesophageal septation. In fact, *Shh* and *Gli1/Gli3* null mutants also present defects in tracheoesophageal septum development, inclu-ding tracheoesophageal fistula and esophageal atresia [Morrisey & Hogan, 2009].

Transforming Growth Factor Beta Family

The transforming growth factor beta (TGF-β) superfamily of cytokines regulates important organogenesis events, such as cell proliferation, differentiation and death. This superfamily can be divided into three subfamilies: TGF-β , BMP and activin. These TGF- ligands bind to their cog-nate receptors on the cell surface and activate downstream cytoplasmatic effectors, the Smads (homologous genes of Drosophila mothers against decapentaplegic) proteins, which translocate into the nucleus and modulate target gene expression [Shi *et al.*, 2009].

<u>TGF- β</u>

There are three **TGF-**β isoforms (TGF-β1, -β2, and -β3) in mammals. All of them plus TGF- β type I and type II receptors, though differentially distributed, are expressed during lung development. In early mouse embryonic lungs, TGF-β1 is expressed in the distal mesenchyme underlying branching points, whereas TGF-β2 is localized in distal epithelium, and TGF-β3 is expressed in proximal mesenchyme and mesothelium [Bragg *et al.*, 2001; Millan *et al.*, 1991].

Endogenous autocrine/paracrine TGF-β signaling via TGF-β type II receptors negatively regulates lung morphogenesis by keeping cell cycle in G_1 arrest [Zhao *et al.*, 1996]. On the other hand, TGF-β signaling via TGF-β type I receptors is responsible for the formation of branch points and localized deposition of ECM components [Heine *et al.*, 1990]. Embryonic lung organ and cell cultures as well as KO models has demonstrated that finely regulated and correct physiologic concentrations and temporospatial distribution of TGF-β1, 2, and 3 are essential for normal lung morphogenesis and defense against lung inflammation [Zhao J *et al.*, 1999; Serra *et al.*, 1994]. Indeed, mice lacking *Tgfb1* develop normally but die within the first month or two of life of aggressive pulmonary inflammation. On the other hand, *Tgfb2* KO die *in utero* of severe cardiac malformations, whereas *Tgfb3* mutants die, during the neonatal period, of lung dysplasia and cleft palate [Kaartinen *et al.*, 1995; Serra *et al.*, 1994].

<u>BMPs</u>

BMPs, with more than 20 family members, have been shown to regulate many developmental processes including lung development [Correia-Pinto *et al.*, 2010; Hogan, 1996]. Indeed, BMPs may play multiple roles in lung development.

BMP-3, -4, -5 and -7 as well as type I BMP receptors are expressed during lung development [Bellusci *et al.*, 1996; Takahashi & Ikeda, 1996]. Signaling through BMP receptors has been involved on promotion of respiratory identity in the foregut. In fact, type I BMP receptors promote tracheal formation via repression of SOX2 and it restricts the site of lung bud initiation [Domyan *et al.*, 2011].

Regarding **BMP-4**, its expression was detected in pulmonary epithelium, with maximal expression at branch tips and in the adjacent mesenchyme [Bellusci *et al.*, 1996]. The exact roles of BMP-4 in lung development remain controversial. BMP-4 has been described as responsible for epithelial proliferation suppression, mediator of branch extension and determinant of branch points [Weaver *et al.,* 2000; Bellusci *et al.*, 1996]. More recently, however, BMP-4 was considered a branching stimulator [Warburton *et al.*, 2005; Bragg *et al.*, 2001]. Nonetheless, overexpression of *Bmp4* at distal budding epithelium induces epithelial proliferation decrease, lung hypoplasia and

distended terminal buds [Bellusci *et al.*, 1996]. FGF-10 is able to induce BMP-4 expression in lung epithelium, acting in concomitance during lung bud outgrowth. In opposition, BMP-4 effects are negatively modulated by BMPs binding proteins, gremlin and noggin [Hyatt *et al.*, 2004; Weaver *et al.*, 2003; Weaver *et al.*, 2000]. Furthermore, BMP signaling is also important in lung vasculogenesis and angiogenesis. In fact, mutations of BMP type II receptor and changes in the expression level of gremlin are associated with primary pulmonary hypertension [Costello *et al.*, 2008; Lane *et al.*, 2000]. Since KO for *Bmp4* and BMP-specific *Smads* cause early embryonic lethality, their functions in lung development *in vivo* still need to be further defined. Nonetheless, it is known that null mutations of *Bmp4* and *noggin* lead to defects in tracheoesophageal septum development, including tracheoesophageal fistula and esophageal atresia [Morrisey & Hogan, 2009; Que *et al.*, 2006.

Wingless Signaling Pathway

In mouse, Wingless (WNT) growth factor family consists of 19 secreted ligands that interact with 10 known frizzled (FZ) receptors and either one of two single-span membrane proteins, the low-density-lipoprotein receptor-related proteins (LRP) 5 and 6 [Tamai *et al.*, 2000]. WNT proteins have been grouped into two classes, canonical (WNT1, WNT3, WNT3a, WNT7a, WNT7b, WNT8) and noncanonical (WNT4, WNT5a, WNT11), according to signal transduction. The canonical pathway involves the WNT binding to FZ receptors and, consequently, the inhibition of β-catenin phosphorylation mediated by glycogensynthase kinase-3β (GSK3β). Hypophosphorylated and stable β-catenin accumulates in the cytoplasm, after which it translocates to the nucleus and interacts with members of TCF/LEF (T Cell Factor/Lymphoid Enhancer Factor) transcription factors, activating transcription of TCF/LEF target genes [Chuang & McMahon, 2003]. Non-canonical pathways consist of the PCP (Planar-Cell-Polarity (PCP)-like pathway), or JNK/AP1 (c-Jun NH(2)-terminal kinase/activating protein 1), and of the PKC/CaMKII/NFAT (Protein Kinase C/Calmodulin kinase II/Nuclear Factor of Activated T cells) dependent transduction [Pongracz & Stockley, 2006]. Moreover, there are two classes of secreted WNT antagonists described: sFRP (secreted Frizzled-related Proteins) and DKK (Dickkopf) [Warburton *et al.*, 2005].

WNT ligands and other components of WNT signaling pathway (β-catenin, FZ, TCF/LEF) have been demonstrated to be expressed during lung development. Indeed, studies of the expression pattern of several WNT proteins suggest that WNT signaling can originate from the epithelium and mesenchyme and can target both tissues in an autocrine/paracrine fashion [Pongracz & Stockley, 2006]. WNT signaling is also important for the normal lung morphogenesis. However, the different results obtained with interference of the WNT signaling cascade (WNT, β-catenin, DKK) makes it

difficult to pinpoint the nature of WNT signaling in lung development [Correia-Pinto *et al.*, 2010].

WNT7b, for instance, is expressed in the lung epithelium with highest expression in the distal region [Pongracz & Stockley, 2006]. In mice, null mutation of *Wnt7b* results in markedly hypoplastic lung and perinatal lethality due to respiratory failure, associated with decrease of mesenchymal cells proliferation and type I pneumocytes differentiation [Rajagopal *et al.*, 2008; Shu *et al.*, 2002]. Regarding **WNT5a**, a ligand for WNT noncanonical pathway, WNT5a have been demonstrated to have a pivotal role in directing the essential coordinated development of pulmonary airway and vasculature. Some authors defense that WNT5a functions during alveolarization, in opposition to WNT canonical pathway, which is more important in earlier lung development [Loscertales *et al.*, 2008]. In fact, *Wnt5a* KO present overexpansion of distal airways, thickened intersaccular interstitium, delayed lung maturation and, consequently, respiratory failure [Li *et al.*, 2002]. Lungs of *Wnt5a* KO animals also present an increase of FGF-10, SHH and BMP-4 expression, which suggests that WNT5a may interact with other essential signaling pathways for lung morphogenesis [Yin *et al.*, 2011; Li *et al.*, 2005].

Interestingly, constitutive activation of WNT canonical pathway in embryonic lung epithelium makes these cells transdifferentiate into intestinal epithelial lineages [Okubo & Hogan, 2004]. Therefore, an appropriate level of WNT signaling in the right place at the right time is essential for normal lung organogenesis [Shi *et al.*, 2009].

Furthermore, WNT signaling pathway has an important role in cleft formation via induction of fibronectin deposition where the splitting must occur. Impaired WNT pathway by DKK (a potent and specific inhibitor of WNT action that is also endogenously secreted by the distal lung epithelium) display altered branching, characterized by unsuccessful cleft formation, enlarged terminal buds and decrease of fibronectin deposition [de Langhe *et al.*, 2005]. Thus, fibronectin is a downstream target of WNT signaling.

Epidermal Growth Factor Family

EGF family consists of EGF, TGF-α and amphiregulin. The members of this family act via EGF receptor (EGFR). The mRNA of EGF family members is synthesized in the mesenchyme, whereas the EGF proteins are expressed in epithelium. Moreover, EGFR is expressed in lung epithelium. Therefore, EGF ligands are produced in the lung mesenchyme and act on epithelium. [Warburton *et al.*, 2000; Ruocco *et al.*, 1996].

Regarding the role of EGF family on lung morphogenesis, several studies have demonstrated that EGF signaling positively modulates lung morphogenesis and cytodifferentiation [Warburton

et al., 2000]. In fact, *Egfr* null mutation provokes a 50% reduction in branching morphogenesis and a neonatal pulmonary lethal phenotype [Miettinen *et al.,* 1997]. Moreover, an *in vivo* study established that maternal treatment with EGF could promote lung morphogenesis and increase the surfactant levels in premature fetal lung [Ma *et al.,* 2009].

Platelet-Derived Growth Factor Family

Platelet-derived growth factor (PDGF) family consists of four different peptides: PDGF-A, PDGF-B, PDGF-C and PDGF-D, which form homo or heterodimers to be active. The PDGF receptors, tyrosine kinase receptors, are of two types: PDGFR-α and PDGFR-β. After binding of PDGF dimers, these receptors dimerize and may combine to generate homo or heterodimers (PDGFR-$\alpha\alpha$, $\alpha\beta$, $\beta\beta$), which have different affinities towards the four PDGFs [Warburton *et al.,* 2000]. The PDGF-AA and PDGF-BB homodimers and PDGFR are present in embryonic mouse lung and have an important role in alveologenesis [Lau *et al.,* 2011; Shi *et al.,* 2009; Warburton *et al.,* 2000]. PDGF-AA is a leading factor in alveolarization. *Pdgfa* homozygous null mutant mice are perinatally lethal, their lungs present lack of alveolar smooth muscle cells, reduction of elastin fibers deposition in the lung parenchyma, and alveolar hypoplasia [Boström *et al.,* 2002]. On the other hand, PDGF-B is crucial for vascular growth and integrity during the alveolar stage [Lindahl *et al.,* 1997].

Insulin-like Growth Factor Family

The insulin-like growth factor (IGF) family consists of two ligands (IGF-I and IGF-II), six IGF binding proteins (IGFBPs 1-6) and two receptors (IGF1R and IGF2R). All members of this family are expressed during lung development. IGF-I is expressed in lung mesenchyme, IGF-II in epithelium, IGF1R presents ubiquitous expression and IGF2R is restricted to pulmonary mesenchyme [Retsch-Bogart *et al.,* 1996; Maitre *et al.,* 1995]. The IGFBPs are differentially regulated during lung development and they may play a role in mediating temporospatial IGF signaling, particularly the regulation of cellular proliferation rates [Warburton *et al.,* 2000]. KO for IGF signaling components do not have a gross defect in primary branching morphogenesis per se, but the lungs are hypoplastic [Liu *et al.,* 1993; DeChiara *et al.,* 1990]. Nonetheless, it is likely that IGF signaling may play a key role in facilitating signaling by other peptide growth factors involved in lung morphogenesis. Indeed, IGF1R signaling function is required for both the mitogenic and transforming activities of the EGF receptor [Warburton *et al.,* 2000].

Moreover, Han *et al* described a possible role for IGF1R during human and rat pulmonary angiogenesis and vasculogenesis. Inhibition of IGF1R signal induced loss of endothelial cells and mesenchymal cell apoptosis. These observations suggest a role of IGF family of growth factors, acting through the IGF1R, as survival factors during normal human lung vascular development [Han *et al.*, 2003].

Pulmonary Neuroendocrine Factors

It has been demonstrated that pulmonary neuroendocrine products, in addition to chemoreception function, have a role in regulation of lung growth and maturation. The earliest PNEC are the first cell type to differentiate within the primitive airway epithelium and can be identified in humans at 8 weeks of gestation. This observation has lead to speculation that PNEC play some role during fetal lung development [McGovern *et al.*, 2010]. In fact, PNEC synthesize and secrete a number of peptides that exhibit pulmonary growth factor activity, namely gastrin-releasing peptide (GRP, the mammalian equivalent of bombesin) and ghrelin [Cutz *et al.*, 2007; Santos *et al.*, 2006; Van Lommel *et al.*, 2001].

Regarding **GRP**, its expression and of its cognate receptor is maximal during the pseugoglandular and canalicular stages [Wang *et al.*, 1996]. It has been demonstrated that GRP stimulates branching morphogenesis and lung maturation both *in vitro* and *in vivo* [Warburton *et al.*, 2000; Emanuel *et al.*, 1999; Aguayo *et al.*, 1994; Sunday *et al.*, 1990].

Concerning **ghrelin**, a strong growth hormone secretagogue, it is expressed in PNEC throughout fetal lung development, reaching a maximal expression during pseudoglandular stage [Santos *et al.*, 2006; Volante *et al.*, 2002]. Moreover, it was demonstrated that ghrelin maternal administration attenuates pulmonary hypoplasia in fetuses with nitrofen-induced congenital diaphragmatic hernia (CDH) [Santos *et al.*, 2006].

1.3.1.3 Hormones

Retinoic Acid

Studies in animal models and humans have revealed that disruption of signaling by retinoic acid (RA), the biologically active form of vitamin A, is associated with developmental abnormalities, including defects in the respiratory system, range from lung hypoplasia to bilateral lung agenesis, and defects in tracheoesophageal septum development, including tracheoesophageal fistula [Chen *et al.,* 2010; Gallot *et al.*, 2005; Malpel *et al.*, 2000].

Retinoids are involved in different stages and phenomena of lung morphogenesis. Moreover, dynamic and differential spatiotemporal expression patterns are present for RA receptors (RAR α, β and γ) and retinoic acid receptor X receptors (RXR α, β and γ) during lung development [Gallot *et al.*, 2005]. RA is involved in formation of lung primordium as well as distal bud outgrowth. RA acts as a major regulatory signal integrating the WNT and TGF-β pathways in the control of FGF-10 induction in the foregut mesoderm. In fact, the activation of WNT signaling required for the emergence of the primordial lung is dependent of local repression of DKK-1 by endogenous RA. Furthermore, RA regulates the expression of genes involved in distal bud outgrowth, such as FGF-10 and SHH [Chen *et al.*, 2010; Gallot *et al.*, 2005].

RA pathway is also involved in differentiation status of the epithelial cells and distal mesenchyme, occurring during the canalicular stage [Cardoso & Lü, 2006; Weaver *et al.*, 2003]. Additionally, RA together with glucocorticoids regulates the synthesis of surfactant. Retinoids have been also involved in promoting alveolization, inducing formation of secondary septae. In fact, alveolar septation is associated with decrease of retinyl ester storage and production of more biologically active molecules such as RA [Gallot *et al.*, 2005; Malpel *et al.*, 2000]. Nonetheless, the three RAR play distinct roles at different times during alveoli development. For instance, RAR-β seems to be a negative factor in the regulation of alveologenesis in the perinatal period, which suggests that RA signaling via RAR-β restricts the number of alveoli being formed during lung morphogenesis. On the other hand, RAR-γ is a positive factor in the regulation of this process and RAR-α is needed for the proper number of alveoli to develop after the perinatal period in mice. In fact, *Rar*β gene mutants mice develop too many alveoli and *Rar*γ gene mutants fail to correctly form alveoli and show a lower number of alveoli compared to wild-type mice [Gudas, 2012; Gallot *et al.*, 2005].

Steroid Hormones

Steroid hormones, namely sex hormones and glucocorticoids, have a relevant role in lung morphogenesis.

More than thirty years ago, a higher incidence of respiratory distress syndrome (RDS) was reported in male *versus* female preterm neonates (1.7:1). Moreover, although surfactant therapy has been successful in decreasing the mortality rate associated to RDS in both sexes, male neonates affected by RDS continue to present a higher incidence of morbidity [Seaborn *et al.*, 2010]. Thus, the role of sex hormones in lung development mechanisms, namely androgens and estrogens, has been studied.

Fetal lung is not only passively exposed to circulating sex hormones but also has the capability to synthesize and inactivate them, and to modulate their action. Indeed, testosterone, androstenedione and estradiol are expressed in canalicular, saccular, and alveolar stages of mouse lungs of both sexes, whereas dihydrotestosterone is detected only in male lungs [Boucher *et al.*, 2010].

During the fetal period, estrogenic effects could be perceived as antagonistic to those of androgens. Nonetheless, since both sex hormones are involved in lung morphogenesis, a balance in their levels must be kept to ensure proper lung development and maturation [Seaborn *et al.*, 2010].

Concerning **androgens**, they have been implicated in a dual role during lung development: i) positive regulators in early branching morphogenesis in both sexes; ii) negative regulators responsible for the observed delay in male lung maturation [Provost *et al.*, 2004; Levesque *et al.*, 2000]. In human, rodents and rabbit, the peak in plasma testosterone level occurs several weeks before lung maturation and, in fact, the surge of surfactant synthesis occurring at mid–late gestation is delayed in males and is linked to androgen levels. In addition to their delaying effects on AECII maturation, androgens stimulate proliferation and fetal lung growth [Seaborn *et al.*, 2010].

On the other hand, **estrogens** have positive effects on surfactant production during fetal development, enhancing the number of AECII and phospholipid content, and stimulating the expression of SP-A and SP-B [Seaborn *et al.*, 2010]. Moreover, estrogens positively modulate alveologenesis during the neonatal period [Carey *et al.*, 2007]. Indeed, estrogens enhance expression of VEGF in primary cultures of fetal lung cells and they have been associated with the sex dimorphism observed in rodent alveologenesis, namely more alveoli and alveolar surface area, per body mass, in females than in males [Seaborn *et al.*, 2010; Trotter *et al.*, 2009]. Both estrogen receptor α (ERα) and β (ERβ) are expressed in fetal lung and are required for the alveoli development, and they regulate alveolar size and number in a nonredundant manner [Seaborn *et al.*, 2010].

Interestingly, lung fluid is also regulated to some extent by sex hormones, with androgens stimulating and estrogens decreasing the activity of the cystic fibrosis transmembrane conductance regulator [Seaborn *et al.*, 2010].

Regarding **glucocorticoids**, they have a relevant role on perinatal lung maturation, regulating the expression of critical genes for respiratory adaptation at birth. Indeed, antenatal glucocorticoids have been widely used for 40 years to stimulate maturation of preterm fetal lungs and improve neonatal lung function [ACOG, 2011; Roberts & Dalziel, 2006; Grier & Halliday, 2004]. Stimulation of the pulmonary surfactant system has been regarded as the most important effect of antenatal glucocorticoids. These hormones/drugs regulate enzymes in AECII that stimulate surfactant lipid syn-

thesis and subsequent release of surfactant. Moreover, glucocorticoids alter production of surfactant proteins. In addition to this effect on surfactant synthesis, they also induce lung architectural changes, accelerating morphologic development and cytodifferentiation of both types of alveolar cells (AECI and AECII). Furthermore, glucocorticoids enhance fetal lung antioxidant enzymes, including catalase, glutathione peroxidase and two superoxide dismutases, and lung fluid absorption, inducing the epithelial sodium channel and the membrane protein sodium/potassium ATPase [Maeda *et al.*, 2007; Grier & Halliday, 2004]. Interestingly, in spite of the use of glucocorticoids to induce lung maturation, they appear to inhibit secondary septation (alveolarization) and vascular development [Bourbon *et al.*, 2009; Morrisey & Hogan, 2009; Maeda *et al.*, 2007; Grier & Halliday, 2004].

1.3.1.4 Extracellular matrix components and intercellular adhesion molecules

ECM components include the interstitial matrix and the basement membrane, which has multiple functions, such as cells support and anchorage, formation of a tissue scaffold, and regulation of intercellular communication. The ECM has an important role in the mediation of epithelial-mesenchymal cell matrix interactions. Interstitial matrix is composed of polysaccharides and fibrous proteins that fill the intercellular spaces, while basement membranes are sheet-like depositions of ECM on which epithelial cells rest. The most important protein components of ECM are laminins, nidogen, type IV collagen, elastin, fibronectin, proteoglycans, tenascin and SPARC (secreted protein, acidic and rich in cysteine) [Smits *et al.*, 2010; Shi *et al.*, 2009; Warburton *et al.*, 2000].

ECM components are differentially expressed during lung morphogenesis. Absence or inhibition of epithelial cells interactions with the basement membrane results in failure of normal lung development [Hilfer, 1996]. Apart from its supportive role of tissue architecture, ECM also plays an active role in modulation of cell proliferation and differentiation during lung development [Shi *et al.*, 2009].

Laminins are a family of glycoproteins implicated in cell adhesion, migration, proliferation and differentiation during tissue development. Laminins are composed of three chains, one central (α) and two laterals (β and γ) that are linked by disulfide [Warburton *et al.*, 2000]. Until to date five α, three β and three γ chain isoforms have been described. This suggests that their combination can lead to many variants of laminin [Nguyen & Senior, 2006]. At least ten laminin variants participate in a temporospatial fashion during lung development [Warburton *et al.*, 2000]. In lung explant culture, α_1 chain isoform laminin has been shown to be important for lung branching morphoge-

nesis and bronchial smooth muscle cell formation [Schuger *et al.*, 1997]. Moreover, mutation of α_5 chain isoform results in a lethal phenotype during fetal life and lung presents poor lobe septation and bronchiolar branching, suggesting that laminin α_5 is essential for normal lobar septation in early lung development, and alveolarization and maturation in late lung development [Nguyen *et al.*, 2005; Nguyen *et al.*, 2002; Warburton *et al.*, 2000]. Until the moment, the mechanisms responsible for the lung phenotypes in mice with laminin mutations are unknown, but it is clear that multiple laminin isoforms are crucial for lung development and those different laminin isoforms exhibit specific, non-overlapping functions [Nguyen & Senior, 2006].

Nidogen is a constituent of the basement membranes that forms a link between laminin and collagen IV. It is actively synthesized by mesenchymal cells during fetal lung development, which suggests that nidogen has a key role in the organization of the basement membrane [Warburton *et al.*, 2000; Dziadek, 1995]. Although inhibition of the interaction of nidogen with laminin affects the progression of lung development *in vitro*, null mutation of *nidogen* does not have significant impact on lung formation [Dong *et al.*, 2002; Dziadek, 1995; Ekblom *et al.*, 1994].

Collagen IV is a key structural component of all basement membranes. The basement membranes of fetal lung present several collagen IV isoforms and these are critical for structural lung integrity. However, the specific role of collagen IV molecules during lung morphogenesis remains still to be fully elucidated [Suki *et al.*, 2005; Warburton *et al.*, 2000].

Elastin, a cross-linked polymer of soluble tropoelastin monomers, is a critical and prominent structural component of the pulmonary ECM. It plays a role in the elastic characteristics of the lung, namely property of recoil to the vascular, conducting airway, and terminal airspace compartments [Smits *et al.*, 2010; Mariani *et al.*, 1997]. Elastin deposition in the primary septa appears to have a spatially instructive role inasmuch as the specific sites of elastic fiber formation correspond precisely to the location of future buds. Moreover, elastin also plays a fundamental role in the control of budding of secondary septa and it is necessary for proper development of alveoli [Shi *et al.*, 2007; Warburton *et al.*, 2000; Mariani *et al.*, 1997].

Fibronectin seems to play an important role in branching morphogenesis by accumulating at clefts, specific points that do not branch [Sakai *et al.*, 2003]. The treatment of developing lung rudiments with anti-fibronectin antibody inhibits branching morphogenesis, while fibronectin supplementation promotes lung branching [Sakai *et al.*, 2003; Roman *et al.*, 1991].

Proteoglycans are deposited within the ECM during early embryonic lung branching morphogenesis. They function as flexible structures in the organization of the basement membrane and

may also play an important role as a reservoir for growth factors, water and cations [Warburton *et al.*, 2000]. Perlecan is a major proteoglycan in the basement membrane and it has also been involved in the control of smooth cell proliferation and differentiation [Roediger *et al.*, 2009; Warburton *et al.*, 2000]. Moreover, both heparan sulfate and chondroitin sulfate proteoglycans are required for lung branching, since FGF-10 signaling is mediated by those proteoglycan [Warburton *et al.*, 2005; Shannon *et al.*, 2003; Izvolsky *et al.*, 2003]. It was already demonstrated that the inhibition of proteoglycan synthesis or treatment with heparitinase severely affects lung branching [Warburton *et al.*, 2005; Toriyama *et al.*, 1997]. In fact, disruption of proteoglycans synthesis results in lung malformation, respiratory distress and neonatal death [Thompson *et al.*, 2010].

Anti-adhesive glycoproteins of the ECM are SPARC and tenascin. **SPARC** has a wide distribution in the basement membrane during lung morphogenesis [Warburton *et al.*, 2005]. Inhibition studies of SPARC interactions demonstrate branching impairment [Strandjord *et al.*, 1995]. On the other hand tenascin, namely **tenascin C**, presents a spatiotemporal distribution in the developing lung either in epithelium or mesenchyme, and it has a regulatory role during branching morphogenesis [Lambropoulou *et al.*, 2009; Kaarteenaho-Wiik *et al.*, 2001; Jones & Jones, 2000]. In fact, some authors defense that tenascin C promotes the penetration into the surrounding mesenchyme and the branching of the growing airways [Roth-Kleiner *et al.*, 2004].

1.3.2 Mechanical stimuli

Physical forces exerted on the developing fetal lung are an important regulator of this process, namely intra-thoracic space, fetal breathing movements, peristaltic airway contractions and lung fluid. Conditions that induce decrease of **intra-thoracic space**, such as thoracic tumors or CDH, lead to pulmonary hypoplasia as result of lung compression [Copland & Post, 2004].

Fetal breathing movements, resulting from episodic diaphragmatic contractions, are a feature of normal fetal life that can be observed as early as 10 weeks of gestation [Copland & Post, 2004]. These movements cause cyclic fluctuation of intratracheal pressure during fetal life and they are required for cell cycle kinetics regulation as well as for biochemical differentiation of Clara cells, type I and type II pneumocytes [Shi *et al.*, 2007; Inanlou *et al.*, 2005]. Experimentally, when fetal breathing movements are disrupted either mechanically, by transecting the fetal cervical spinal cord above phrenic motoneurons, or genetically, in the *myogenin* null mouse that lacks normal skeletal muscle fibers, lung hypoplasia occurs, namely decrease of lung weight and DNA content

compared to control animals [Copland & Post, 2004; Tseng *et al.*, 2000; Liggins *et al.*, 1981].

Spontaneous and coordinated **peristaltic airway contractions** are rhythmic narrowings of the airways, caused by contractions of the surrounding airway smooth muscle cells. These contractions produce a pulsatile distal-driven movement of lung liquid, which might cause an expansion of the end lung buds, thereby stretching the epithelial layer lining the buds [Schittny *et al.*, 2000]. These peristaltic contractions have been shown to play an important role in embryonic lung branching morphogenesis [Jesudason *et al.*, 2005].

Concerning **lung fluid** and its intraluminal hydraulic pressure, *in utero* the lung is a hydraulic, fluid-filled system. This lung fluid has two sources: amniotic fluid and secretions of the epithelial cells into the airway lumen, which are osmotically driven by active chloride secretion, through chloride channels. This gives rise to a continuous forward flow of lung liquid that drains into the amniotic fluid. The larynx and nasopharynx, where the partial occlusion of the vocal cords acts as a one way valve, generate back pressure to partially inflate the growing lungs and maintains an intraluminal hydraulic pressure of approximately 1.5 cm water in the airways [Shi *et al.*, 2007]. This physiological circulation of lung fluid filling the air spaces is critical to lung development. In fact, if it is disturbed lung growth and maturation is impaired. For instance, excess fluid drainage during fetal life or decrease of fluid pressure, due to oligohydramnios or premature rupture of the membranes, are associated with lung hypoplasia [Shi *et al.*, 2007; Wilson *et al.*, 2007; Copland & Post, 2004]. In opposition, prenatal tracheal occlusion increases luminal pressure and expansion and, consequently, enhances the rate of branching but negatively affects epithelial cell differentiation [Unbekandt *et al.*, 2008; Wilson *et al.*, 2007; Flecknoe *et al.*, 2000]. Similar to effects on airway branching, lung distention has also been shown to play a critical role in coordinating angiogenesis during lung development [Tschumperlin *et al.*, 2010; Cloutier *et al.*, 2008].

Taken together, these observations demonstrate the importance of mechanical forces for proper lung development. Mechanotransduction, that is meaning the conversion of mechanical forces into biochemical signals, appears to be related with stimulation of gene expression of growth factors and their receptors. However, the mechanisms by which pressure is sensed have not yet been determined, but stress-activated ion channels, stress-induced activation of protein tyrosine kinases and ECM-integrin-cytoskeletal pathways have received the most attention [Morrisey & Hogan, 2009; Copland & Post, 2007; Liu & Post, 2000].

1.3.3 Chemical factors

The fetus develops in physiological **hypoxic environment** of the uterus. Several studies have shown that the low-fetal oxygen environment is beneficial for embryo development and for cardiovascular and kidney organogenesis [Rajatapiti *et al.*, 2010; Chen *et al.*, 1999; Yue & Tomanek, 1999; Loughna *et al.*, 1998]. Only recently, it has studied the influence of oxygen on fetal lung development. *In vitro* studies have shown that hypoxia positively regulates lung branching morphogenesis [McGovern *et al.*, 2010; Gebb & Jones, 2003]. Indeed, a low-oxygen environment (1.5% or 3% versus 21% of oxygen) enhances the rate of branching of both distal lung epithelium and vasculature, and increase VEGF and tenascin C expression [Rajatapiti *et al.*, 2010; van Tuyl *et al.*, 2005; Gebb & Jones, 2003]. Only a study, using whole lung organ culture, did not find significant branching acceleration under hypoxic condition [Shinkai *et al.*, 2005]. Moreover, the relevance of oxygen in lung development is demonstrated by hypoxia-inducible factor 2α (**HIF-2α**). HIF-2α is an oxygen-regulated transcription factor expressed in endothelial and type II pneumocytes, which expression increases toward the end of gestation. *In vivo* experiments with newborn mice expressing an AECII conditional mutant of *Hif2α* revealed that HIF-2α is a key regulator of alveolar maturation and the production of phospholipids. In fact, these newborns are born alive but quickly succumb to respiratory distress and their lungs present dilated alveoli covered with enlarged, aberrant AECII and a diminished number of AECI. Furthermore, the AECII lack two crucial enzymes involved in the metabolism of glycogen into surfactant lipids [Huang *et al.*, 2012].

Lung development occurs in a **hypercalcaemic environment** (free ionized plasma calcium concentration $[Ca^{2+}]_o$ of the fetus is higher than of the adult). Recently, Finney *el al* demonstrated that the extracellular calcium-sensing receptor (CaR), which monitored $[Ca^{2+}]_o$, is expressed in fetal mouse lung epithelium. Moreover, it was demonstrated that CaR expression is developmentally regulated, with an expression peak at 12.5 dpc and a subsequent decrease by 18 dpc, after which the receptor is absent [Finney *et al.*, 2008]. Using a lung explant culture model, it was verified that lung branching morphogenesis is sensitive to $[Ca^{2+}]_o$. High levels of $[Ca^{2+}]_o$, found in fetal plasma, induce suppressive effects on lung branching morphogenesis and stimulates lung expansion by increasing fluid secretion into the lumen of the developing lung. Thus, fetal $[Ca^{2+}]_o$, acting through a developmentally regulated CaR, is an important extrinsic factor that modulates the intrinsic lung developmental program and prevents hyperplastic lung disease *in utero* [Finney *et al.*, 2008; Wilson, 2008].

2. Glycoprotein 130 Family of Cytokines

The gp130 dependent family of cytokines or interleukin-6 (IL-6) family of cytokines is a group of functionally and structurally related proteins that comprises IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and cardiotrophin-like cytokine (CLC) [White & Stephens, 2011; Heinrich *et al.*, 2003]. Recently, IL-27 and neuropoietin (NP) had been also added to this cytokines family [White & Stephens, 2011; White & Stephens, 2010; Murakami *et al.*, 2004]. These cytokines are grouped in same family, since all exert their action by the bind to plasma membrane receptor complexes containing the common signal transducing receptor chain gp130. This is the characteristic that unify these cytokines on the same biochemical family [Carbia-Nagashima & Arzt, 2004; Heinrich *et al.*, 2003]. However, each cytokine interacts with a specific receptor that is a complex of receptor subunits. Classically, these subunits are characterized as α chains (ligand-specific and non-signalling: IL6Rα, IL11Rα and CNTFRα) and chains with transduction signal functions (gp130, LIFR and OSMR). According to the cytokine, its receptor is composed by different combinations of these subunits. Thus, the receptor complex for gp130 family of cytokines consists of (i) gp130 homodimers with a ligand-specific α chain for IL-6 and IL-11; (ii) gp130 heterodimers (gp130/LIFR) without specific α chain for LIF and OSM; (iii) or gp130 heterodimers with a ligand-specific α chain (CNTFRα) for CNTF and CLC or α chain-like for CT-1) [White & Stephens, 2011; Murakami *et al.*, 2004; Heinrich *et al.*, 2003]. Until the moment, the α chain recruited by CT-1 has not been characterized [White & Stephens, 2011]. Interestingly, human OSM has the exceptional capability to recruit two different receptor complexes, gp130/LIFR heterodimers (type I OSM receptor) and gp130/OSMR (type II OSM receptor), while mouse OSM binds to gp130/OSMR only (Figure 4) [White & Stephens, 2011; Murakami *et al.*, 2004; Heinrich *et al.*, 2003]. Regarding the new members of this cytokines family, NP was also reported to utilize CNTFRα/gp130/LIFR tri-partite receptor complex [Derouet *et al.*, 2004], however recent studies demonstrated that NP signaling is not mediated by LIFR signaling [White & Stephens, 2010]. On the other hand, IL-27 engages a gp130/WSX-1 (IL27Rα) heterodimeric receptor complex [Pflanz *et al.*, 2004].

After activation of the specific receptor complex, signal transduction involves the activation of Janus kinase (JAK) and the subsequent recruitment of signal transducers and activators of transcription (STAT) proteins, mainly STAT3 and, to a lesser extent, STAT1 [Heinrich *et al.*, 2003; Tanaka & Miyajima, 2003]. STAT1 has been shown to be induced by IL-27 [Takeda *et al.*, 2003].

Alternatively, gp130 cytokine family can also initiate cell signaling via other signaling pathways, including the mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3 kinase (PI3K) cascades [Mihara *et al.*, 2012; Carbia-Nagashima & Arzt, 2004; Heinrich *et al.*, 2003].

The gp130 dependent family of cytokines activates target genes involved in differentiation, survival, apoptosis and proliferation [Heinrich *et al.*, 2003]. Therefore, these cytokines regulate a variety of complex biological processes, including hematopoiesis, immune response, inflammation, mammalian fertility, liver and neuronal regeneration, myocardial development, pituitary proliferation, bone homeostasis, adipocyte differentiation and function, and embryonic development [Mihara *et al.*, 2012; Fujio *et al.*, 2011; White & Stephens, 2011; Carbia-Nagashima & Arzt, 2004; Heinrich *et al.*, 2003].
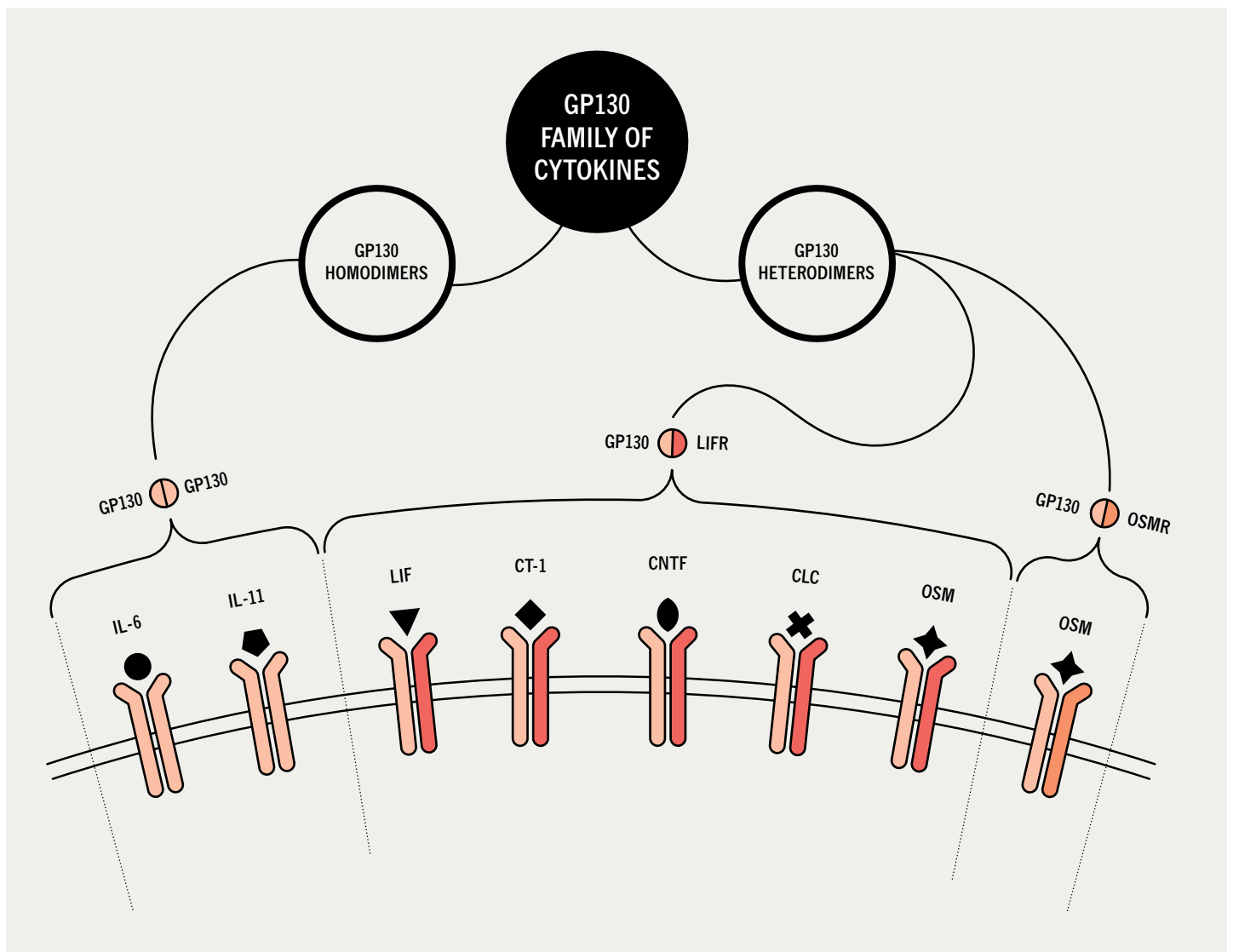
Figure 4. Illustration of receptors of gp130 family of cytokines. IL-6 and IL-11 receptors are gp130 homodimers, whereas LIF, CT-1, CNTF, CLC and OSM receptors are gp130/LIFR heterodimers. Human OSM receptor could also be a different receptor complex, a gp130/OSMR heterodimer.

Absence of common gp130 signal transducer in gp130 KO mice causes embryonic lethality and animals are not viable, exhibiting disrupted placental architecture, hypoplastic development and a decrease in fetal liver hematopoiesis [Fasnacht & Müller, 2008; Wang *et al.*, 2003], and highlighting a role for gp130 signaling during development. Conditional gp130 KO mice, where gp130 was inactivated by conditional gene targeting after birth, exhibited neurological, cardiac, hematopoietic, immunological, hepatic, and pulmonary defects, confirming the widespread importance of gp130 signaling. Regarding lung, it was demonstrated that a conditional gp130-mutant mice develop lung emphysema with increasing age [Betz *et al*, 1998]. Furthermore, lung epithelial cell-specific gp130 deficient mice established a role for gp130 signaling in the repair of the bronchiolar epithelium following injury, influencing epithelial cell migration, density, and shape [Fasnacht & Müller, 2008; Kida *et al.*, 2008]. Moreover, LIFRα deletion (a receptor subunit part of the receptor complexes for LIF, CNTF, CT-1, CLC and OSM) also causes perinatal death with multiple defects [Fasnacht & Müller, 2008; Wang *et al.*, 2003; Betz *et al*, 1998; Ware *et al.*, 1995]. On the other hand, mice lacking individual members of gp130-type cytokines displayed milder phenotype than expected which is most likely due to the similarity and redundancy of downstream events induced by gp130-dependent cytokines [Fasnacht & Müller, 2008]. In fact, as these cytokines share gp130 as a common signal transducer, they present redundant biological activities even though they also exhibit specific biological activities [Carbia-Nagashima & Arzt, 2004; Heinrich *et al.*, 2003]. Moreover, considering that gp130 is ubiquitously expressed, the time and place at which gp130 functions in vivo appears to be mainly determined by expression of the respective specific receptor chains and the cytokines themselves, which synthesis and release are spatially and temporally restricted [Carbia-Nagashima & Arzt, 2004].

In the following sections the roles of classic cytokines of this family will be briefly reviewed.

2.1 Interleukin-6

IL-6 is a pleiotropic cytokine with important roles on acute inflammatory response, infection, haematopoiesis, cell growth, differentiation, survival, apoptosis and proliferation [Mihara *et al.*, 2012; Heinrich *et al.*, 2003; Dame & Juul, 2000]. The α chain ligand-specific of IL-6 receptor is widely distributed in developing human fetuses, which suggest an IL-6 nonhematopoietic role in the developing fetus [Dame & Juul, 2000]. Indeed, IL-6 signaling has been involved embryonic submandibular gland development [Melnick *et al.*, 2001] and lung maturation [Jobe & Ikegami, 2001]. Regarding

this point, it has been proved that IL-6 promotes fetal lung maturation by inducing SP synthesis and, consequently, it improves lung mechanics and gas exchange [Jobe & Ikegami, 2001; Shimoya *et al.*, 2000]. Moreover, IL-6 also plays an important modulatory role in lung angiogenesis, a fundamental process in fetal lung morphogenesis [McClintock & Wagner, 2005]. In opposition, in adult mice, it has been demonstrated that IL-6 is a main causative agent of emphysema, via induction of apoptosis in the lung [Ruwanpura *et al.*, 2011].

IL-6 KO mice present impaired hematopoiesis, defects in acute phase protein synthesis, compromise of the bactericidal and antiviral response, and defects in liver regeneration and bone homeostasis [Bluethmann *et al.*, 1994; Poli *et al.*, 1994].

2.2 Interleukin-11

IL-11, acting either alone or in synergy with other cytokines, is capable of stimulating megakaryopoiesis, thrombopoiesis, myelopoiesis and lymphopoiesis *in vitro*. Other activities of IL-11, outside of the hematopoietic system, include inhibition of adipogenesis, induction of hepatocyte production of acute phase proteins, induction of neuronal differentiation, and as a potential regulator of cartilage and bone function [White & Stephens, 2011; Suga *et al.*, 2004; Zheng *et al.*, 2001; Davidson *et al.*, 1997]. Growing evidence indicates an important role of IL-11 in epithelial tissue biology and epithelial regeneration. In fact, IL-11 has been found to have mucosal protective effects in the setting of mucosal injury. These effects have been investigated most thoroughly in the gastrointestinal tract, where IL-11 stimulates proliferation and differentiation of intestinal cells and prevent apoptosis of epithelial cells. Consequently, IL-11 protects small intestinal cells following cytoablative treatments, reduces experimental mucositis, and ameliorates injury in models of inflammatory bowel disease [Opal *et al.*, 2003; Dickinson *et al.*, 2000]. IL-11 has also been shown to improve survival after thoracic irradiation [Wang *et al.*, 2006; Zheng *et al.*, 2001]. Furthermore, IL-11 attenuates nephrotoxic nephritis [Lai *at al.*, 2001] and it decreases severity of acute necrotizing pancreatitis in mice [Shimizu *et al.*, 2000]. Regarding the lung, IL-11 is produced by a variety of structural cells (fibroblasts, epithelial cells, human airway smooth muscle cells) and eosinophils in response to a variety of stimuli [Zheng *et al.*, 2001; Minshall *et al.*, 2000]. Actually, IL-11 induces tissue fibrosis and is over-expressed in asthma context, while inhibits antigen-induced inflammation. This suggests that IL-11 acts as a healing cytokine in

the asthmatic airway and in other tissues. Interestingly, IL-11 provides protective effects against oxidant-mediated injury in fetal and adult lung [Waxman *et al.*, 2003; Waxman *et al.*, 1998].

Despite all these IL-11 functions, mice deficient in IL-11Rα only present female sterility [Robb *et al.*, 1998].

2.3 Leukemia Inhibitory Factor

LIF is a multifunctional glycoprotein cytokine that regulates many cellular responses such as proliferation, differentiation and survival in different cellular types [Heinrich *et al.*, 2003; Metcalf, 2003; Auernhammer & Melmed, 2000]. Indeed, LIF displays several biological activities ranging from the classic hematopoietic and inflammatory actions to effects on blastocyst implantation, kidney development, neural development, bone cell metabolism, adipocyte lipid transport and energy homeostasis, muscle satellite cell proliferation, heart hypertrophy, retinal vascularization and endocrine actions [McColm *et al.*, 2006; Kurdi *et al.*, 2005; Metcalf, 2003; Auernhammer & Melmed, 2000]. LIF deficient animals are born normal and they only exhibit female sterility due to defective blastocyst implantation, postnatal growth retardation and hematopoiesis defects [Pichel *et al.*, 2003; Betz *et al.*, 1998].

During fetal development, LIF expression was already described in the ureteric bud [Plisov *et al.*, 2001], skin, skeletal muscle, heart, brain, liver and gut [Robertson *et al.*, 1993] supporting a role for this cytokine in the normal development of several organ systems. For instance, LIF has an inhibitory effect in fetal kidney development [Bard & Ross, 1991] as well as in embryonic stem cells [Williams *et al.*, 1988].

Regarding the lung, numerous lung structural cell types express LIF and the LIFR, suggesting previously unrecognized roles for this cytokine in lung biology [Knight, 2001]. Several studies have investigated LIF's role in the pathophysiology of different lung diseases, such as acute respiratory distress syndrome [Jorens *et al.*, 1996], asthma [Xiong *et al.*, 2007], chronic airway inflammation [Knight, 2001] and inflammation induced by tobacco [Jorens *et al.*, 1996]. It was already described that LIF, with IGF-I, regulates lung maturation. In fact, absence of LIF in addition to IGF-I null mutant mice aggravates pulmonary immaturity and mice die due to respiratory failure. LIF/IGF-I double deficient embryos present lung hypoplasia and defective differentiation of the alveolar epithelium and vasculogenesis [Moreno-Barriuso *et al.*, 2006; Pichel *et al.*, 2003].

2.4 Oncostatin M

OSM is closely related structurally, genetically and functionally to LIF. In fact, it has been suggested that OSM and LIF evolved by gene duplication relatively recently [Tanaka & Miyajima, 2003]. OSM can modulate a variety of biological processes and has some functional overlap with LIF, such as growth regulation, differentiation, gene expression, and cell survival. However, OSM is also a unique cytokine that plays an important role for various biological systems. These specific OSM effects appear to be mediated by specific oncostatin receptor (gp130/OSMR) [White & Stephens, 2011; Tanaka & Miyajima, 2003].

Regarding these OSM specific functions, OSM modulates growth of malign and benign cells either positively (endothelial-like, hematopoietic, Sertoli and Leydig cells) or negatively (solid tissue tumor cells, normal mammary and breast epithelial cells,) depending on the target cells [Tanaka & Miyajima, 2003; Grant *et al.*, 2001; Miyajima *et al.*, 2000; Hara *et al.*, 1998]. OSM plays some important roles in the hematopoiesis [Tamura *et al.*, 2002a], liver development [Morikawa *et al.*, 2005; Kinoshita & Miyajima, 2002], nervous system development [Morikawa *et al.*, 2005], testis morphogenesis [Teerds *et al.*, 2007], inflammatory responses namely in fibroblasts, endothelial cells, and lung epithelial cells [Morikawa *et al.*, 2005; Tamura *et al.*, 2002b], and tissue remodeling [Znoyko *et al.*, 2005]. Regarding the lung, OSM is a potent mediator of lung inflammation and extracellular matrix accumulation, and is up-regulated in patients with idiopathic pulmonary fibrosis and scleroderma [Mozaffarian *et al.*, 2008].

Despite all OSM functions, OSMR-deficient mice develop normally, presenting alterations in hematopoiesis as well as liver regeneration [Tanaka *et al.*, 2003]. OSM-deficient mice exhibit a reduction in responses to mechanical and thermal stimuli [Morikawa *et al.*, 2005]. Interestingly, OSM overexpression is deleterious during mouse development [Tanaka & Miyajima, 2003].

During fetal life, OSM is expressed in hematopoietic tissues (bone marrow, liver, thymus and spleen) and in choroid plexus, and limb [Tamura *et al.*, 2002a]. After birth, it is detectable in other organs, such as lymph node, bone, heart, kidney, small intestine, skin, nasal cavity, and lung [Morikawa *et al.*, 2005; Tamura *et al.*, 2002b]. In the lung, the signals are detected in alveolar epithelium and bronchiolar epithelium [Tamura *et al.*, 2002b]. The OSMR/gp130 receptor complex is widely expressed on a variety of cell types, including epithelial cells, fibroblasts, chondrocytes, hepatocytes, and some neurons [Mozaffarian *et al.*, 2008].

2.5 Cardiotrophin-1

CT-1 was recently discovered substance, which affects most of organ systems. Firstly, it was demonstrated that CT-1 is expressed at high levels in the myocardium during the course of cardiogenesis and supports embryonic cardiomyocyte survival and hypertrophy [White & Stephens, 2011; Jougasaki, 2010; Stejskal & Ruzicka, 2008]. In cardiovascular system, CT-1 also plays an important cardioprotective effect on myocardial damage [Ruixing et al., 2007], is overexpressed in individuals with heart failure [Talwar et al., 2000] and hypertensive heart disease [López et al., 2007], and it has been proposed as prognostic factor in these pathologies [Jougasaki, 2010; Stejskal & Ruzicka, 2008].

It was already demonstrated that CT-1 is expressed in adult heart, skeletal muscle, liver, lung, kidney, dorsal root ganglia, ovary, colon, prostate and testis as well as in fetal heart, kidney and lung [White & Stephens, 2011; Jougasaki, 2010; Stejskal & Ruzicka, 2008; Zhou et al., 2003]. This CT-1 expression pattern suggests that it exerts pleiotropic actions in other organ systems, namely hypertrophic and cytoprotective actions [Jougasaki, 2010]. Indeed, CT-1 is a hepatocyte survival factor and is involved in liver regeneration [Iñiguez et al., 2006], is a potent regulator of signaling in adipocytes [Zvonic et al., 2004], plays an important role in the pathophysiology of atherosclerosis [Ichiki et al., 2008], has relevant anti-inflammatory effects namely in a model of endotoxin-induced acute lung injury [Pulido et al., 1999], decreases pulmonary arterial pressure in a hypoxic pulmonary hypertension model [Nomura et al., 2003], is important in normal motoneuron development [Pennica et al., 1996] and regeneration of the skeletal muscle [Nishikawa et al., 2005], induces osteoclast differentiation and activation [Richards et al., 2000], and promotes photoreceptor survival [Song et al., 2003]. Furthermore, CT-1 has been related with chronic asthma, contributing to airway wall thickening through reduction of apoptosis rates and induction of hypertrophy of airway smooth muscle cells [Zhou et al., 2003].

2.6 Ciliary Neurotrophic Factor

CNTF is a 22-kDa nonsecreted cytosolic cytokine synthesized by Schwann cells in the periphery and by neurons and astrocytes centrally that was originally identified for its role in the differentiation and/or survival of different neuronal cell types [White & Stephens, 2011; Rezende et al., 2009; Sango et al., 2008; Ozog et al., 2004]. Its specific receptor, CNTFRα, is located on

neuronal cell membranes and CNTF seems to be released only by damaged cells [Vlotides *et al.*, 2004]. CNTF expression and synthesis are dramatically increased by trauma, inflammation and ischaemia [Stanley *et al.*, 2000]. Indeed, CNTF acts as a lesion factor, preventing neuronal cell death and facilitating axonal regeneration after nerve injury [Sango *et al.*, 2008]. Furthermore, CNTF significantly delays cell death in models of photoreceptor degeneration [Wahlin *et al.*, 2000], and form a part of the feedback loop regulating the hypothalamo-pituitary-gonadal axis [Stanley *et al.*, 2000]. Interestingly, this cytokine is suggested to be anti-diabetogenic, since it improves peripheral sensitivity to insulin, reduces insulin secretion, adiposity, and body weight [Rezende *et al.*, 2009; Ahima *et al.*, 2006]. In addition, CNTF promotes the survival of rat pancreatic islets, albeit not affecting its differentiation [Rezende *et al.*, 2009]. However, CNTF deficient mice exhibit only very mild neuronal problems as gradual loss of motoneurons, resulting in minor muscle weakness [Takahashi *et al.*, 1994; Masu *et al.*, 1993]. In opposition, mice deficient in CNTFRα display profound motoneuron deficits and die perinatally due to inability to suckle, suggesting that additional cytokine signal through CNTFRα and compensate for the loss of CNTF in CNTF deficient mice [Elson *et al.*, 2000; DeChiara *et al.*, 1995].

It was already described that CNTF is widely expressed in the adult brain, heart, lung, liver, kidney and testis, in addition to preferential expression in the sciatic nerve [Ohta *et al.*, 1996]. Regarding embryonic development, CNTF is specifically expressed in rat pineal gland and eyes [Hata *et al.*, 2002].

2.7 Cardiotrophin-like cytokine

CLC [also known as novel neurotophin-1/B cell-stimulating factor-3 (NNT-1/BSF- 3)] is a newly identified gp130-related cytokine with highest homology to CNTF and CT-1 [Forger *et al.*, 2003; Uemura *et al.*, 2002]. CLC can be actively secreted from cells due to complex formation with the soluble type I cytokine receptor cytokine-like factor-1 (CLF-1) or soluble CNTFR (sCNTFR) [Vlotides *et al.*, 2004; Forger *et al.*, 2003]. Interestingly, mice with a targeted deletion of the *clf* gene exhibit a phenotype similar to CNTFRα KO mice [Alexander *et al.*, 1999]. Thus, CLC/CLF-1 composite cytokine is a second ligand for CNTFRα [Elson *et al.*, 2000].

CLC is mainly expressed in secondary lymphoid organs, such as lymph nodes and spleen, but has also been detected in numerous other organ systems including bone marrow, ovary, placenta, pituitary, liver, kidney, colon, heart, lung and pancreas [Vlotides *et al.*, 2004; Senaldi

et al., 1999]. During development, CLC is expressed in lung, kidney, brain, tooth, epithelia and skeletal muscles. In the lung, CLC is expressed faintly in distal airway epithelium whereas CLF is strongly expressed in distal and proximal epithelia [de Bovis *et al.*, 2005]. This CLC expression pattern suggests important biological roles in cellular functions.

CLC has been shown to act as a potent neurotrophic factor, B cell stimulatory agent [Senaldi *et al.*, 2002; Senaldi *et al.*, 1999] and neuroimmunoendocrine modulator of corticotroph function [White & Stephens, 2011; Vlotides *et al.*, 2004; Elson *et al.*, 2000]. Indeed, CLC supports the survival of embryonic motor and sympathetic neurons [Forger *et al.*, 2003; Elson *et al.*, 2000; Senaldi *et al.*, 1999] and induces astrocyte differentiation of fetal neuroepithelial cells [Uemura *et al.*, 2002]. CLC/CLF-1 complex also promotes mesenchymal to epithelial conversion and nephrogenesis [Schmidt-Ott *et al.*, 2005].

2.8 Interleukin-27 and Neuropoietin

Recently, IL-27 and neuropoietin were also included in the gp130 dependent family of cytokines. IL-27 is important in immune response regulation since it has broad inhibitory effects on Th_1, Th_2, and Th_{17} subsets of T cells [Jankowski *et al.*, 2010]. Moreover, it has been described that IL-27 is involved in processes such as neuronal growth, bone maintenance, cardiac development, angiogenesis regulation and intestinal epithelial barrier protection [Diegelmann *et al.*, 2012; White & Stephens, 2011; Villarino *et al.*, 2004].

Regarding neuropoietin, it has been characterized as a regulator of central nervous system development, with the ability to induce neuroepithelial cells differentiation into astrocytes [White & Stephens, 2011; White & Stephens, 2010; Derouet *et al.*, 2004]. Moreover, neuropoietin was shown to inhibit adipogenesis and to have ability to modulate insulin sensitivity [White & Stephens, 2010].

3. Renin-Angiotensin System

Since the first identification of renin by Tigerstedt and Bergmann in 1898, the renin-angiotensin system (RAS) has been extensively studied. The RAS is classically known as an endocrine system regulating blood pressure, electrolyte and fluid homeostasis [Paul *et al.*, 2006; Lavoie & Sigmund, 2003]. This classical RAS consists of several key components, namely angiotensinogen (the hepatic derived precursor), two critical enzymes, renin (secreted from the juxtaglomerular apparatus of the kidney) and angiotensin-converting enzyme (ACE, pulmonary-bound metalloproteinase), whose sequential actions produce the decapeptide angiotensin I and the physiologically active octapeptide, angiotensin II (ANG II), respectively. ANG II operates on two G protein-coupled receptors, the ANG II type 1 (AT_1) and type 2 (AT_2) receptors [Goyal *et al.*, 2011; Paul *et al.*, 2006].

AT_1 receptor has been described to be expressed during both fetal and postnatal life, while AT_2 receptor expression is almost solely in the fetus and dramatically decreases after birth, being restricted to a few organs and at low levels, such as brain, adrenal, heart, kidney, myometrium and ovary [de Gasparo *et al.*, 2000; Morrell *et al.*, 1996; Shanmugam *et al.*, 1996]. Indeed, an increase of AT_2 receptor expression during adult life has been only observed under pathological conditions, such as vascular injury, myocardial infarction, heart failure, renal failure, brain ischaemia, skin wounds and sciatic or optic nerve transsection [Kaschina & Unger, 2003].

ANG II has an important role in vascular tone regulation and it is one of a growing number of peptide hormones that have been involved in cellular growth and apoptosis regulation [Lemarié & Schiffrin, 2010; Paul *et al.*, 2006]. An opposite action of AT_1 and AT_2 receptors has been shown both *in vitro* and *in vivo* studies. Whereas AT_1 mediates the vasoconstrictor and growth promoting effects of ANG II, AT_2 has been shown to mediate vasodilator effects, to exert inhibitory effects in cell proliferation, growth and hypertrophy, and to promote apoptosis [Lemarié & Schiffrin, 2010; Chassagne *et al.*, 2000]. For instance, AT_2-mediated actions that counteract AT_1 effects have been described on endothelial [Stoll *et al.*, 1995] and vascular smooth muscle cells proliferation [Akishita *et al.*, 1999], heart hypertrophy [Inagami & Senbonmatsu, 2001], phosphatidylinositol hydrolysis in rat skin fibroblasts [Gyurko *et al.*, 1992], and fetal kidney development [Yosypiv, 2011]. The dual actions of ANG II on its receptors, i.e. this broader and functional antagonism between AT_1 and AT_2 receptors, with respect to tissues growth, could be physiological interpreted as a basis of a RAS homeostasis at the tissue level. This mechanism might control organ development, differentiation and remodeling, for instance, in cases where excessive growth, induced by growth factors, needs to be controlled.

The signaling pathways of the AT_1 receptor include classical G-protein-coupled cascades lead-ing to activation of MAPK and PI3K/Akt cascades, and JAK/STAT pathway [Stoll & Unger, 2001]. The elucidation of the nature of AT_2 signaling remains enigmatic and still is a matter of intense research efforts. However, while ANG II via AT_1 receptors mediates their growth actions through kinases activation, a hallmark of AT_2 signaling on growth suppressing actions appears to be more related with the activation of specific phosphatases causing protein dephosphorylation. These phosphatases can lead to the inhibition of MAPK [namely extracellular signal-regulated kinases-1 and -2 (ERK1/2 or p44/42) and JNK], PI3K/Akt and STAT pathways, and activation of p38 kinase and nuclear factor (NF)-$\kappa\beta$ [Lemarié & Schiffrin, 2010; Yosypiv, 2011; Stoll & Unger, 2001]. The stimulation of the AT_2 receptor may involve two other mechanisms: activation of bradykinin/ nitric oxide/ cyclic guanosine 3',5'-monophosphate pathway, and stimulation of phospholipase A_2 and release of arachidonic acid [Lemarié & Schiffrin, 2010].

The current view of the RAS had to be expanded significantly by more recent findings that increased complexity of the system. Exciting new concepts, such as the identification of new peptides [ANG (1-7)] [Santos *et al.*, 2000], new enzymes (serine protease chymase, ACE-2) [Arakawa & Urata, 2000; Donoghue *et al.*, 2000], novel receptors (AT_4) [Swanson *et al.*, 1992], and signal transduction pathways involved [Sayeski & Bernstein, 2001; de Gasparo *et al.*, 2000], have been derived from recent studies.

Moreover, the introduction of the **local tissue RAS** concept, not requiring hormone secre-tion into the systemic circulation, also has changed our view of the RAS. This local RAS concept was based on findings of RAS components in unlikely places (for instance, the presence of renin in the brain) where the endocrine actions of the system could not explain the findings [Kaparianos & Argyropoulou, 2011; Paul *et al.*, 2006]. The definition of local RAS requires that all components necessary for the biosynthesis of the biologically active peptide, ANG II, as well as receptors for this product, are present within the tissue. Furthermore, the biologically active product needs to be regulated within the tissue, independently of the systemic circulation [Carey & Siragy, 2003].

The first local RAS was described on kidney [Yosypiv, 2011; Gomez *et al.*, 1988]. In this sequence, during the last two decades, evidences have demonstrated the presence of functional local RASs with autocrine/paracrine/intracrine actions in several other organs or systems, such as nervous system and sensory organs, heart, blood vessels, adrenal glands, reproductive tract, digestive organs, skin, adipose tissue, and lymphatic organs [De Mello & Frohlich, 2011; Vaajanen & Vapaatalo, 2011; Leung, 2007; Paul *et al.*, 2006; Engeli *et al.*, 2000]. If there is a common de-

nominator for the physiological role of these local systems, it is the preservation of a homeostasis, at the tissue level, between opposing effects mediated by the RAS, such as growth promotion (via AT_1) and inhibition (via AT_2). The dual actions of ANG II on its receptors can be considered as a basis for this balance [Paul *et al.*, 2006]. Regarding the cardiac RAS, for instance, the predominant physiological role appears to be the maintenance of an appropriate cellular milieu balancing stimuli inducing and inhibiting cell growth and proliferation, as well as mediating adaptive responses to myocardial stress, for example, after myocyte stretch [De Mello & Frohlich, 2011; Paul *et al.*, 2006].

Moreover, it has been also demonstrated that local ANG II formation and its tissue-specific effects on growth and differentiation are extremely important for embryonic and fetal development [Paul et al., 2006; Hu *et al.*, 2004; Morrell *et al.*, 1996; Shanmugam *et al.*, 1996; Grady et al., 1991]. The different RAS components were already described to be widely expressed on extraembryonic fetal tissues, as chorion, decidua and placenta, and in different developing organs, displaying specific spatial and temporal distribution during gestation, which suggest a possible role for ANG II in organogenesis [Leung, 2007; Paul *et al.*, 2006; Morrell *et al.*, 1996; Shanmugam *et al.*, 1996]. Indeed, ANG II has already been demonstrated as an important modulator of fetal kidney, heart, vasculature, adrenal gland, liver and brain development [Yosypiv, 2011; Paul *et al.*, 2006].

Regarding lung morphogenesis, there is some evidence that lung expresses ACE as well as AT_1 and AT_2 receptors during fetal development. In rat, ACE expression was observed in endothelium of hilar pulmonary arteries since 15 dpc, whereas AT_1 mRNA was expressed by non-epithelial undifferentiated mesenchyme on 17 dpc [Morrell *et al.*, 1996] and AT_2 mRNA was expressed in pulmonary artery since 15 dpc and in bronchi and trachea since 17 dpc [Shanmugam *et al.*, 1996]. Interestingly, ANG II has been demonstrated to potentiate the mitogenic effect of EGF [Norman *et al.*, 1987] and increase expression of PDGF [Grady *et al.*, 1991; Naftilan *et al.*, 1988], both positive regulators of branching morphogenesis. Furthermore, it is also well known that the maternal administration of angiotensin converting-enzyme inhibitors (ACE-I) or angiotensin receptor-antagonists (ARA) against hypertension, during gestation, induces several congenital anomalies, such as oligohydramnios, intrauterine growth retardation, hypocalvaria, renal dysplasia, renal failure, pulmonary hypoplasia and death. In addition to the oligohydramnios induced by these pharmacological drugs, other underlying mechanism for lung hypoplasia development still remains unknown [Walfisch *et al.*, 2011; Quan, 2006; Cooper *et al.*, 2006].

Although the widespread expression and functions of RAS components either during fetal or adult life, the AT_1 receptor KO phenotype is only characterized by *ex utero* survival rate decrease,

low body weight gain, hypotension, and abnormal kidney morphology [Kaschina & Unger, 2003]. On the other hand, AT_2 receptor KO phenotype revealed subtle deficiencies, such as impaired drinking responses to water deprivation, increased vasopressor responses to injection of ANG II, reduced body temperature, spontaneous movements and exploratory behavior [Stoll & Unger, 2001; Akishita *et al.*, 1999; Hein *et al.,* 1995]. Indeed, histological sections of heart, kidney, brain, aorta, adrenal, lung, spleen, ovary, uterus, pancreas, eye, skeletal muscle and blood did not reveal any differences in morphology between non-mutant and hemizygous or homozygous AT_2 mutant mice [Hein *et al.*, 1995]. However, it is necessary to stress that the KO approach may induce a phenotype that is a mixture of both lack of receptor function and compensatory events and mechanisms during development and later life [Stoll & Unger, 2001].

4. Congenital Diaphragmatic Hernia

Severe respiratory failure in the newborn remains a main cause of neonatal death. Among the causes of severe respiratory failure in the newborn, CDH remains the most life threatening [Gosche *et al.*, 2005].

In the history of Medicine, the earliest recordings of diaphragmatic hernia comes from Hippocrates (460-370 BC), who declared that it was caused by traumatic perforations, with ensuing herniation of abdominal viscera into the thoracic cavity, and subsequent death [Coar, 1822]. On the other hand, the first CDH description occurred only in 1679 by Lazarus Riverius, as an incidental postmortem finding in 24 years-old man [Riverius, 1679]. The first pediatric case of CDH was recorded in 1701 by Sir Charles Holt, who described the clinical and postmortem findings of an infant with 2 months-old with CDH [Holt, 1701].

Actually, CDH is considered a severe developmental anomaly, with a mean incidence of 1:2.500 live births, which etiology remains poorly understood [Keller *et al.*, 2010; van den Hout *et al.*, 2009; Gosche *et al.*, 2005]. This congenital anomaly is characterized by a diaphragmatic defect that allows intra-thoracic herniation of abdominal organs and consequently maldevelopment of the alveoli and pulmonary vessels. The diaphragmatic defect may differ in its localization, dimensions and type and, consequently, it associates to different degrees of pulmonary hypoplasia and clinical severity. In humans, four types of diaphragmatic defects can be recognized: a posterolateral or Bochdalek type, an anterior Morgagni type, a central hernia or septum transversum type, and diaphragmatic eventration type (an incomplete diaphragmatic muscular consolidation). The Bochdalek hernia is the most common diaphragmatic defect (about 70% of the cases) [van Loenhout *et al.*, 2009]. Since the diaphragmatic closure on the right side occurs earlier than the left, it occurs on the left side in 85-90% of cases, which is the form that is associated with worse prognosis. CDH occurs in 13% of cases on the right side and only 2% are bilateral [Rottier & Tibboel, 2005; Gallot *et al.*, 2005].

The clinical presentation of CDH infants is very heterogeneous and the outcome of newborns is sometimes quite surprising. At one extreme of the spectrum of the disease there are newborns minimally affected with excellent prognosis after surgical correction of the diaphragmatic defect. At the opposite, there are severe CDH cases with multiples associated anomalies, which determine a poor prognosis that could even result in fetal demise. In the middle range, they are found CDH classic infants with severe pulmonary hypoplasia and PH that require intensive and sophisticated treatment in addition to the straightforward surgical diaphragmatic

correction. These newborns represent the greatest challenge for those caring CDH infants, because if they survive to the critical neonatal period, lung will growth progressively and resolution of increased vascular resistance will occur with good long term prognosis.

4.1 CDH Etiology

Classically, CDH has been explained based on 4 different theories: i) the diaphragmatic malformation is linked to abnormal adjacent lung development, due to the absence of critical signals emanating from adjacent lung; ii) the diaphragmatic defect is secondary to abnormal muscle innervations by the ipsilateral phrenic nerve; iii) the diaphragmatic hernia is secondary to atypical myotube formation, resulting in a fragile diaphragm that could rupture during the fetal life; iv) CDH formation is due to an abnormal or delayed closure of the pleuroperitoneal canal secondary to blockage by the intestine returning into the abdominal cavity [Gosche *et al.*, 2005]. A fifth most recent theory, proposed by Babiuk *et al* suggests that the amuscular mesenchymal component of the pleuroperitoneal fold, likely derived from the somatopleure, is defective and does not provide a full foundation for the formation of diaphragmatic musculature. Thus, the error arises from an amuscular mesenchymal malformation [Babiuk *et al.*, 2003].

Regardless of the theories that explain the CDH embryonic origin, its molecular dynamics and genetic defects are yet unknown. It probably results from genetic predisposition and/or environmental factors. Among environmental factors, the study of the vitamin A deficiency effects in humans and teratogenic animal models suggest that the retinoid signaling pathway is important in CDH etiology [Clugston *et al.*, 2010; van Loenhout *et al.*, 2009; Kling & Schnitzer, 2007; Mey *et al.*, 2003; Thébaud *et al.*, 1999]. Regarding the genetic factors, the possible involvement of different genes in CDH has been suggested by the analysis of both chromosomal abnormalities (found in 10-30% of human infants with CDH) and transgenic mice. There are a lot of genes whose deletion has been associated with true diaphragmatic defects, such as: Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), retinoid receptors RARα and β type, retinal dehydrogenase (RALDH) 1, 2 and 3, Stimulated by retinoic acid gene 6 homolog (STRA6), Wilm's Tumor 1 (Wt1), Slit3, Roundabout (ROBO1), Glypican-3 (GPC3), Fibulin-4 (FBLN4), collagen, Delta-like 3, the Lunatic Fringe and other Notch pathway factors. On the other hand, there are other genes that are associated with defects in the diaphragmatic muscle fibers, such as Fog2 (Friend of GATA 2), GATA4, Pax3, HGF and c-Met, Grb2-associated binding 1 (Gab1), Lysyl oxidase (Lox), Single-

minded homolog 2 (Sim2), Myogenic differentiation 1 (MyoD) and dystrophin, and fibrillin-1 (FBN1) [reviewed by Brady *et al.*, 2011; Klaassens *et al.*, 2009; Holder *et al.*, 2007].

It is necessary to stress that CDH can occur as an isolated defect or may have other coexistent congenital defects, or it may even be a part of syndromes with complex malformations. In fact, in about 10-20% of affected fetuses, CDH is associated with a chromosomal abnormality, mainly trisomy 18, trisomy 13, and Pallister- Killian syndrome (mosaicism for tetrasomy 12p) [Holder *et al.*, 2007]. CDH presents frequent association with other defects, mainly cardiac, renal and skeletal, and multiple malformation syndromes (e.g., Fryns syndrome, de Lange's syndrome, and Marfan syndrome) [Holder *et al.*, 2007].

One inquisitive aspect in the CDH patophysiology is the presence of bilateral pulmonary hypoplasia from early stages of lung development. Actually, it is well established that the diaphragmatic defect is independent, as well as previous or simultaneous to the pulmonary hypoplasia [Babiuk & Greer, 2002]. Thus, convincing evidences suggest that there being a common mechanism underlying the pathogenesis of CDH that targets primordial diaphragm and lung development in parallel. This highlights the hypothesis that an insult occurs in a critical point of the embryonic development, affecting other organs than diaphragm, like the lung and even the heart. Interestingly, from animal studies, it has been demonstrated that fetal lung hypoplasia in CDH results from complex dynamic processes that can be divided in early and late gestational determinant, according to the dual-hit hypothesis [Keijzer *et al.*, 2000]. In this hypothesis the pathogenesis of pulmonary hypoplasia in CDH is explained by two developmental insults. The first insult occurs early in development (early determinants), before diaphragm development take place, and appears to be secondary to molecular disturbances. This early insult affects both lungs equally during pseudoglandular and canalicular stages of lung development. The second insult (late determinants) occurs after the establishment of the diaphragmatic defect. It seems to be primarily related to mechanical factors, mediated by visceral thoracic herniation, and affect essentially later stages of lung development of the affected side [Keijzer *et al.*, 2000]. Thus, it results in greater impairment of the development of the ipsilateral lung than of the contralateral one.

4.2 CDH Pathophysiology

CDH was originally regarded as an anatomic disease (solely related to the diaphragmatic defect) and, consequently, a surgical emergence. It was believed that the postnatal respiratory insufficiency and failure was secondary to lung compression induced by the herniated organs into the thorax, often including small intestine, colon, stomach and more rarely the spleen and liver, pancreas, adrenals and kidneys. Thus, the surgical reduction of the hernia and the defect closure allowed lung re-expansion [Deprest & De Coppi, 2012; Chinoy, 2002].

However, during 90th years, the knowledge of CDH pathophysiology progressed and it became clear that lung hypoplasia, abnormal arteriolar muscularization and consecutive persistent pulmonary hypertension (PH) associated with this disorder were the key determinants of mortality [Luong *et al.*, 2011; Keller *et al.*, 2010; van den Hout *et al.*, 2009]. Contradictory results about lung maturity and surfactant status in CDH have been published [van Tuyl *et al.*, 2003; Thébaud *et al.*, 2001; Keijzer *et al.*, 2000], but one of the most recent studies performed with human CDH fetuses demonstrated that surfactant maturation is not delayed [Boucherat *et al.*, 2007]. In this way, CDH treatment has evolved to the concept of physiological emergence [Luong *et al.*, 2011; Masumoto *et al.*, 2009]. It was demonstrated that early surgery repair is associated with unfavorable changes in lung compliance and gas exchange. Thus, several authors have advocate and shown benefit to delayed surgical repair of CDH, after clinical stabilization [Deprest & De Coppi, 2012; Masumoto *et al.*, 2009; Charlton *et al.*, 1991]. In our days, initial therapy is directed toward hemodynamic stabilization and respiratory support. This awareness results in improvement in therapeutics, like pulmonary vasodilators (inhaled nitric oxide), sophisticated ventilation strategies (high frequency oscillatory ventilation) and extra corporeal membrane oxygenation (ECMO) [de Buys Roessingh & Dinh-Xuan, 2009; Bartlett, 2005].

Despite the improvements in understanding CDH pathophysiology and advances in neonatal care, in the most severely affected subset of CDH newborn, the prognosis remains poor, with a mortality and morbidity rates that remains exceedingly high [Luong *et al.*, 2011; Keller *el al.*, 2010; van den Hout *et al.*, 2009; Colvin *et al.*, 2005]. Reports of the CDH mortality rate range from 32 to 62%, with some specialized tertiary care centers achieving survival rates of 70% or more [DeKoninck *et al.*, 2011; Stege *et al.*, 2003]. Unfortunately, there is significant long-term morbidity among survivors, mainly pulmonary (including bronchopulmonary dysplasia, asthmatic symptoms and recurrent respiratory tract infections) and gastrointestinal (reflux disease, oral aversion, and growth problems) [Rocha *et al.*, 2011; Peetsold *et al.*, 2009; Bagolan & Morini, 2007].

4.3 CDH Animal Models

Since the etiology and pathogenesis of CDH and its associated anomalies are still largely unknown, several animal models have been proposed to study CDH. The animal models available to study CDH include: i) surgical models in the rabbit and sheep; ii) teratogen-induced models in the rat and mouse; iii) and genetic (KO) mouse models [van Loenhout *et al.*, 2009; Kling & Schnitzer, 2007; Clugston *et al.*, 2006].

Surgical models are based on a surgical intervention making a diaphragmatic defect in fetal rabbits and sheep. Two variants of the surgical CDH animal model exist. The first consists in surgically creation of diaphragmatic defect and position of abdominal bowel into the chest, in fetal lamb and rabbits. The second model consists in inflation of a balloon in the thoracic cavity of fetal lambs [van Loenhout *et al.*, 2009]. Surgical animal models are useful in investigating interventional therapies, such as the corticosteroids administration, *in utero* repair of the diaphragmatic defect and tracheal occlusion [Bratu *et al.*, 2001]. Unfortunately, the diaphragmatic defect is created relatively late in gestation and certain pulmonary changes seen in human CDH might have occurred prior to this time. Therefore, these surgical models are less instructive in studying the etiology and pathogenesis of CDH, as well as the other associated anomalies (such as cardiac anomalies) [van Loenhout *et al.*, 2009].

Regarding the **teratogenic** animal models, CDH can be induced by administration of a pan-RAR antagonist (BMS493) or RALDH2 inhibitors, such as 2,4-dichlorophenyl-p-nitro-phenylether (nitrofen; an herbicide), 4-biphenyl carboxylic acid (BPCA; a breakdown product of a thromboxane-A_2 receptor antagonist), bisdiamine (an inhibitor of alcohol dehydrogenase), or SB-210661 (a benzofuranyl urea derivative developed for inhibiting 5-lipoxygenase) [Clugston *et al.*, 2010; van Loenhout *et al.*, 2009; Kling & Schnitzer, 2007; Mey *et al.*, 2003; Thébaud *et al.*, 2001; Thébaud *et al.*, 1999; Tasaka *et al.*, 1992].

The **nitrofen** model is the most extensively used for the study of CDH. This model is based in its administration to pregnant dams on 9.5 dpc when normal lung and diaphragm development are just about to begin. Originally, nitrofen was used as an herbicide and, in spite of the extensive use of nitrofen in agriculture, its teratogenetic effects have never been shown to play a role in human CDH. In toxicology screens in adult rats, no apparent problems were observed. However, the nitrofen administration during midgestation to pregnant dams causes developmental anomalies that reasonably replicate the major abnormalities and the pathophysiology described in human

CDH [van Loenhout *et al.*, 2009; Montedonico *et al.*, 2008; Kling & Schnitzer, 2007]. In fact, in this model, the specific location and extent of the diaphragmatic defects are very comparable, but also the similarities in the CDH-associated anomalies, including lung hypoplasia and PH, and cardiovascular and skeletal defects, are impressive too [van Loenhout *et al.*, 2009; Migliazza *et al.*, 1999a; Migliazza *et al.*, 1999b; Tenbrinck *et al.*, 1990]. Therefore, the nitrofen-induced CDH model is one of the best to investigate the etiology, pathogenesis and therapeutic options in CDH.

Although the mechanism by which nitrofen induces the diaphragmatic defect and lung hypoplasia remains to be elucidated, recent evidences suggest the involvement of abnormalities linked with the retinoid signaling pathway in this model and also in human CDH etiology [Montedonico *et al.*, 2008; Kling & Schnitzer, 2007; Gallot *et al.*, 2005; Greer *et al.*, 2003]. The first evidence that CDH could be connected to perturbations in the retinoid signaling pathway was obtained already in 1941 by Andersen, who described diaphragmatic hernias in embryos of pregnant rats on a vitamin-A-deficient diet [Andersen, 1949]. This effect of maternal vitamin A deficiency was confirmed by Wilson *et al* in 1953 [Wilson *et al.*, 1953]. Moreover, CDH and lung hypoplasia have been also described in RARα and β compound KO, as well as in mice with the deletion of the RXR ligand activation domain [Kling & Schnitzer, 2007; Mascrez *et al.*, 1998]. One clinical study demonstrated the presence of decreased plasma levels of retinol and retinol-binding protein in human CDH, suggesting a possible deterioration of retinol transport across the placenta [Major *et al.*, 1998]. Furthermore, CDH has been observed in patients with deletions on the 15q chromosome, which contains the encoding gene for cellular retinoic acid binding protein (CRABP1) [van Loenhout *et al.*, 2009; Holder *et al.*, 2007; Major *et al.*, 1998]. Additionally, administration of RA to nitrofen-treated lung explants demonstrated an increase in lung growth and partially rescued the hypoplasia [Montedonico *et al.*, 2006]. *In vivo*, exogenous administration of retinoids (vitamin A or RA) improves lung growth and maturation, increases fetal survival and decreases the incidence of CDH, in the nitrofen-induced CDH model [Montedonico *et al.*, 2006; Baptista *et al.*, 2005; Babiuk *et al.*, 2004; Thébaud *et al.*, 1999]. Regarding this teratogen model, it was already demonstrated that nitrofen inhibits RALDH2, a key enzyme responsible for the conversion of retinal to retinoic acid [Mey *et al.*, 2003].

Besides the retinoid signaling pathway, another pathway implicated in CDH is the thyroid hormone signaling pathway. Nitrofen, triidothyronine (T_3) and thyroxine (T_4) have similar chemical structures. Therefore, it is possible that nitrofen influences both diaphragm formation and lung development by interfering with thyroid hormone homeostasis [van Loenhout *et al.*, 2009;

Brandsma *et al*, 1994]. Nonetheless, a clear relation between the thyroid signaling pathway and lung or diaphragm defects has not been demonstrated so far.

In summary, although the nitrofen model appears to be the best model available since the timing of the developmental insult is similar to that in humans, a large disadvantage is that the significance of the potential teratogenic effects of nitrofen in rodents has never been demonstrated in humans. Moreover, although increasing evidence of the etiology of CDH points towards a disturbance in the retinoid signaling and/or thyroid signaling pathways, the nitrofen model has not resolved the pathogenesis of CDH [van Loenhout *et al.*, 2009].

Concerning **genetic** models, the use of KO mice technology allowed linking several expected and unexpected genes to CDH. Indeed, several reports of diaphragmatic defects in genetically engineered mutant mice have been described, introducing potential genetic models for the study of CDH. Diaphragmatic defects have been observed in *Wt1* null-mutant mice, in *RAR*α and β_2 double null-mutant mice, in *MyoR* and *capsulin* double null-mutant mice, in mice with *COUP-TFII* conditionally inactivated in mesenchyme tissues, in single null mutant mice for *Gli2* and *Gli3* as well as the double mutant, in *Slit3* null mutant mice, in *Fog2* mutant mice, in *Gata4* KO mice, and i*n PDGFR*α null mice models [van Loenhout *et al.*, 2009; Clugston *et al.*, 2006]. However, translation to the CDH human situation has not been made for all these genes. Moreover, the observed phenotype in CDH is so variable that it is potentially not due to a single gene mutation, but the result of multiple gene mutations. The different genes involved in different signaling pathways that have been shown to be important for normal embryonic development might be involved.

4.4 CDH Clinical Approach

CDH prenatal diagnosis, which occurs in at least two out of three cases, is possible from routine obstetric screening from 15 weeks of gestation [DeKoninck *et al.*, 2011]. The magnetic resonance imaging (MRI) is indicated for fetal differential diagnosis with other birth defects. After confirmation of prenatal diagnosis of CDH is necessary ruling out chromosomal abnormalities/malformations. In fact, the presence of chromosomal abnormalities and/or other structural malformations, which occurs in around 40%, is associated with a worse prognosis, with mortality exceeding 90% [Deprest *et al.*, 2011; Stege *et al.*, 2003]. In these cases, several authors propose the medical termination of pregnancy. In the other cases, it is necessary stratify the prognosis [reviewed by Doné *et al.*, 2008]. In addition to the chromosome and/or other malformations are

several prognostic factors that have been proposed for follow-up of CDH fetuses, including the upper versus lower position of the left hepatic lobe, the right lung area to head circumference ratio (LHR), gestational age at the time of prenatal diagnosis, the presence of polyhydramnios, the lung area to thorax transverse area ratio, the stomach in intra-thoracic position, side and size of the defect, the hypoplasia degree of the abdominal cavity, the left ventricular hypoplasia degree, the impedance of the left pulmonary artery, the acceleration time to right ventricular ejection time ratio, and the evaluation of fetal lung volumetry by 2D ultrasound, 3D or MRI [reviewed by Gucciardo et al., 2008]. However, many of these possible prognostic factors have not been universally accepted. The two prognostic factors that have obtained more consistent results are the LHR (expressed as a function of what is expected in a gestational aged control; observed / expected LHR) [Alfaraj et al., 2011; Jani et al., 2009; Jani et al., 2007] and liver position [Mayer et al., 2011; Knox et al., 2010].

Based upon in these parameters, CDH fetuses can be now prenatally stratified into low and high-risk groups. The low-risk fetuses should be managed expectantly during pregnancy [DeKoninck et al., 2011; Mayer et al., 2011]. In opposition, for the high-risk fetuses (i.e. fetuses which prognosis is predictably poor with postnatal treatment available), the consensus is that something needs to be done before birth in order to total or partially revert the fetal lung hypoplasia. Hence, antenatal therapies that promote fetal lung growth remain an appealing approach to improve survival of high-risk CDH fetuses [Deprest & De Coppi, 2012].

Regarding fetal surgical interventions, such as open fetal repair of the diaphragmatic defect and fetoscopic endoluminal tracheal occlusion (FETO), it is recognized that might improve lung growth. However, these techniques are invasive, technically demanding, limited by the maternal and fetal risks, and its efficacy is still not determined, with controversial results in survival and morbidity rates [Jani et al., 2009; Harrisson et al., 2003]. Recently, a brasilian group described in a randomized controlled trial with 41 patients that FETO improves neonatal survival in cases with isolated severe CDH [Ruano et al., 2012]. Moreover, the FETO consortium demonstrated that FETO in severe CDH is associated with a high incidence of spontaneous preterm premature rupture of membranes and preterm delivery, but a substantial improvement in survival. In fact, survival rate increased in severe cases with leftsided CDH from 24.1% to 49.1%, and in right-sided CDH from 0% to 35.3% [Jani et al., 2009]. Subsequently, a randomized trial comparing expectant management during pregnancy and FETO, followed by standardized neonatal care (TOTAL, the Tracheal Occlusion to Accelerate Lung growth-trial) has been set up and is currently underway [DeKoninck et al., 2011].

At the same time, less invasive approaches such as antenatal pharmacological treatment to stimulate lung growth before birth and to treat PH are also under investigation [Luong *et al.*, 2011; Santos *et al.*, 2006; Baptista *et al.*, 2005; Thébaud *et al.*, 1999]. For instance, Santos *et al* described that maternal administration of ghrelin attenuates pulmonary hypoplasia in nitrofen-induced CDH model [Santos *et al.*, 2006]. Furthermore, Luong *et al* demonstrated recently that antenatal sildenafil treatment attenuates PH in experimental CDH [Luong *et al.*, 2011. However, until the moment, any of the above strategies had resulted in significant clinical impact.

Aims and Thesis Layout

Pulmonary development is a complex process involving two different cellular phenomena, growth and maturation. The growth process occurs through fundamental cross-talk interactions between epithelial and mesenchymal tissues, airway and vasculature, involving a multitude of effectors including growth factors, hormones, extracellular matrix interactions, and mechanical stimuli. During the last decades, the regulating mechanisms of lung growth have been unraveled. However, regardless of the advanced knowledge of normal lung growth, there are several possible regulators and determinants that need to be investigated.

Lung pathologies are an epidemiological relevant issue. Indeed, there are several pediatric and adult diseases characterized by lung hypoplasia or dysplasia, involving important morbidity and mortality rates, around the world. The understanding of the effectors and lung growth regulatory mechanisms has clinical relevance since it can open new perspectives in the treatment of these lung diseases as well as modulation of lung repair.

The present dissertation was developed according to this rationale: to clarify novel physiological regulators of normal lung morphogenesis, expecting to contribute for the development of new therapeutic or preventive strategies for lung diseases. For someone who is a resident of Gynecology and Obstetrics, an especial interest in congenital lung diseases is natural. Moreover, since long, our lab has focused research on the pathophysiology and development of potential prenatal therapeutics for CDH.

Thus, the following specific aims were pursued in this thesis:

i. To investigate the role of inflammatory cytokines, namely gp130 family of cytokines, on fetal lung growth;

ii. To study the existence and functional relevance of a local RAS during fetal lung development;

iii. To demonstrate the intrinsic ability of fetal lung to regulate its growth and to investigate possible effectors responsible for this mechanism;

iv. To establish new therapeutic strategies for fetal lung hypoplasia, in context of CDH.

The present PhD thesis is organized into nine different chapters. In **Chapter I**, just before the Aims and Thesis Layout, a General Introduction to the thesis theme has been presented, including the most important regulators and mechanisms of fetal lung growth, general concepts regarding the novel lung regulators here investigated (gp130 family of cytokines and RAS), and abnormal pulmonary development and pathophysiology that characterizes CDH. The following five chapters present the most important experimental work of this dissertation, addressing the questions raised in the aims, which resulted in published or submitted papers to international peer-reviewed scientific journals. **Chapter II** presents the first manuscript published from the scope of this work entitled *'IL-6 is constitutively expressed during lung morphogenesis and enhances fetal lung explant branching'*. In **Chapter III** is presented the article *'Leukemia inhibitory factor in rat fetal lung development: expression and functional studies'*. The research manuscript *'The role of gp130 family of cytokines on fetal lung development'*, which has been submitted to a peer-reviewed journal, is attached in **Chapter IV**. **Chapter V** presents the article *'Intrinsic catch-up growth of hypoplastic fetal lungs is mediated by IL-6'*. Finally, the manuscript *'Local fetal lung renin-angiotensin system as a target to treat congenital diaphragmatic hernia'* is presented in **Chapter VI**. **Chapter VII** is a general discussion of the most relevant results of this PhD work. A compendium of the major conclusions drawn from this work is presented along with the future perspectives in **Chapter VIII**. In the end of the thesis, in **Chapter IX**, all the references from the entire write up are presented in alphabetical order.
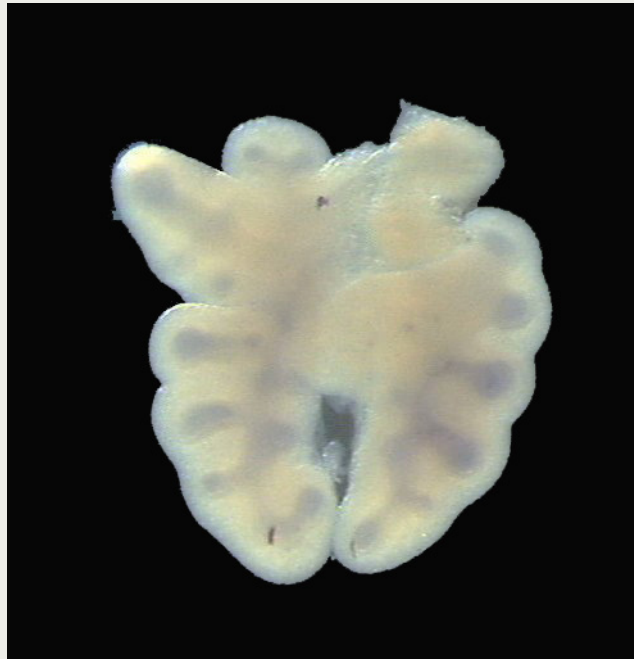
CHAPTER II
IL-6 AND FETAL LUNG GROWTH

IL-6 is Constitutively Expressed During Lung Morphogenesis and Enhances Fetal Lung Explant Branching

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IL-6 Is Constitutively Expressed During Lung Morphogenesis and Enhances Fetal Lung Explant Branching

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ABSTRACT: Previous studies have shown that chorioamnionitis, with increased IL-6, promotes fetal lung maturation and decreases the incidence of respiratory distress syndrome in premature neonates. However, the expression pattern and the effects of IL-6 on fetal lung growth mechanisms remain unknown. IL-6 expression was assessed by *in situ* hybridization and by real-time PCR between 14.5 and 21.5 d postconception. Normal and nitrofen-induced hypoplastic lung explants were cultured with increasing IL-6 doses or IL-6 neutralizing antibodies. Branching, cellular proliferation (Ki-67) and MAPK phosphorylation in fetal lung explants were analyzed. Pulmonary primitive epithelium expressed IL-6 constitutively throughout all gestational ages, displaying highest levels during earliest stages. In normal and hypoplastic lung explants, IL-6 neutralizing antibodies significantly reduced, whereas IL-6 supplementation induced a biphasic effect (lower doses increased, while the highest dose did not accomplish additional effect) on branching and cellular proliferation. IL-6 enhanced p38-MAPK phosphorylation without changing MEK1/2 and JNK pathways. The present study suggests a physiological role for IL-6 on pulmonary branching mechanisms most likely involving p38-MAPK intracellular signalling pathway. (*Pediatr Res* **60:** 530–536, 2006)

During the last decades, the regulating mechanisms of lung branching have been unraveled. This morphogenic process occurs through fundamental cross-talk interactions between epithelial and mesenchymal tissues *via* extremely complex processes, involving a multitude of effectors including growth factors, extracellular matrix interactions, and hormones (1). The understanding of these mechanisms has clinical relevance since it can open new perspectives in the treatment of fetal lung hypoplasia as well as modulation of lung repair.

IL-6 is a pleiotropic cytokine with important roles on acute inflammatory response, infection, hematopoiesis, regulation of bone absorption, cell growth, differentiation, survival, apoptosis, and proliferation (2–4). Several studies have emphasized the importance of IL-6 signaling in several processes of branching organs such as embryonic submandibular gland development (5,6), mammary gland remodeling (7), benign and malign prostate growth (8,9), and lung maturation (10).

In fact, several clinical and animal-based studies suggest that antenatal exposure to inflammatory mediators may improve lung volume and compliance as well as accelerate fetal lung maturation (10). In humans, it was demonstrated that IL-6 elevation in fetuses with chorioamnionitis promoted fetal lung maturation by enhancing surfactant protein A (SP-A) synthesis. In fact, fetal IL-6 is a regulatory cytokine of pulmonary surfactant proteins and plays an important role in lung maturity decreasing the incidence of respiratory distress syndrome in preterm neonates (11). In different animal models such as the rat (12), the rabbit (10), and the sheep (13), it was shown that intra-amniotic injection of endotoxin or continuous administration of IL-6 improved lung function by increasing expression of surfactant apoprotein mRNA (12,14,15).

Recently, it was demonstrated that IL-6 plays an important modulatory role in lung angiogenesis (16), a fundamental process in fetal lung morphogenesis (17). Moreover, Dame and Juul (4) demonstrated that IL-6 receptor was widely distributed in developing human fetuses, including in bronchial epithelial cells. Such findings suggest an IL-6 nonhematopoietic role in the developing fetus.

IL-6 exerts its action *via* the signal transducer gp130 leading to the activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) and MAPK cascades (2). Many of the effectors that modulate fetal lung branching morphogenesis seem to activate signaling pathways that converge into the mitogen-activated protein MAPK (18). There are three major families of MAPK: the extracellular signal-regulated kinases-1 and -2 (ERK1/2), c-Jun NH$_2$-terminal kinases (JNK), and p38 kinase.

Although several reports demonstrated that IL-6 can modulate lung development, the IL-6 expression pattern as well as its effects during early lung development are largely unknown. Therefore, in the current study, IL-6 expression during antenatal lung development was characterized. Furthermore, the effects of IL-6 supplementation or IL-6 neutralizing antibodies in normal and hypoplastic fetal lung explants were evaluated.

Abbreviations: D$_0$, day 0; **D$_4$,** day 4; **dpc,** days postconception; **ISH,** *in situ* hybridization; **MAPK,** mitogen-activated protein kinase

Additionally, the potential activation of MAPK pathway by IL-6 was investigated.

MATERIALS AND METHODS

Animal experiments were performed according to the Portuguese law for animal welfare. Animals were housed in an accredited mouse house and treated as specified by the recommendations of the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (National Institutes of Health Publication No.85-23, revised 1996).

Animal model and experimental design. Sprague-Dawley female rats (225g; Charles-River, Barcelona, Spain) were maintained in appropriate cages under controlled conditions and fed with commercial solid food. The rats were mated and checked daily for vaginal plug. The day of plugging was defined as gestational d 0.5 for time dating purposes. Nine and a half dpc pregnant rats were exposed to 100 mg of nitrofen (2,4-dichlorophenyl-p-nitrophenylether) dissolved in 1 mL of olive oil administered by gavage (nitrofen-induced hypoplastic lungs: nitrofen group) or with an equal volume of vehicle (normal lungs: normal group) (19). For *in situ* hybridization and PCR studies, normal lungs were used, whereas for fetal lung explant cultures normal and nitrofen-induced hypoplastic lungs were used.

From the normal group, fetuses were removed by cesarean section at 14.5, 15.5, 17.5, 19.5, and 21.5 dpc. Fetuses at 14.5 dpc were processed *in toto* for whole mount hybridization studies. Fetuses older than 14.5 dpc were killed by decapitation and lungs were excised, processed, and embedded in paraffin for *in situ* hybridization. Random left lung samples were collected for the quantification of IL-6 expression by real-time PCR. These samples were immersed in RNAlater (QIAGEN GmbH, Hilden, Germany) and stored at $-80°C$.

Fetuses harvested at 13.5 dpc from normal and nitrofen groups were dissected and lungs collected for explant culture. After 4 d culture, explants from each group were collected either for immunohistochemistry or Western blot analysis.

ISH studies. Digoxigenin-labeled IL-6 probe was synthesized from a 901 bp fragment of the coding sequence of IL-6, inserted on a pBluescript KS-vector (kindly supplied by Dr. Georg H. Fey, University of Erlangen, Nurnberg, Germany). The plasmid was linearized with *Bam*HI and T3 RNA polymerase was used to synthesize the antisense riboprobe. The *in vitro* transcription reaction was performed using DIG RNA Labeling Mix (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions.

Whole mount. Embryos were fixed overnight at 4°C in 4% paraformaldehyde (PFA), rinsed in $1\times$ PBS, dehydrated through a methanol series, and stored in 100% methanol at –20°C. Whole mount ISH was performed as previously described (20). Embryos were visualized as whole mounts in PBT (PBS, 0.1% Tween20) under binocular microscopy (Leica MZFLIII; Leica Geosystems AG, Heerbrugg, Switzerland), and photographed using Sony 3CCD Color Video Camera.

Paraffin sections. Tissue sections (10 μm thick) were processed for ISH as previously described by Strahle *et al.* (21). ISH slides were observed and photographed on light microscope with an Axiocam color video camera (Carl Zeiss GmbH, Jena, Germany).

RNA extraction and reverse transcription. Total mRNA from left lung samples of 30 fetuses (15.5 dpc, $n = 9$; 17.5 dpc, $n = 7$; 19.5 dpc, $n = 7$; 21.5 dpc, $n = 7$) was extracted using the RNeasy Mini Kit Protect (QIAGEN GmbH). Total mRNA quantification was done by spectrophotometry (Bio-Photometer, Eppendorf AG, Hamburg, Germany).

Reverse transcription was performed as previously described by Santos *et al.* (22).

Quantitative real-time PCR. Quantitative real-timePCR was performed as previously described by Santos *et al.* (22).

Primer design was based on available sequences in *GenBank* (NCBI-NLM-PubMed-Gene). All the primers are intron-spanning (Table 1). For IL-6 and β-actin primer sets, standard amplification curves (ST curves) were made with randomly selected cDNA samples setting $r = 0.99$. In all the samples IL-6 expression was normalized for β-actin.

Fetal lung explant cultures. Harvesting and dissection of 13.5 dpc lungs was made in DPBS (Cambrex, East Rutherford, NJ) under a dissection microscope (Leica MZFLIII). The lungs were transferred to Nucleopore membranes with an 8 μm pore size (Whatman, Clifton, NJ) and incubated in a 12-well culture plates from Costar (Corning, NY). The membranes were presoaked in DMEM (Cambrex) for 1 h before the explants were placed on them. Floating cultures of the explants were incubated in 200 μL of 50% DMEM, 50% nutrient mixture F-12 (Invitrogen, Carlsbad, CA) supplemented with 100 μg/mL streptomycin, 100 units/mL penicillin (Invitrogen), 0.25 mg/mL ascorbic acid (Sigma Chemical, Poole, Dorset, UK) and 10% FCS (Invitrogen). The fetal lung explants were incubated in a 5% CO_2 incubator at 37°C for 96 h, and the medium was replaced every 48 h. The branching morphogenesis was monitored daily by photographing the explants. At d 0 (D_0: 0 h) and d 4 (D_4: 96 h) of culture, the total number of peripheral airway buds (branching) in all lung explants was determined, whereas the epithelial perimeter was measured using AxionVision Rel. 4.3 (Carl Zeiss GmbH). The results of branching and epithelial perimeter were expressed as D_4/D_0 ratio.

IL-6 supplementation studies. Twelve normal and 12 nitrofen-hypoplastic lung explants were used as control (0 pg/mL). Additionally, recombinant rat IL-6 (R & D Systems, Minneapolis, MN) was added daily to normal and nitrofen-induced hypoplastic lung explants to achieve a final concentration of 0.01, 0.1, 1, 10, 100 pg/mL (normal lungs: 0.01, $n = 8$; 0.1, $n = 8$; 1, $n = 8$; 10, $n = 10$; 100, $n = 8$. Hypoplastic lungs: 0.01, $n = 10$; 0.1, $n = 9$; 1, $n = 9$; 10, $n = 12$; 100, $n = 8$).

IL-6 neutralizing studies. Normal explants were treated daily with IgG anti-rat IL-6 neutralizing antibody ($n = 12$) at concentration of 1.0 μg/mL (30 times the concentration previously shown to suppress IL-6 effect) (R & D Systems). Nonspecific effects were evaluated by adding identical concentration of normal goat IgG control antibody ($n = 10$) (R & D Systems).

Proliferation assay by immunohistochemistry. Nontreated (0 pg/mL) and IL-6 treated (10 and 100 pg/mL) normal and nitrofen explants as well as normal explants treated with normal goat IgG and IgG anti-rat IL-6 neutralizing antibodies (interruption studies) were processed for proliferation assay (5 per each group). Ki-67 immunostainings were performed on formalin-fixed and paraffin-embedded lung explants. Sections (5 μm) were placed on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). The primary antibody, a monoclonal rabbit serum anti-Ki-67 (NeoMarkers, Fremont, CA), was used in a 1:200 dilution. After dewaxing in xylene and rehydration in ethanol, the samples were incubated in 3% hydrogen peroxide in methanol to quench endogenous peroxidase. Antigen retrieval was achieved by boiling in 10 mM citrate buffer followed by cool down at room temperature. Samples were blocked with 5% BSA (Roche Molecular Biochemicals). Incubation of the primary antibody occurred at 4°C overnight. Negative control reactions included omission of the primary antibody. Incubation with the UltraVision detection system anti-polyvalent horseradish peroxidase (Lab Vision Corporation, Fremont, CA) was carried accordingly to manufacturer's instructions. To visualize the peroxidase activities in sections, diaminobenzidine tetrahydrochloride was used. Sections were counterstained with hematoxylin.

For cell proliferation quantification, epithelial and mesenchymal Ki-67 positive and negative cells were counted at $\times 20$ magnification (Olympus DP 70, Cell P; Olympus, Hamburg, Germany) in four independent peripheral areas per explant. Results are presented as percentage of the ratio of positive-to-total cells normalized for lung section area.

Western blot analysis. Nontreated (0 pg/mL) and 10 pg/mL IL-6 treated normal explants were processed for Western blot analysis. Proteins from lung explants were obtained according to Kling *et al.* (18). Five micrograms of protein were loaded onto 12.5% acrylamide minigels, electrophoresed at 100 V at room temperature, and then transferred to Hybond-C Extra (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Blots were probed with p38, p44/42 (ERK1/2), JNK, phospho-p38 (dp-p38), phospho-p44/42 (dp-ERK1/2), phospho-JNK (dp-JNK) MAb (New England Biolabs, US) according to manufacturer's instructions. For loading control, blots were probed with β-tubulin MAb (Santa Cruz Biotechnology Inc., US). Afterward blots were incubated with a secondary horseradish peroxidase conjugate (New England

Table 1. *Primers used for quantitative PCR*

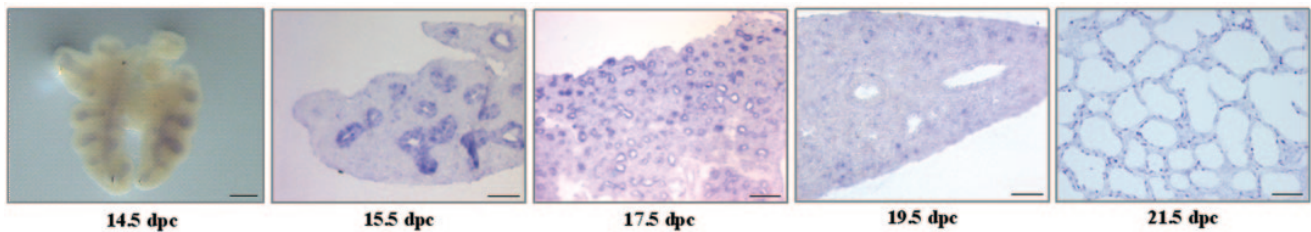| Gene	Accession number	Primer set	Product size (bp)
IL-6	NM_012589	5′-CAA GAG ACT TCC AGC CAG-3′	141
		5′-CTC CGA CTT GTG AAG TGG T-3′	
β-actin	NM_031144	5′-GAT TTG GCA CCA CAC TTT CTA CA-3′	114
		5′-ATC TGG GTC ATC TTT TCA CGG TTG G-3′	

Figure 1. IL-6 expression pattern during normal rat lung development. ISH studies showed that in normal fetal lung, IL-6 expression was localized to the primitive airway epithelium. The positive IL-6 signal was detected during all the studied developmental ages, predominantly during the pseudoglandular phase (14.5 and 15.5 dpc). Scale bar: 14.5 dpc = 4960 μm; 15.5, 17.5, 19.5, and 21.5 dpc = 100 μm.

Biolabs, Beverly, MA), developed with Super Signal West Femto Substrate (Pierce Biotechnology, Inc., Rockford, IL) and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech, Inc.).

Statistical analysis. All quantitative data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA (real-time PCR and morphometric explants studies) or three-way ANOVA (proliferation studies) on ranks and the Student-Newman-Keuls test was used for post-test analysis. For IL-6 neutralizing studies (morphometric and proliferation studies), t test analysis was used. Statistical significance was set at $p < 0.05$.

RESULTS

IL-6 expression pattern during normal pulmonary development. ISH studies revealed that IL-6 was expressed throughout all studied gestational ages in fetal lung (Fig. 1). Figure 1 illustrates IL-6 mRNA predominantly expressed in primitive lung epithelium presenting significant expression since the earliest studied stage of rat lung development (14.5 dpc). IL-6 mRNA levels appeared highest during pseudoglandular stage (14.5 and 15.5 dpc) decreasing during subsequent stages of lung development.

Real-time PCR analysis. The ISH results of IL-6 expression prompted us to perform real-time PCR to quantify relative expression of IL-6 levels during normal lung development (Fig. 2). The mRNA levels of β-actin were not significantly altered during gestation and were subsequently used to normalize the expression data for IL-6. In Figure 2, mRNA levels

of IL-6 normalized to β-actin are presented. In normal fetal lung development, IL-6 was expressed in all studied gestational ages. IL-6 mRNA levels were significantly higher in the earliest assessed gestational age (15.5 dpc), which corresponds to the pseudoglandular stage of lung development. In the subsequent stages of lung development, as suggested by ISH results, IL-6 mRNA levels decreased.

IL-6 supplementation studies. To evaluate IL-6 role during lung morphogenesis, normal and hypoplastic fetal lung explants were treated with different doses of recombinant rat IL-6. In Figure 3, representative examples of normal (Fig. 3*A*) and hypoplastic (Fig. 3*B*) fetal lung explants treated with increasing IL-6 doses are illustrated. IL-6 appears to have an enhancing effect on lung explants growth, except with the highest dose.

The results of morphometric analysis on fetal lung explants are summarized in Figure 4. In normal and nitrofen explants, increasing IL-6 doses induced a biphasic effect on total number of peripheral airway buds as well as on epithelial perimeter. Lower IL-6 doses enhanced explant growth, whereas the highest dose did not induce significant effect. In normal explants (Fig. 4, *A* and *C*), IL-6 treatment induced maximal growth rates at 10 pg/mL, whereas in nitrofen explants, the total number of peripheral airway buds (Fig. 4*B*) was maximal at 10 pg/mL and epithelial perimeter (Fig. 4*D*) at 1 pg/mL. The number of peripheral airway buds and the epithelial

Figure 2. IL-6 mRNA levels during normal fetal rat lung development, expressed in arbitrary units normalized for β-actin (15.5 dpc: $n = 9$; 17.5 dpc: $n = 7$; 19.5 dpc: $n = 7$; 15.5 dpc: $n = 7$). *$p < 0.05$ *vs* 15.5 dpc, § $p < 0.05$ *vs* 17.5 dpc, ¶ $p < 0.05$ *vs* 19.5 dpc.
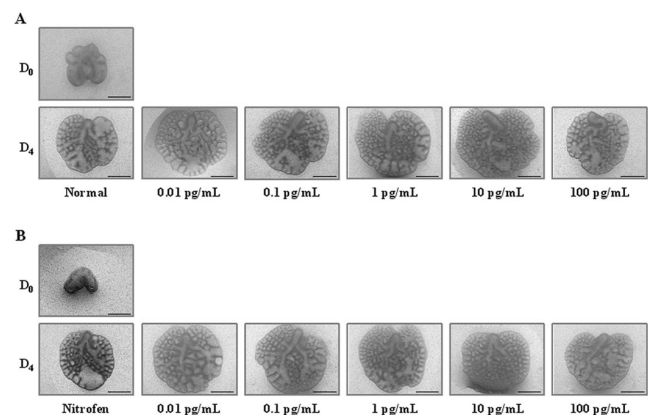
Figure 3. Branching morphogenesis in rat lung explant system. (*A*) Normal lung explants. (*B*) Nitrofen-hypoplastic lung explants. *Upper panel* is representative of lungs at 13.5 dpc at culture d 0 (D$_0$). *Bottom panel* represents lungs, treated with different IL-6 doses, at d 4 (D$_4$). Scale bar = 6349 μm (all images at same magnification).
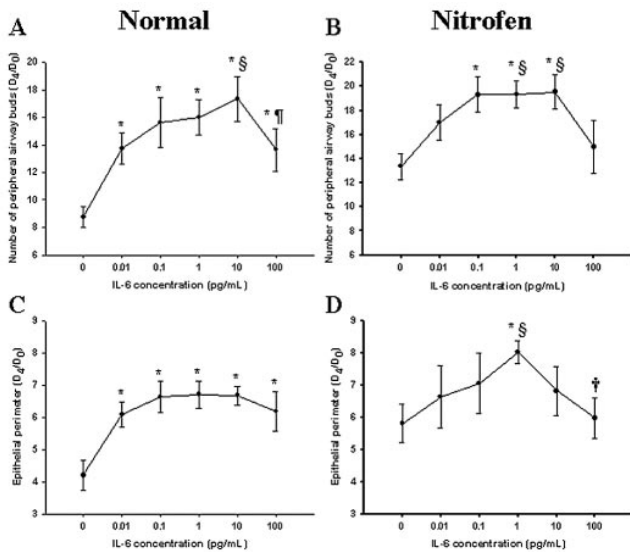
Figure 4. Morphometric analysis of lung explant cultures treated with different IL-6 doses. (*A*) Number of total airway buds in normal explants. (*B*) Number of total airway buds in hypoplastic explants. (*C*) Epithelial perimeter in normal explants. (*D*) Epithelial perimeter in hypoplastic explants. Results are expressed as D_4/D_0 ratio (Normal lungs: 0, $n = 12$; 0.01, $n = 8$; 0.1, $n = 8$; 1, $n = 8$; 10, $n = 10$; 100, $n = 8$. Hypoplastic lungs: 0, $n = 12$; 0.01, $n = 10$; 0.1, $n = 9$; 1, $n = 9$; 10, $n = 12$; 100, $n = 8$). *$p < 0.05$ *vs* IL-6 at 0 pg/mL, § $p < 0.05$ *vs* IL-6 at 0.01 pg/mL, † $p < 0.05$ *vs* IL-6 at 1 pg/mL, ¶ $p < 0.05$ *vs* IL-6 at 10 pg/mL.

Figure 5. Cellular proliferation assay in normal and nitrofen explants by Ki-67 immunohistochemistry. (*A*) Nontreated normal explants. (*B*) 10 pg/mL IL-6 treated normal explants. (*C*) 100 pg/mL IL-6 treated normal explants. (*D*) Nontreated hypoplastic explants. (*E*) 10 pg/mL IL-6 treated hypoplastic explants. (*F*) 100 pg/mL IL-6 treated hypoplastic explants (five lungs analyzed per each group). The bottom graphics presents epithelial and mesenchymal cellular proliferation quantification. Results are expressed as percentage of positive-to-total cells ratio normalized for mm². Scale bar = 100 μm (all images at same magnification). *$p < 0.05$ *vs* normal or nitrofen explants nontreated, ¶ $p < 0.05$ *vs* IL-6 at 10 pg/mL. ns, not statistically significant.

perimeter were significantly reduced in nitrofen when compared with normal explants at D_0 (Table 2). However, in the absence of IL-6 treatment and after 4 d in culture no significant differences between normal and nitrofen groups were observed. After IL-6 treatment, D_4/D_0 ratio of the number of peripheral airway buds (Fig. 4*B*) and epithelial perimeter (Fig. 4*D*) was always higher in the nitrofen group than in the normal group, independently of IL-6 dose.

Figure 5 shows cellular proliferation assay in normal and nitrofen explants nontreated and treated with either 10 pg/mL (dose of maximal effect) or 100 pg/mL (dose with lesser effect). Treatment with 10 pg/mL of IL-6 induced a significant increase on proliferation rate in normal and nitrofen explants, whereas in explants treated with 100 pg/mL of IL-6 a significant decrease is evident in such proliferation when compared with 10 pg/mL. No significant differences between normal and nitrofen groups were observed, although there is a statistically significant interaction between variables: groups × IL-6 doses

Table 2. *Number of peripheral airway buds and epithelial perimeter in normal and nitrofen groups, at d 0 and 4 of culture*

Group	Number of peripheral airway buds		Epithelial perimeter	
	D_0	D_4	D_0	D_4
Normal	6 ± 0	54.8 ± 4.9	7054 ± 255	31 512 ± 3887
Nitrofen	4.33 ± 0.42	51.8 ± 4.76	5046 ± 294	29 222 ± 3522
p Value	<0.01	NS	<0.01	NS

NS, not statistically significant.

Figure 6. IL-6 neutralizing studies in normal lung explants treated with normal goat IgG (control $n = 10$) and IgG anti-rat IL-6 antibodies ($n = 12$). **A**: Branching morphogenesis (upper panel) and morphometric analysis of lung growth (middle and bottom panels). **B**: Cellular proliferation assay (5 lungs analyzed per each group). Scale bar A = 6349 μm, B = 100 μm. $p < 0.01$: * *vs*. goat IgG.

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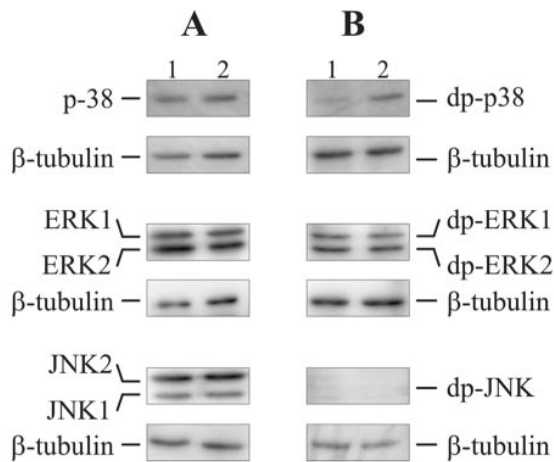
Figure 7. MAPK activities in nontreated (*1*) and treated with IL-6 at 10 pg/mL (*2*) lung explants. Western blot analysis of MAPK with MAbs to p38, ERK1/2 and JNK1/2 (*A*) and to diphosphorylated forms of p38 (dp-p38), ERK1/2 (dp-ERK1/2) and SAPK/JNK (dp-JNK1/2) (*B*). Control loading was performed using β-tubulin (55 KDa). ERK1 and 2 correspond to 44 and 42 KDa, respectively. JNK1 and 2 correspond to 46 and 54 KDa, respectively.

($p < 0.001$). Finally, no significant differences in proliferation rate between epithelium and mesenchyme were observed.

IL-6 neutralizing studies. In Figure 6, results of morphometric analysis (Fig. 6*A, left panels*) and proliferation studies (Fig. 6*B, right panels*) in normal lung explants treated with normal goat IgG (control) and IgG anti-rat IL-6 antibodies are shown. IL-6 blocking antibodies significantly reduced the number of peripheral airway buds, epithelial perimeter and epithelial and mesenchymal cellular proliferation rate.

MAPK signaling pathway. Normal lung explants treated with IL-6 at 10 pg/mL (dose with maximal effect), presented (semi-quantitative analysis) an enhanced p-38 kinase phosphorylation when compared with nontreated explants. No differences were observed in MEK1/2 and SAPK/JNK phosphorylation (Fig. 7).

DISCUSSION

This study demonstrated that IL-6 was constitutively expressed in pulmonary primitive epithelium, during all studied gestational ages of lung development, presenting highest levels of expression during pseudoglandular stage. Blocking IL-6 significantly reduced branching and cellular proliferation rate in normal explants, whereas IL-6 supplementation, either in normal or hypoplastic fetal lung explants, induced a biphasic effect on branching and cellular proliferation rate, with increased effect at lower doses, while no additional effect was obtained with the highest dose. These effects of IL-6 are most likely mediated through MAPK signaling pathway *via* p38.

IL-6 is a multifunctional inflammatory cytokine with important roles in cell proliferation, differentiation, growth, and apoptosis (2–4). Several clinical and experimental studies have investigated IL-6 role in pathophysiology of different lung diseases. In the adult, IL-6 has a critical role on inflammatory pathogenesis of acute respiratory distress syndrome

(23), asthma (24), chronic obstructive pulmonary disease (25), and non-small-cell lung cancer (26). IL-6 role during prenatal and neonatal period has been studied since decreased risk of respiratory distress syndrome in preterm neonates was demonstrated (10,11). In fact, it has been proved that inflammatory mediators, namely IL-6, promote fetal lung maturation by inducing SP-A expression and protein synthesis consequently improving lung mechanics and gas exchange (10,11). Studies carried on different animal models of intra-amniotic inflammation corroborated these observations in neonates exposed to chorioamnionitis (12–15,27,28).

Pulmonary development is a complex process involving two different cellular phenomena, growth and maturation (1). All previous studies concerning the effect of IL-6 on prenatal lung development had focused mainly in maturation processes in a pathologic inflammatory context (11–15,27,28). In this study, however, it was demonstrated that IL-6 was constitutively expressed in normal primitive lung epithelium throughout the studied ages. Interestingly, the epithelial IL-6 expression is in agreement with the findings previously documented in adult lung (29). In adults, epithelial expression was also observed in other branching organs such as salivary glands (30), prostate (8,9), and mammary gland (7). However, IL-6 expression in fetuses was only described during submandibular gland morphogenesis (5,6). In fact, Melnick *et al.* (5) showed a significant increase in IL-6 mRNA with progressive salivary gland development. Conversely, the current study demonstrated that in fetal lung the highest IL-6 mRNA levels occurred during early pseudoglandular stage decreasing progressively in subsequent stages of lung development.

Hypothesizing that IL-6 has a role during lung branching morphogenesis, normal fetal lung explants were cultured with increasing doses of IL-6 or with IL-6 neutralizing antibodies. Furthermore, IL-6 effect in nitrofen-induced hypoplastic lungs was also studied since it is well established that these lungs have restricted branching (31,32). This work clearly demonstrated that IL-6 supplementation induced a biphasic pattern on branching either in normal or hypoplastic explants. In fact, lower doses of IL-6 induced an increase in growth rate (total number of peripheral airways buds and epithelial perimeter) of normal and hypoplastic lung explants, whereas highest dose did not accomplish additional effect. These observations were corroborated by proliferation studies, which demonstrated that 10 pg/mL of IL-6 (maximal effect on branching) significantly increased Ki-67 epithelial and mesenchymal positive cells. On the other hand, 100 pg/mL of IL-6 induced no significant changes in proliferation rate when compared with nontreated explants. Interestingly, it seems that IL-6's effects were exacerbated in nitrofen explants. Although nitrofen explants are smaller at D_0, after 4 d in culture, no significant differences between normal and nitrofen explants could be detected (Table 2). The ability of hypoplastic explants to grow in culture with higher rates than normal explants had already been previously observed (33), however, the explanation for this phenomenon remains to be elucidated. A possible explanation consists in hypoplastic lungs eliciting up-regulation of some effectors as documented for neuroendocrine cellular products (33). Interestingly, it was also reported that end-gestation

human hypoplastic lungs present higher production of tumor necrosis factor-α than normal lungs (34).

Regarding the biphasic pattern of IL-6 effects on branching rate, it is interesting to stress that previous studies relating inflammation, lung maturation, and incidence of respiratory distress syndrome (RDS) report a similar pattern. In fact, although intrauterine infection seems to protect very premature infants against RDS, high quantities of pro-inflammatory cytokines cause serious disease (35). On the other hand, our findings suggest that IL-6 is constitutively produced during the earliest gestational stages, which may contribute for the explanation of enhanced fetal lung tolerance to inflammation during early gestation when compared with later gestation (26).

Although IL-6 knockout mice do not seem to have significant lung development abnormalities (36–38), this study clearly suggests a physiologic role of IL-6 in lung branching morphogenesis. Although previous studies on IL-6 and lung development emphasized maturational process, the highest expression of IL-6 on the early fetal lung development stages suggests that the physiologic role for IL-6 is most likely linked to branching.

Previous reports demonstrated epithelial expression of functional IL-6 receptor during fetal lung development (4,39). The mechanism responsible for IL-6 enhancing effects during lung branching morphogenesis is most likely dependent on gp130 signaling pathway through Stat3 and/or MAPK (2–4). In fact, it seems that IL-6 signaling through Stat3 is important to normal submandibular gland morphogenesis in the mouse (5) as well as in human prostate cancer cell growth (40). Additionally, adult rat mammary gland remodeling depends on gp130 signaling through Stat3 and MAPK (7). None of the previous studies implied JAK/STAT signaling pathway in fetal lung growth mechanisms. In contrast, MAPK signaling was recently suggested as a crucial pathway for fetal lung development (18). Thus, MAPK pathway activation by IL-6 in fetal lung development was investigated. Explants treated with 10 pg/mL IL-6 were selected due to its maximal effect on growth. These experiments clearly show activation of p38-MAPK by IL-6 treatment, whereas no significant activation of MEK1/2 and JNK pathways was observed.

In conclusion, IL-6 epithelial expression throughout fetal lung development as well as the present *in vitro* studies suggest a physiologic role for endogenous IL-6 on pulmonary branching mechanisms most likely involving p38-MAPK intracellular pathway.

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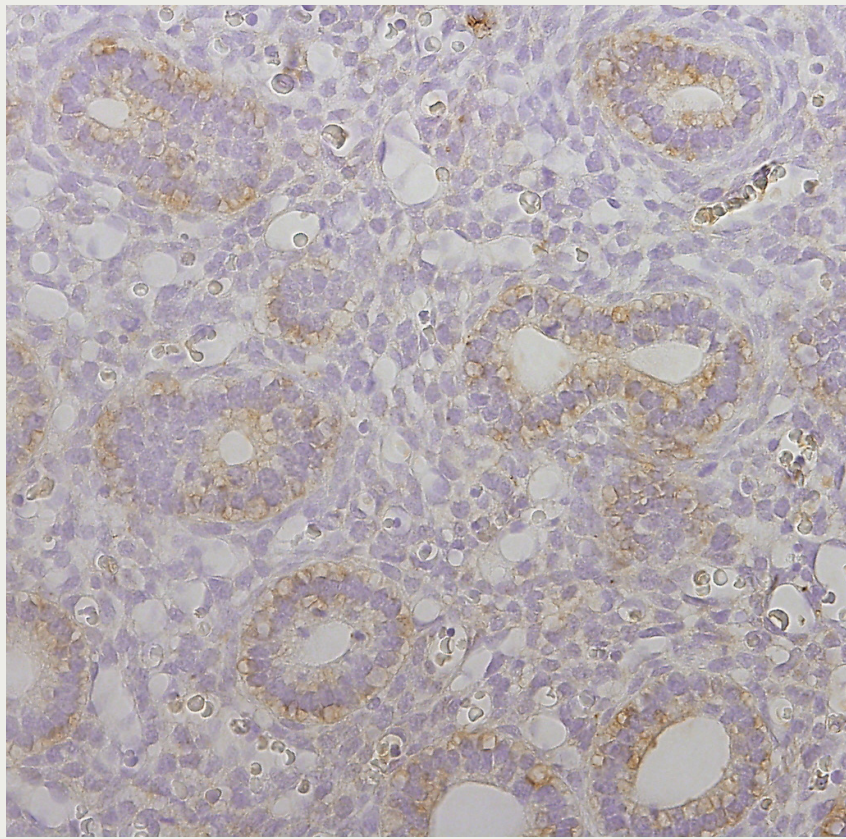
CHAPTER III
LIF AND FETAL LUNG DEVELOPMENT

Leukemia Inhibitory Factor
in Rat Fetal Lung Development:
Expression and Functional Studies

Cristina Nogueira-Silva, Paulina Piairo, Emanuel Carvalho-Dias,

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PLoS one

Leukemia Inhibitory Factor in Rat Fetal Lung Development: Expression and Functional Studies

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Abstract

Background: Leukemia inhibitory factor (LIF) and interleukin-6 (IL-6) are members of the family of the glycoprotein 130 (gp130)-type cytokines. These cytokines share gp130 as a common signal transducer, which explains why they show some functional redundancy. Recently, it was demonstrated that IL-6 promotes fetal lung branching. Additionally, LIF has been implicated in developmental processes of some branching organs. Thus, in this study LIF expression pattern and its effects on fetal rat lung morphogenesis were assessed.

Methodology/Principal Findings: LIF and its subunit receptor LIFRα expression levels were evaluated by immunohisto-chemistry and western blot in fetal rat lungs of different gestational ages, ranging from 13.5 to 21.5 days post-conception. Throughout all gestational ages studied, LIF was constitutively expressed in pulmonary epithelium, whereas LIFRα was first mainly expressed in the mesenchyme, but after pseudoglandular stage it was also observed in epithelial cells. These results point to a LIF epithelium-mesenchyme cross-talk, which is known to be important for lung branching process. Regarding functional studies, fetal lung explants were cultured with increasing doses of LIF or LIF neutralizing antibodies during 4 days. MAPK, AKT, and STAT3 phosphorylation in the treated lung explants was analyzed. LIF supplementation significantly inhibited lung growth in spite of an increase in p44/42 phosphorylation. On the other hand, LIF inhibition significantly stimulated lung growth via p38 and Akt pathways.

Conclusions/Significance: The present study describes that LIF and its subunit receptor LIFRα are constitu-tively expressed during fetal lung development and that they have an inhibitory physiological role on fetal lung branching.

Introduction

Fetal lung development is a complex process, involving several effectors such as growth factors, extracellular matrix interactions, hormones and as, recently described inflammatory mediators [1–3]. In fact, it was already demonstrated that interleukin-6 (IL-6) promotes fetal lung maturation [4–6] and also that IL-6 is constitutively expressed in pulmonary primitive epithelium and enhances fetal lung branching [3].

IL-6 is one of the members of the family of the glycoprotein 130 (gp130)-type cytokines. This family comprises IL-6, leukemia inhibitory factor (LIF), IL-11, oncostatin M, ciliary neurotrophic factor (CNTF), cardiotropin-1 (CT-1) and cardiotrophin-like cytokine. These cytokines share the membrane glycoprotein gp130 as a common signal transducer which explains the fact that these show some functional redundancy even though they also exhibit specific biological activities [7,8].

LIF is a pleiotropic cytokine, that exists in both soluble and matrix-bound forms, and that binds to a heterodimer LIF receptor alpha subunit (LIFRα)/gp130. Signal transduction involves the activation of Janus kinase (JAK) and the subsequent recruitment of signal transducers and activators of transcription (STAT) proteins, mainly STAT3. Alternatively, LIF can also initiate cell signaling via the mitogen-activated protein kinase (MAPK) cascade [7–10]. Moreover, LIF displays several biological activities ranging from the classic differentiation of myeloid leukemic cells into macro-phage lineage to effects on proliferation of primordial germ cells, maintenance of embryonic stem cell pluripotentiality, endometrial decidualization, blastocyst implantation, neural development, bone cell metabolism, adipocyte lipid and energy homeostasis, muscle satellite cell proliferation, heart hypertrophy, inhibition of retinal vascularization and inflammation [9–13]. Furthermore, several studies have emphasized the importance of LIF signaling in several processes of branching organs. For instance, this cytokine

inhibits fetal nephrons formation [14–16], induces mammary gland involution [17], decreases thyroid tumors growth [18,19] and increases pancreatic regeneration [20]. It was already described that LIF, together with insulin-like growth factor I (IGF-I), regulates lung maturation. In fact, absence of LIF in addition to IGF-I null mutant mice aggravates pulmonary immaturity. Indeed, LIF/IGF-I double deficient embryos present lung hypoplasia and defective differentiation of the alveolar epithelium and vasculogenesis [21].

Regardless of several evidences in literature that point towards a possible involvement of LIF during fetal lung development, LIF expression pattern as well as its effects during lung morphogenesis are largely unknown. Despite LIF/IGF-I double deficient mice lung phenotype, LIF knockout mice have no significant abnormal lung features [21,22]. However, in the current study, it was demonstrated that LIF and LIFRα were constitutively expressed during fetal lung development and that in vitro LIF supplementation significantly inhibited lung growth, likely through p44/42 pathway.

Results

LIF and LIFRα expression pattern during fetal lung development

The immunohistochemistry (IHC) and western blot studies revealed that LIF and LIFRα protein were expressed throughout all studied gestational ages in fetal lung (Figure 1 and 2). LIF was mainly expressed in bronchiolar and also in alveolar epithelium from 13.5 days post-conception (dpc) until term. At 19.5 and 21.5 dpc, mesenchymal tissue also displayed scattered LIF positive cells (Figure 1A and 1B). These LIF expression results prompted us to perform a western blot analysis in order to quantify the relative expression of LIF levels during normal lung development. LIF expression levels were minimal at 13.5 dpc, increased slightly throughout gestation and reached its maximum at the end of gestation (21.5 dpc) (Figure 1C and 1D).

Regarding LIFRα, early in gestation, protein expression was restricted to the mesenchyme (Figure 2A and 2B). Throughout fetal lung development, namely from 17.5 dpc onwards, LIFRα mesenchymal expression decreased and epithelial expression was observed until term (Figure 2A and 2B). LIFRα expression levels remained constant during fetal lung development (Figure 2C and 2D).

Role of LIF in fetal lung development

In order to evaluate the role of LIF on lung morphogenesis, fetal lung explants were treated daily with increasing concentrations of recombinant LIF. In Figure 3A, representative examples of fetal lung explants treated with increasing LIF concentrations, after 4 days in culture, are illustrated. LIF appears to have a dose-effect inhibitory action on lung explants growth. In fact, a decrease in the total number of peripheral airway buds (Figure 3B), epithelial perimeter (Figure 3C), area (Figure 3D) and external perimeter (Figure 3E) of lung explants was observed in all concentrations
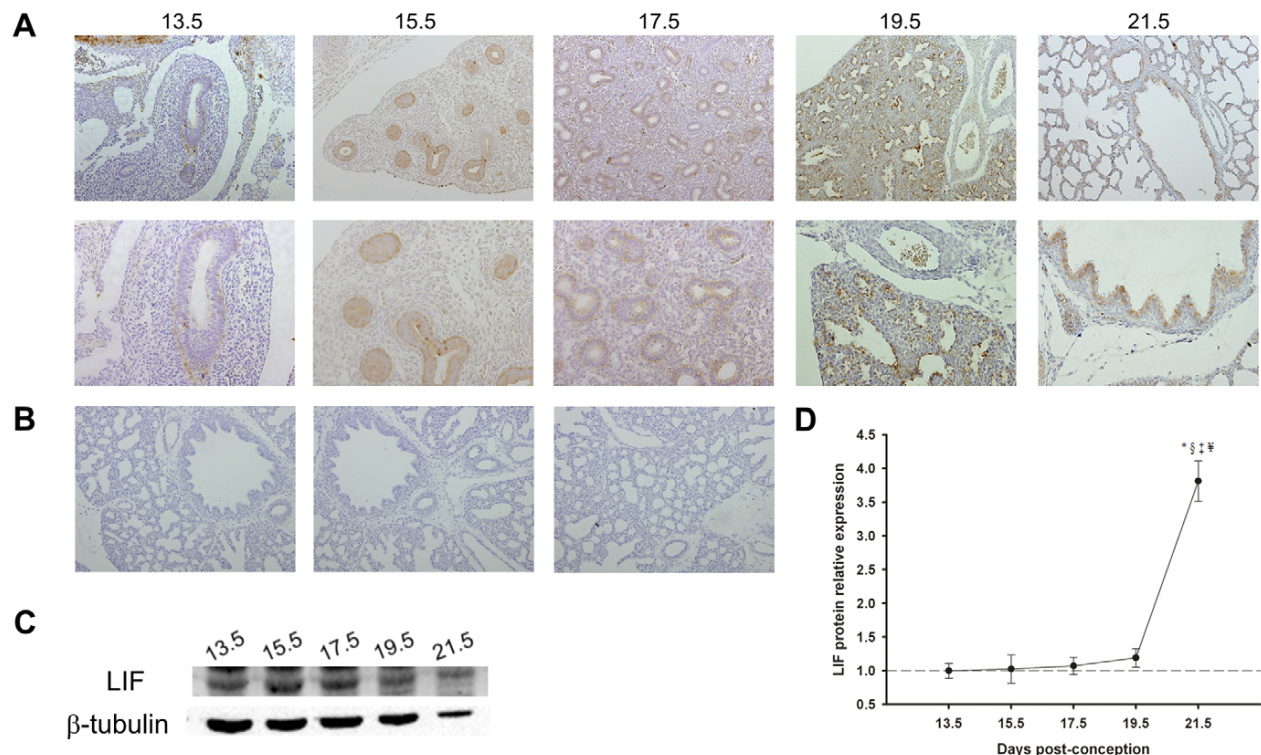

Figure 1. LIF expression pattern during fetal lung development (from 13.5 until 21.5 dpc). (A) IHC studies revealed that LIF expression was localized to airway epithelium. Original magnification: Upper panel – ×100; Bottom panel – ×200. (B) IHC negative controls: Left – omission of the primary antibody; Center – non-immune goat IgG isotype control; Right – simultaneous omission of the primary and secondary antibodies. In all negative controls immunoreactive LIF staining was not observed. (C) Western blot analysis of LIF throughout gestation (45 kDa). Control loading was performed using β-tubulin (55 kDa). (D) Relative LIF protein levels expressed in arbitrary units normalized for β-tubulin. p<0.05: *vs. 13.5 dpc, § vs. 15.5 dpc, ‡ vs. 17.5 dpc, ¥ vs. 19.5 dpc.
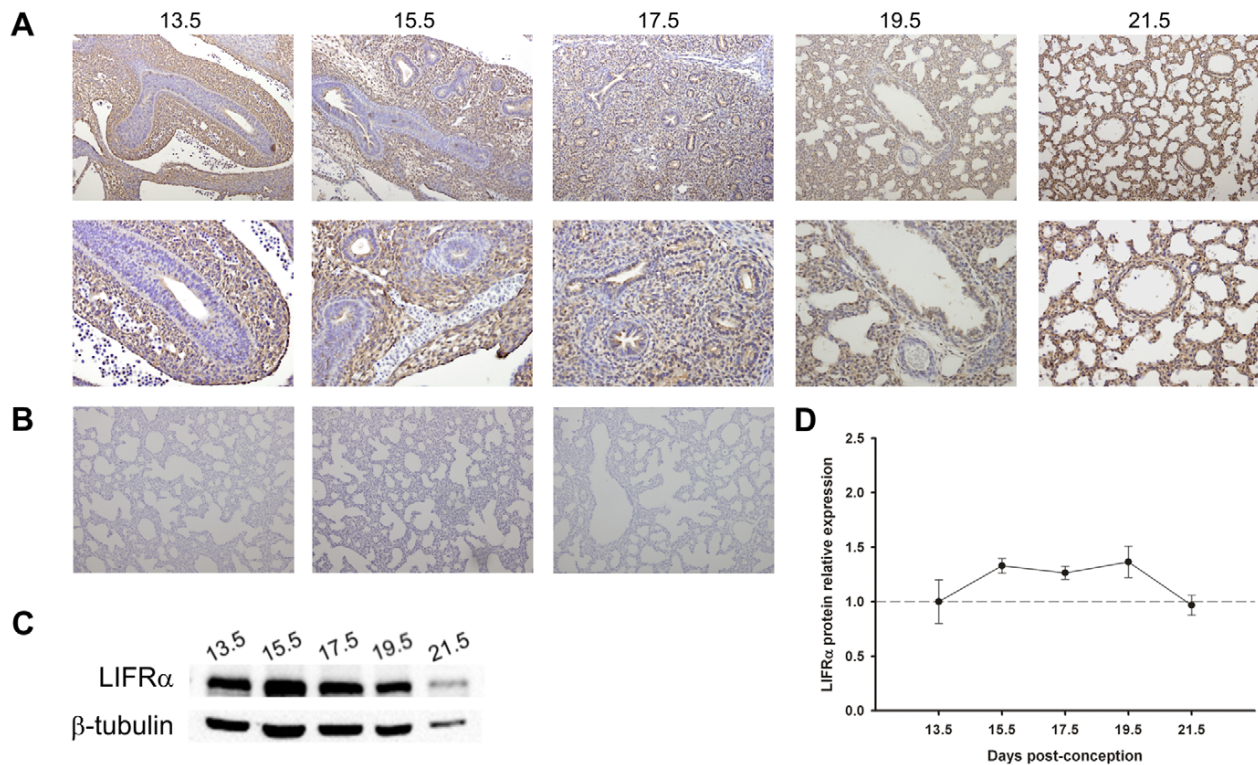doi:10.1371/journal.pone.0030517.g001

Figure 2. LIFRα expression pattern during fetal lung development (from 13.5 until 21.5 dpc). (A) IHC studies revealed that LIFRα was first mainly expressed in the mesenchyme, but after pseudoglandular stage it was also observed in epithelial cells throughout gestation. Original magnification: Upper panel – ×100; Bottom panel – ×200. (B) IHC negative controls: Left – omission of the primary antibody; Center – non-immune goat IgG isotype control; Right – simultaneous omission of the primary and secondary antibodies. In all negative controls immunoreactive LIFRα staining was not observed. (C) Western blot analysis of LIFRα throughout the gestation (190 kDa). Control loading was performed using β-tubulin (55 kDa). (D) Relative LIFRα protein levels expressed in arbitrary units normalized for β-tubulin. No significant difference was observed between gestational ages.
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tested, this effect is most significant in the highest LIF concentrations studied, 20 and 40 ng/mL.

In Figure 4 it is shown the morphometric analysis of lung explants treated with control IgG, anti-LIF IgG antibodies or FGF-10. LIF inhibition significantly stimulated lung branching (Figure 4B) and epithelial perimeter (Figure 4C) in a similar way as FGF-10. No differences were observed on lung explant area (Figure 4D) or external perimeter (Figure 4E).

Pooled samples of lung explants (n = 15) treated with LIF at 40 ng/mL (selected due to its maximal effect on inhibition of explants growth) were evaluated for modulation of MAPK, Akt and STAT3 pathways (Figure 5). Lung growth inhibition induced by LIF significantly stimulated p44/42 phosphorylation (Figure 5). On the other hand, the increase on lung branching induced by blocking LIF action significantly stimulated p38 and Akt phosphorylation (Figure 6).

Discussion

This study demonstrated that LIF was constitutively expressed in pulmonary epithelium, whereas its subunit receptor, LIFRα, was expressed in the mesenchyme until the pseudoglandular stage and in epithelial cells, at later stages. Moreover, LIF supplementation significantly inhibited lung growth most likely through p44/42 pathway. On the other hand, LIF inhibition significantly

increased lung branching and stimulated p38 and Akt phosphorylation.

LIF is a multifunctional glycoprotein cytokine that regulates many cellular responses such as proliferation, differentiation and survival in different cellular types [8–13]. Regarding the lung, several studies have investigated LIF's role in the pathophysiology of different lung diseases, such as acute respiratory distress syndrome [23], asthma [24], chronic airway inflammation [25] and inflammation induced by tobacco [23]. In fact, LIF is well known to induce the synthesis and activation of phospholipase A_2 in bronchial epithelial cells [12] and it has protective effects in the setting of hyperoxic lung injury [26]. Concerning LIF's putative role during prenatal lung period, all previous studies had focused mainly in maturation processes.

In the current study, it was demonstrated that LIF was expressed in primitive lung epithelium, since early stages of lung development (13.5 dpc), a moment in which predominantly occurs growth phenomena. LIF expression in bronchiolar and also in alveolar epithelium is in agreement with previous findings documented in adult human lung [25,27,28]. In adults, epithelial expression was also observed in other branching organs such as mammary gland [29] and pancreas [20]. During murine fetal development, LIF expression was already described in the ureteric bud [15], skin, skeletal muscle, heart, brain, liver and gut [30,31] supporting a role for this cytokine in the normal development of
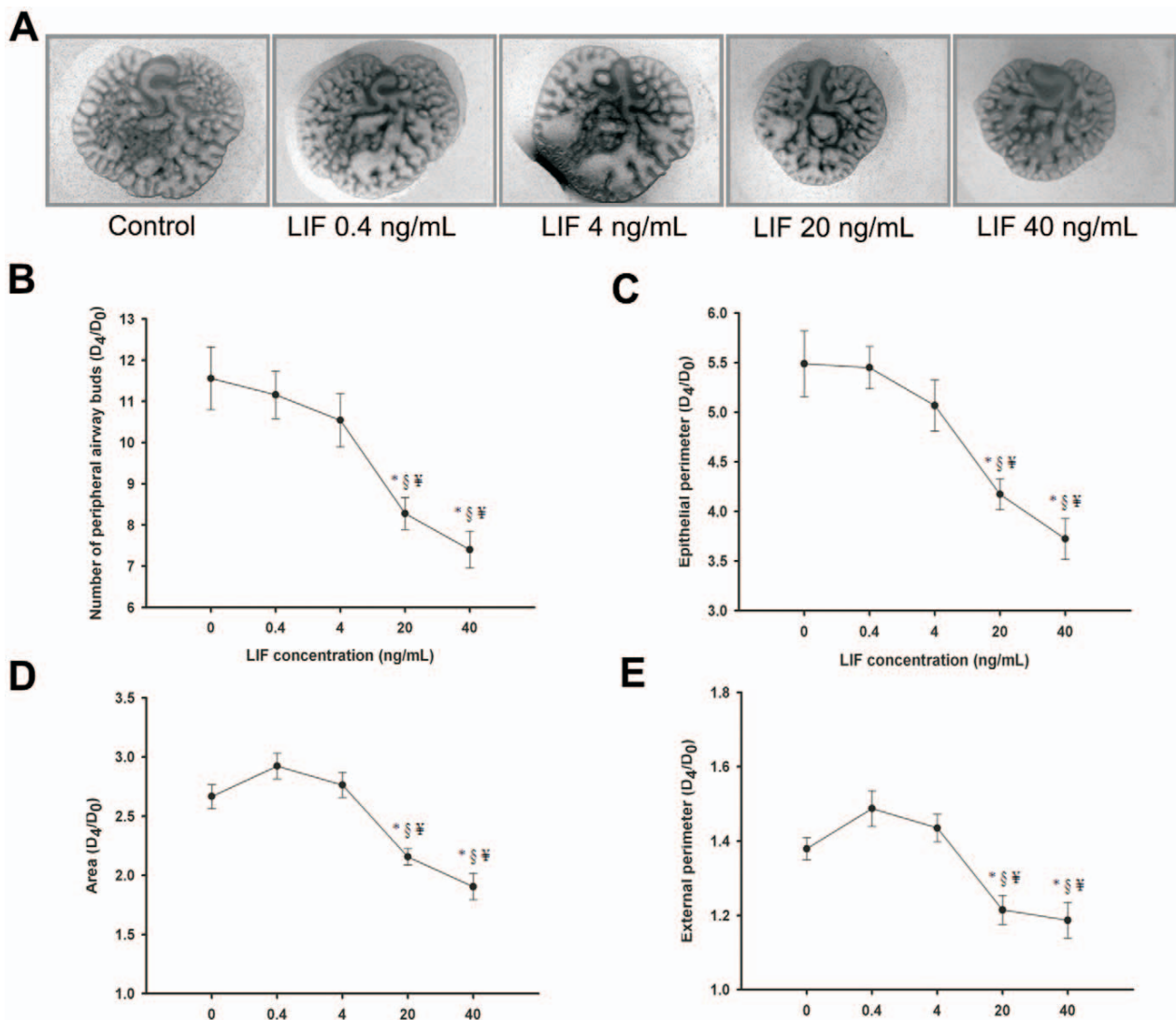
Figure 3. LIF supplementation studies in a fetal lung explant culture system. (A) Representative examples of fetal lung explants treated daily with increasing concentrations of recombinant LIF, after 4 days in culture. Original magnification ×25. (B) Number of total airway buds; (C) Epithelial perimeter; (D) Area; (E) External perimeter of lung explants treated with LIF. LIF significantly inhibited lung growth. Results are expressed as ratio of day 4 (D_4) and day 0 (D_0) of culture (D_4/D_0 ratio). $p < 0.05$: * vs. LIF at 0 ng/mL (control), § vs. LIF at 0.4 ng/mL, ¥ vs. LIF at 4 ng/mL.
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several organ systems. Moreover, LIF expression was observed in all studied gestational ages and its expression levels increased by the end of the gestation, which is in accordance with the literature [31].

Regarding LIFRα, it was expressed in constant levels throughout gestation: first mainly in lung mesenchyme, and after pseudoglandular stage it was predominantly observed in the epithelium until term. LIFRα is known to be expressed in numerous human lung structural cell types, namely fibroblasts, bronchial smooth muscle cells and airway epithelial cells [25,27,28]. Interestingly, during fetal kidney morphogenesis, LIFRα was first detected in metanephric mesenchyme and later in newly formed tubules [15]. It is necessary to stress that the occurrence of epithelial LIF and mesenchymal LIFRα expression, since early stages of lung development and until the end of pseudoglandular stage, points to a LIF epithelium-mesenchyme

interaction during this stage of lung development, which is mainly characterized by successive branching phenomena. It is well described that lung development occurs through specific cross-talk interactions between epithelium and mesenchyme [1]. Therefore, LIF and LIFRα interaction might possibly be a new player on lung branching mechanisms. Nonetheless, the highest LIF expression was observed at the end of the gestation which also suggests a physiological role for LIF in lung maturation, as already demonstrated in literature [21,22].

In order to evaluate LIF's role in lung branching morphogenesis, fetal lung explants were cultured with increasing doses of LIF or LIF neutralizing antibodies. LIF supplementation inhibited lung growth, whereas LIF blockage stimulated lung branching. Interestingly, the blockage of LIF activity increased the number of peripheral airways buds and epithelial perimeter in a similar way as FGF-10, a classical and very important lung growth factor that
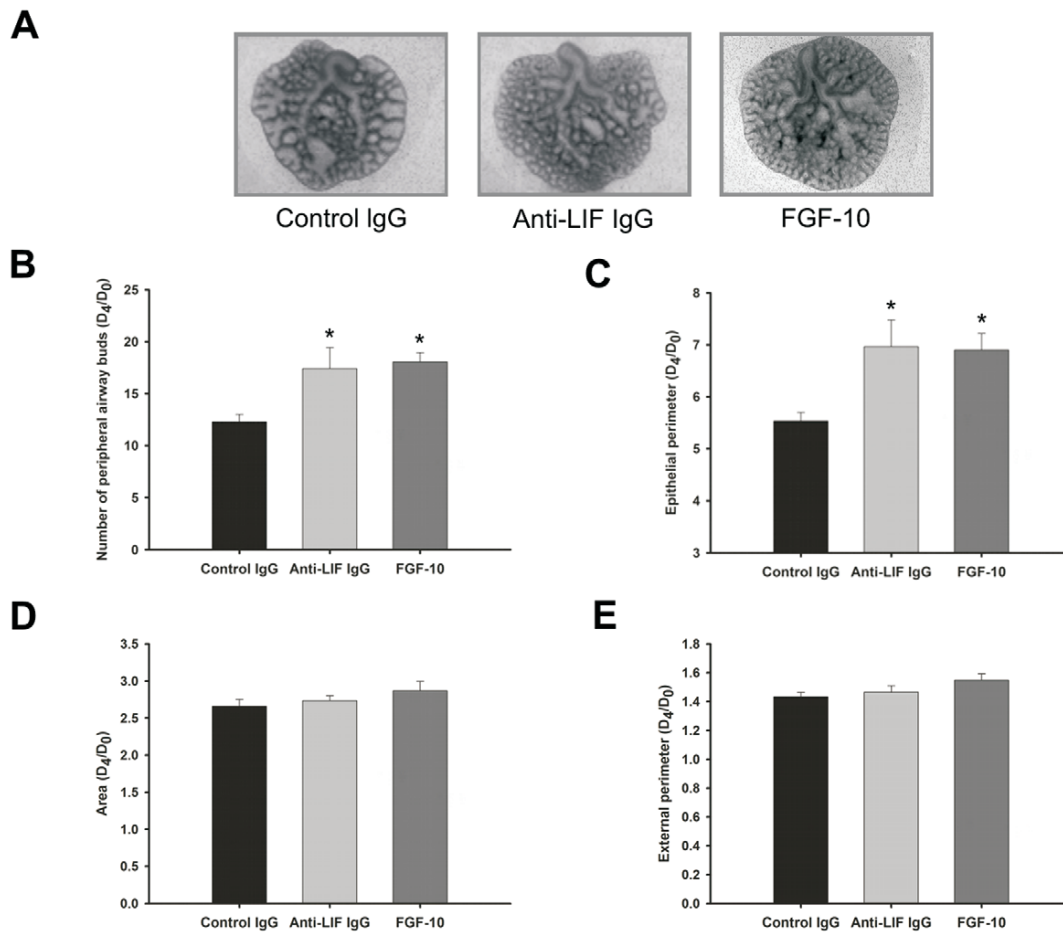
Figure 4. LIF neutralizing studies in a fetal lung explant culture system. (A) Representative examples of lung explants treated daily with normal IgG (control; 1 μg/mL), anti-LIF IgG (1 μg/mL) or FGF-10 (500 ng/mL), after 4 days in culture. Original magnification ×25. (B) Number of total airway buds; (C) Epithelial perimeter; (D) Area; (E) External perimeter of treated lung explants. Inhibition of LIF action significantly stimulated lung branching in a similar way than FGF-10. Results are expressed as D_4/D_0 ratio. $p < 0.05$: * vs. control IgG.
doi:10.1371/journal.pone.0030517.g004

is known to increase lung branching [32,33]. LIF inhibitory effect had already been described in fetal kidney development [16] and in the proliferation of human normal and malign breast epithelial cells [34] which, like the lung, develop by branching events. Moreover, LIF inhibitory effect was also described on other cell types such as embryonic stem cells [35], bone [36], endothelial [11] and neuronal cells lines [37]. However, LIF stimulates duct cell proliferation in adult pancreas [18]. Interestingly, this LIF inhibitory effect on fetal lung branching opposes the stimulatory effect described for IL-6 [3], also a member of family of the gp130-type cytokines.

Although this *in vitro* study clearly suggests a physiological role for LIF on lung branching, LIF knockout mice do not seem to have abnormal lung features when compared to normal littermates [21,22]. Indeed, LIF knockout mice are born normal and they only exhibit defective blastocyst implantation, postnatal growth retardation and hematopoiesis defects [22,38,39]. The absence of significant lung development abnormalities in LIF knockout mice does not mean a minor role for LIF during lung morphogenesis *in vivo*. In fact, as demonstrated for several phenomena, mice lacking individual members of gp130-type cytokines displayed milder

phenotype than expected, which is most likely due to the similarity and redundancy of downstream events induced by gp130-dependent cytokines [38]. On the other hand, LIFRα deletion causes perinatal death with multiple defects [22,38,40–42]. However, it is necessary to stress that this receptor subunit is a part of the receptor complexes for LIF, CNTF and CT-1 [8]. Moreover, it is necessary to stress that it is recognized that the knockout approach also bears intrinsic problems, since the ensuing phenotype may consist of compensatory events during development and later life [43]. In opposition to LIF-deficient lungs, the additional absence of LIF in the IGF-I-null background of mutant mice aggravates the prenatal pulmonary immature phenotype and these mice die due to respiratory failure [21,22]. These results point to a possible LIF role on lung maturation, but only involving also IGF-I. Furthermore, lung development is a complex process involving two different cellular phenomena, maturation and growth [1], and in the context of present work LIF's involvement was studied with regard to the growth phenomena.

In different cells and tissues, LIF signaling is mediated mainly by JAK/STAT3, p44/42 kinase and PI3K/Akt pathways [7–10,17]. Interestingly, many of the effectors that modulate fetal lung
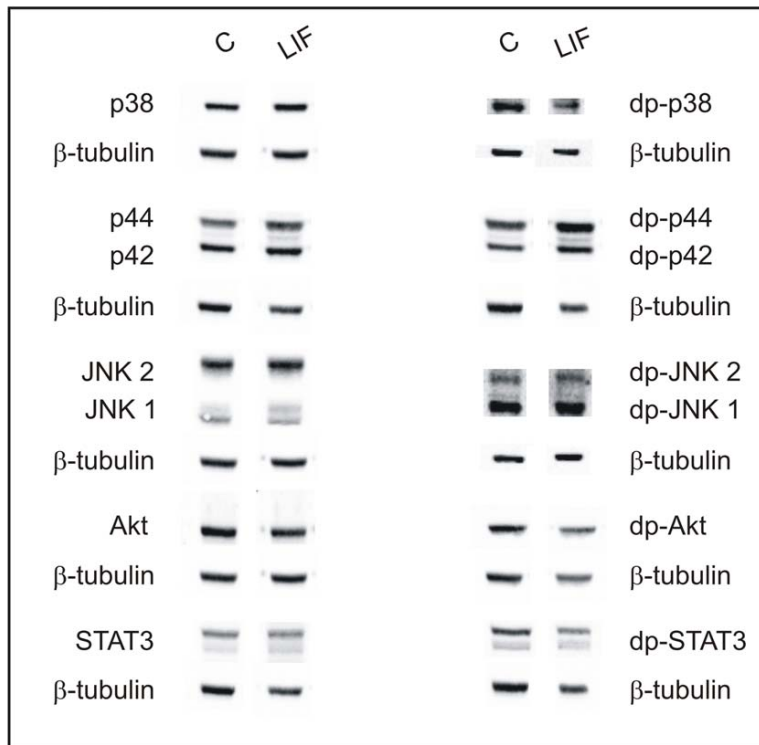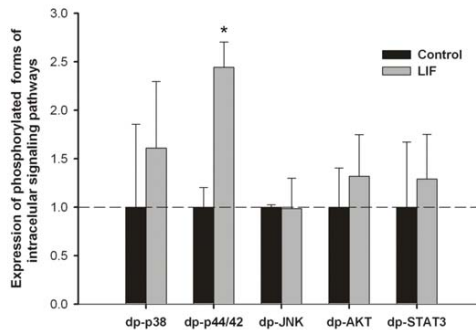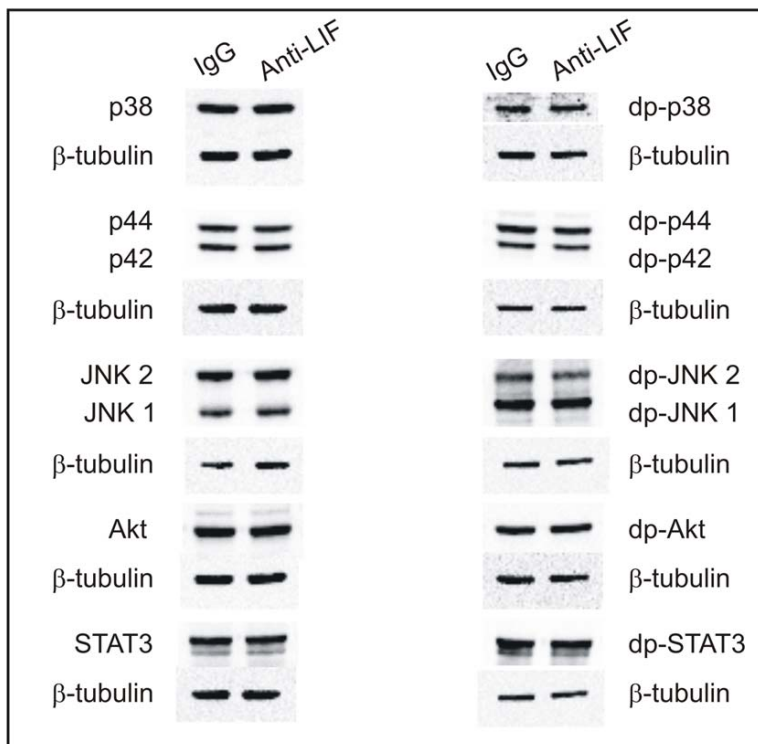
Figure 5. Analysis of intracellular signaling pathways that mediates LIF actions on lung growth. (A) Western blot analysis of p38, p44/42, JNK1/2, Akt and STAT3, and to diphosphorylated forms of p38 (dp-p38), p44/42 (dp-p44/42), SAPK/JNK (dp-JNK1/2), Akt (dp-Akt) and STAT3 (dp-STAT3) in control lung explants (C) and treated with LIF at 40 ng/mL (LIF). Control loading was performed using β-tubulin (55 kDa). p38 corresponds to 38 kDa. p44/42 correspond to 44 and 42 kDa, respectively. JNK1 and 2 correspond to 46 and 54 kDa, respectively. Akt corresponds to 60 kDa. STAT3 corresponds to two bands, 79 and 86 kDa. (B) Semi-quantitative analysis of expression of phosphorylated forms of these intracellular signaling pathways. Results are presented as arbitrary units normalized for β-tubulin. $p < 0.05$: * vs. control.
doi:10.1371/journal.pone.0030517.g005

branching morphogenesis seem to activate signaling pathways that converge into the MAPK and PI3K/Akt cascades [44]. Thus, MAPK, PI3K/Akt and STAT3 pathway modulation by LIF and anti-LIF in fetal lung development was investigated. The inhibition of lung growth induced by LIF significantly stimulated p44/42 phosphorylation and did not significantly change p38, JNK, Akt or STAT3 pathways. In contrast to these results, in lung development, the p44/42 pathway has been involved in stimulation of branching morphogenesis. Indeed, Kling *et al* demonstrated that p44/42 inhibition reduces branching morphogenesis and causes mesenchymal cell apoptosis in fetal rat lungs

[44]. According to the literature, it is not the first association between LIF, p44/42 pathway and growth inhibition/arrest. In fact, in human medullary thyroid cancer cells, it was demonstrated that p44/42 pathway induces autocrine-paracrine growth inhibition via LIF [19]. Moreover, the absence of LIF in the IGF-1 null mice, which induces changes on pulmonary maturation and vasculogenesis, also induces activation of p44/42 [21]. Furthermore, the LIF action mediated by p44/42 was already demonstrated in earlier mammary gland development [17] and myoblasts [45]. Thus, considering that lung explants were incubated during 4 days, p44/42 stimulation might not be the
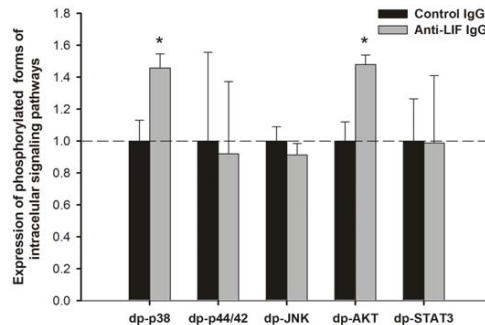
Figure 6. Analysis of intracellular signaling pathways that mediates anti-LIF actions on lung branching. (A) Western blot analysis of p38, p44/42, JNK1/2, Akt and STAT3, and to diphosphorylated forms of p38 (dp-p38), p44/42 (dp-p44/42), SAPK/JNK (dp-JNK1/2), Akt (dp-Akt) and STAT3 (dp-STAT3) in control IgG lung explants (IgG) and treated with anti-LIF IgG (Anti-LIF). Control loading was performed using β-tubulin (55 kDa). (B) Semi-quantitative analysis of expression of phosphorylated forms of these intracellular signaling pathways. Results are presented as arbitrary units normalized for β-tubulin. $p < 0.05$: * vs. IgG.
doi:10.1371/journal.pone.0030517.g006

direct pathway that mediates LIF action, but the result of crosstalks of other non-canonical intracellular pathway. Regarding anti-LIF treatment, the increase on lung branching observed was mediated by stimulation of p38 and Akt phosphorylation. PI3K/Akt pathway is a classical stimulator of lung branching [44,46]. Moreover, p38 was already demonstrated as mediator of lung branching [3,47]. Interestingly, it was demonstrated that IL-6, another a gp130-type cytokines, also enhances lung branching via p38 phosphorylation [3].

In this study, it was demonstrated fetal LIF inhibits lung branching, in opposition to IL-6 (another cytokine of the gp130 family). Although cytokines of the gp130 family share a common

signal transducer, these findings suggest specific biological activities for each cytokine on lung development. In fact, specific characteristics are emerging for each member of this family, brought about mainly through restricted temporal and spatial release, differential expression of cell surface receptors, and different signaling patterns between gp130 homodimers and heterodimers [25]. Interestingly, LIF receptor is a gp130 heterodimer, whereas IL-6 receptor is a gp130 homodimer. Thus, this raises the hypothesis that the effect, on lung development, of cytokine signaling through gp130 heterodimers might be different and even opposite to gp130 homodimers. Although this hypothesis needs to be verified, this specificity can possibly represent a

regulatory mechanism of lung morphogenesis, intrinsic to this family of cytokines, in order to achieve the correct lung growth.

In conclusion, LIF and LIFRα expression during fetal lung development as well as the *in vitro* studies presented in this work suggest an inhibitory physiological role for endogenous LIF, mediated by epithelial-mesenchymal interactions, on pulmonary branching mechanisms.

Materials and Methods

This study was carried out in strict accordance with the recommendations in the 'Guide for the Care and Use of Laboratory Animals', published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). Animal experiments were also performed according to the Portuguese law for animal welfare and the protocol was approved by the Committee on the Ethics of Animal Experiments of the Life and Health Sciences Research Institute of the University of Minho (DGV 022162 - 520/000/000/2006). Moreover, all efforts were made to minimize animal suffering.

Animal model and experimental design

Sprague-Dawley female rats (225 g; Charles-River, Spain) were maintained in appropriate cages under temperature-controlled room (22–23°C) on a 12 hours light: 12 hours dark cycle, and fed with commercial solid food. The rats were mated and checked daily for vaginal plug. The day of plugging was defined as gestational day 0.5 for time dating purposes. Fetuses were removed by caesarean section at 13.5, 15.5, 17.5, 19.5 and 21.5 dpc (days post-conception) and sacrificed by decapitation. Lungs were dissected and processed for IHC and western blot analysis. To perform fetal lung explant cultures fetuses were harvested at 13.5 dpc and their lungs dissected.

IHC

LIF and LIFRα immunostainings were performed on formalin-fixed and paraffin-embedded lungs of different gestational ages (13.5–21.5dpc). Sections (5 μm) were placed on SuperFrost®Plus slides (Menzel-Glaser, Germany). LIF antibody (sc-1336; Santa Cruz Biotechnology Inc., USA) was used in a 1:50 dilution and LIFRα antibody (sc-659; Santa Cruz Biotechnology Inc.) in a 1:50 dilution. After dewaxing in xylene and rehydration in ethanol, the samples were incubated in 3% hydrogen peroxide in methanol to quench endogenous peroxidase. Antigen retrieval was achieved by boiling in 10 mM citrate buffer followed by cool down at room temperature. Samples were blocked with 5% BSA (Roche, Germany). Incubation of the primary antibody occurred at 4°C overnight. Negative control reactions included omission of the primary antibody, the simultaneous omission of the primary and secondary antibodies, and a non-immune goat IgG isotype control diluted to a matching concentration as the primary antibody, instead of the primary antibody. Incubation with the goat ImmunoCruz™ Staining System (sc-2053; Santa Cruz Biotechnology Inc.) or with the UltraVision detection system anti-polyvalent horseradish peroxidase (Lab Vision Corporation, USA) was carried according to manufacturer's instructions (for LIF and LIFR immunostainings, respectively). To visualize the peroxidase activities in sections, diaminobenzidine tetrahydrochloride (Dako, Denmark) was used. Sections were counterstained with hematoxyline. The slides were observed and photographed with Olympus BX61 microscope (Olympus, Japan). The pictures presented are representative of 6 animals (n = 6) and 12 samples for each gestational age, and three independent experiments were performed.

Western blot analysis

Different pooled lung samples for each gestational age (13.5–21.5 dpc) and cultured lung explants were processed for western blot analysis. Proteins were obtained according to Kling *et al* [44]. Ten or twenty five μg of protein were loaded onto 10% or 7.5% acrylamide minigels (respectively), electrophoresed at 100 V at room temperature and then transferred to nitrocellulose membranes (Hybond™ -C Extra, GE Healthcare Life Sciences, UK). Blots were probed with LIF and LIFRα polyclonal antibodies (Santa Cruz Biotechnology Inc.) according to manufacturer's instructions (1:250 and 1:500, respectively). For loading control, blots were probed with β-tubulin MAb (1:200000, Abcam, UK). Afterwards blots were incubated with a secondary horseradish peroxidase conjugate (Santa Cruz Biotechnology Inc.), developed with Super Signal®West Femto Substrate (Pierce Biotechnology, USA) and the chemiluminescent signal was captured using the Chemidoc XRS (Bio-Rad, USA). Quantitative analysis was performed with Quantity One 4.6.5 1-D Analysis Software (Bio-Rad). Three independent experiments were performed (n = 3).

Fetal lung explant cultures

Harvesting and dissection of 13.5 dpc lungs was made in DPBS (Lonza, Switzerland) under a dissection microscope (Leica MZFLIII, Switzerland). The lungs were transferred to Nucleopore membranes with an 8 μm pore size (Whatman, USA) and incubated in a 24-well culture plates (Nunc, Denmark). The membranes were pre-soaked in DMEM (Invitrogen, UK) for 1 hour before the explants were placed on them. Floating cultures of the explants were incubated in 200 μl of 50% DMEM, 50% nutrient mixture F-12 (Gibco, USA) supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin (Gibco), 0.25 mg/mL ascorbic acid (Sigma-Aldrich, USA) and 10% FCS (Gibco). The fetal lung explants were incubated in a 5% CO_2 incubator at 37°C for 96 hours, and the medium was replaced every 48 hours. The branching morphogenesis was monitored daily by photographing the explants. At day 0 (D_0: 0 hours) and day 4 (D_4: 96 hours) of culture, the total number of peripheral airway buds (branching) in all lung explants was determined, whereas the explants area, epithelial perimeter and external perimeter were measured using AxionVision Rel. 4.3 (Carl Zeiss, Germany). These results were expressed as D_4/D_0 ratio.

LIF supplementation studies

Twelve lung explants were used as control (0 ng/mL). Additionally, recombinant LIF (Sigma-Aldrich) was daily added to lung explants in order to achieve a final concentration of 0.4, 4, 20 and 40 ng/mL (0.4 n = 15; 4 n = 12; 20 n = 12; 40 n = 15).

After 4 days in culture, control and LIF treated lung explants (at 40 ng/mL) were processed for western blot analysis of non-phosphorylated and phosphorylated forms of p38, p44/42, JNK, Akt and STAT3 (Cell Signaling Technology Inc., USA) according to the method described above. Four independent experiments were performed (n = 4).

LIF neutralizing studies

Lung explants were treated daily with IgG anti-LIF neutralizing antibody (n = 12) at concentration of 1.0 μg/mL (R&D Systems, USA). Non-specific effects were evaluated by daily addition of identical concentration of normal IgG control antibody (n = 9; R&D Systems). Moreover, lung explants were also treated with FGF-10 that was added daily to the culture medium at a concentration of 500 ng/mL (n = 9; R&D Systems).

After 4 days in culture, normal IgG and anti-LIF IgG treated lung explants were processed for western blot analysis of the intracellular signaling pathways, as described above (n = 4).

Statistical Analysis

All quantitative data are presented as mean ± SEM. Statistical analysis was performed using the statistical software SigmaStat (version 3.5; Systat Software Inc., USA). For LIF and LIFRα expression levels evaluation and supplementation studies one-way ANOVA was used. For LIF neutralizing studies and intracellular signaling pathways analysis t-test analysis was used. The Student-Newman-Keuls test was used for post-test analysis. Statistical significance was set at $p < 0.05$.

Author Contributions

Conceived and designed the experiments: CN-S EC-D JC-P. Performed the experiments: CN-S PP EC-D FOP RSM. Analyzed the data: CN-S PP JC-P. Wrote the paper: CN-S PP RSM JC-P.

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GP130 FAMILY OF CYTOKINES

IL-6,
IL-11

LIF, CT-1,
CNTF, CLC,
OSM

HOMODIMER
RECEPTOR

HETERODIMER
RECEPTOR
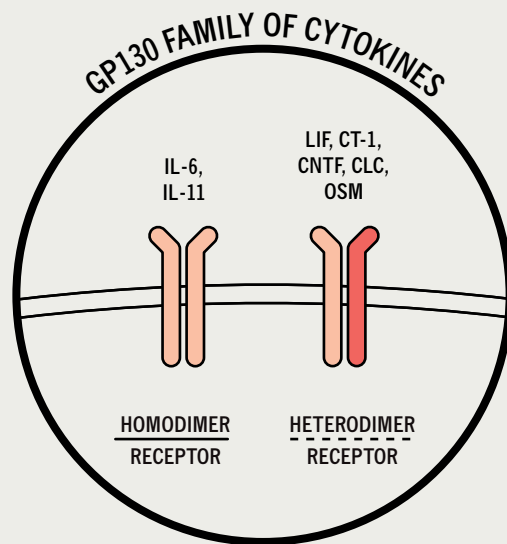
CHAPTER IV
GP130 FAMILY OF CYTOKINES AND FETAL LUNG GROWTH

Results presented in this chapter constitute a manuscript that is submitted
to a peer-reviewed journal:

The role of GP130 family of cytokines on fetal lungo development

**Cristina Nogueira-Silva, Paulina Piairo, Emanuel Carvalho-Dias,
Carla Veiga, Rute S Moura, and Jorge Correia-Pinto**

The Role of Glycoprotein 130 Family of Cytokines on fetal lung development

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Abstract

The glycoprotein 130 (gp130) dependent family of cytokines comprises interleukin-6 (IL-6), IL-11, leukemia inhibitory factor (LIF), cardiotrophin-like cytokine (CLC), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and oncostatin M (OSM). These cytokines share the membrane gp130 as a common signal transducer. Recently, it was demonstrated that IL-6 promotes, whereas LIF inhibits fetal lung branching. Thus, in this study, the effects on fetal lung morphogenesis of the other classical members of the gp130-type cytokines (IL-11, CLC, CNTF, CT-1 and OSM) were investigated. Fetal rat lung explants were cultured *in vitro* with increasing concentrations of IL-11, CLC, CNTF, CT-1 and OSM. Treated lung explants were morphometrically analyzed and assessed for MAPK, PI3K/AKT and STAT3 signaling modifications. IL-11, which similarly to IL-6 acts through a gp130 homodimer receptor, significantly stimulated lung growth via p38 phosphorylation. On the other hand, CLC, CNTF, CT-1 and OSM, whose receptors are gp130 heterodimers, inhibited lung growth acting in different signal-transducing pathways. Thus, the present study demonstrated that although cytokines of the gp130 family share a common signal transducer, there are specific biological activities for each cytokine on lung development. Indeed, cytokine signaling through gp130 homodimers stimulate, whereas cytokine signaling through gp130 heterodimers inhibit lung branching.

Keywords: cardiotrophin-1; cardiotrophin-like cytokine; ciliary neurotrophic factor; fetal lung development; glycoprotein 130; interleukin-11; oncostatin M.

Introduction

Normal lung development is particularly dependent on tightly regulated signaling networks, triggered by both its classically known effectors, such as growth factors, extracellular matrix molecules and hormones, and by its recently implicated regulatory factors like inflammatory cytokines (1-4).

The glycoprotein 130 (gp130) dependent family of cytokines or interleukin 6 (IL-6) family of cytokines is quite a large group of structurally related cytokines that includes IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), and oncostatin M (OSM) (5, 6). Other family members have recently emerged (IL-27 and neuropoietin), thus it is likely that the currently defined gp130 cytokine family is not complete (5, 7, 8). These small proteins are grouped in same family, since all signal through a common signal transducing receptor chain, the gp130. However, each cytokine interacts with a specific receptor that is a complex of receptor subunits. Thus, the multimeric receptor complex for gp130 family of cytokines consists of (i) gp130 homodimers with a ligand-specific α chain for IL-6 and IL-11; (ii) gp130 heterodimers (gp130/LIFR and gp130/OSMR) without specific α chain for LIF and OSM; (iii) or gp130 heterodimers with a ligand-specific α chain (CNTFRα) for CNTF and CLC or α chain-like for CT-1 (5-7). Until the moment, the α chain recruited by CT-1 has not been characterized (5).

The gp130 cytokine receptors signal directly through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, particularly STAT3 and STAT1 (6, 9). Alternatively, gp130 cytokine family can also initiate cell signaling via other signaling pathways, including the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K/Akt) cascades (6, 10, 11). Through these pathways, gp130 cytokine signaling activates target genes involved in several cellular responses namely, cell differentiation, survival, apoptosis and proliferation.

Adding to their reputation as classical regulators of immune response and inflammation, these cytokines are also well known for their regulatory role in diverse biological processes including, hematopoiesis, mammalian fertility, liver and neuronal regeneration, myocardial development, pituitary proliferation, bone homeostasis, adipocyte differentiation and function, and embryonic development (5, 6, 10-12). Concerning lung development, IL-6 was demonstrated to have an enhancing effect on lung explant growth and proved to be an important regulator of normal lung growth, whereas in opposition to IL-6, LIF was found to inhibit lung branching (3, 4,

13). Such evidences lead us to speculate that other members of gp130 family of cytokines might be involved on normal lung development. Moreover, these cytokines present some functional redundancy, even though they also exhibit specific biological activities (6, 11). Therefore, we proposed to investigate the role of other gp130 family of cytokines on fetal lung growth.

Materials and methods

This study was carried out in strict accordance with the recommendations in the 'Guide for the Care and Use of Laboratory Animals', published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). Animal experiments were also performed according to the Portuguese law for animal welfare and the protocol was approved by the Committee on the Ethics of Animal Experiments of the Life and Health Sciences Research Institute of the University of Minho (DGV 022162 - 520/000/000/2006). Moreover, all efforts were made to minimize animal suffering.

Animal model and experimental design

Sprague-Dawley female rats (225 g; Charles-River, Spain) were maintained in appropriate cages under temperature-controlled room (22–23°C) on 12 hours light: 12 hours dark cycle, and fed with commercial solid food. The rats were mated and checked daily for vaginal plug. The day of plugging was defined as gestational day 0.5 for time dating purposes. Fetuses were removed by caesarean section at 13.5 dpc (days post-conception), sacrificed by decapitation and their lungs dissected for fetal lung explant cultures.

Fetal lung explant cultures

Harvesting and dissection of 13.5 dpc lungs was made in DPBS (Lonza, Switzerland) under a dissection microscope (Leica MZFLIII, Switzerland). The lungs were transferred to Nucleopore membranes with an 8 m pore size (Whatman, USA), previously presoaked in DMEM (Invitrogen, UK) for 1 hour, and incubated in a 24-well culture plates (Nunc, Denmark). Floating cultures of the explants were incubated in 200 L of 50% DMEM, 50% nutrient mixture F-12 (Gibco, USA) supplemented with 100g/mL streptomycin, 100 units/mL penicillin (Gibco), 0.25 mg/mL ascorbic acid (Sigma-Aldrich, USA) and 10% FCS (Gibco). The fetal lung explants were incubated in a 5% CO_2 incubator at 37°C for 96

hours, and the medium was replaced every 48 hours. The branching morphogenesis was monitored daily by photographing the explants. At day 0 (D0: 0 hours) and day 4 (D4: 96 hours) of culture, the total number of peripheral airway buds (branching) in all lung explants was determined, whereas the explants area, epithelial perimeter and external perimeter were measured using AxionVision Rel. 4.3 (Carl Zeiss, Germany). These results were expressed as D4/D0 ratio.

IL-11, CLC, CNTF, CT-1 and OSM supplementation studies

In vitro cultures were daily supplemented with several doses of recombinant IL-11 (0.1; 1; 10; 100 pg/mL), CLC (0.003; 0.03; 0.3; 3; 30 nM), CNTF (0.1; 1; 10; 100; 1000 ng/mL), CT-1 (0.1; 1; 10; 100; 200 ng/mL), and OSM (0.1; 1; 10; 100 ng/mL). All recombinant proteins were purchased from R&D Systems, USA. Per each tested dose at least nine, often more, fetal lung explants were used, likewise twelve lung explants were used as control. For CLC and CT-1 supplementation studies the control explants were supplemented with 4 mM of sterile HCL (according to manufacturer's instructions of recombinant proteins reconstitution).

After 4 days in culture, control and cytokine treated lung explants at selected concentrations (IL-11 at 0.1 pg/mL; CLC at 30 nM; CNTF at 1000 ng/mL; CT-1 at 200 ng/mL; OSM at 100 ng/mL) were processed for western blot analysis of nonphosphorylated and phosphorylated forms of p38, p44/42, JNK, Akt and STAT3 (Cell Signaling Technology Inc., USA) according to the method described below.

Western blot analysis

Pooled samples of the cultured lung explants were processed for western blot analysis. Proteins were obtained according to Kling et al (14). Ten g of protein were loaded onto 10% acrylamide minigels, electrophoresed at 100 V at room temperature and then transferred to nitrocellulose membranes (HybondTM -C Extra, GE Healthcare Life Sciences, UK). Blots were probed with antibodies to non-phosporylated and phosporylated forms of p38, p44/42 (ERK1/2), JNK, Akt and STAT3 (1:1000; Cell Signaling Technology Inc., USA) according to the manufacturer's instructions. For loading control, blots were probed with β-tubulin (1:150000, Abcam, UK). Afterwards blots were incubated with a secondary horseradish peroxidase conjugate (Santa Cruz Biotechnology Inc.), developed with Super SignalHWest Femto Substrate (Pierce Biotechnology, USA) and the chemiluminescent signal was captured using the Chemidoc XRS (Bio-Rad, USA).

Quantitative analysis was performed with Quantity One 4.6.5 1-D Analysis Software (Bio-Rad). Three independent experiments were performed (n = 3).

Statistical analysis

All quantitative data are presented as mean ± SEM. Statistical analysis was performed using the statistical software SigmaStat (version 3.5; Systat Software Inc., USA). For supplementation studies one-way ANOVA was used and for intracellular signaling pathways analysis t-test was used. The Student-Newman-Keuls test was used for post-test analysis. Statistical significance was set at p 0.05.

Results

Role of gp130 cytokines on fetal lung development

This study aimed to clarify the role of gp130 dependent family of cytokines on lung morphogenesis. Thus, fetal lung explants cultured *in vitro* were treated daily with increasing concentrations of recombinant IL-11, CLC, CNTF, CT-1 and OSM. In Figure 1A representative examples of fetal lung explants treated with increasing IL-11 concentrations, after 4 days in culture, are illustrated. IL-11 appears to have an enhancing effect on lung explants growth which is maximal at the lowest concentration tested, 0.1 pg/mL. In fact, an increase in the total number of peripheral airway buds (Figure 1B), epithelial perimeter (Figure 1C), area (Figure 1D) and external perimeter (Figure 1E) of lung explants was observed in all concentrations tested, except the highest, 100 pg/mL. In all the above mentioned morphometric parameters, explants treated with the highest dose presented the most similar effect to the control explants. In detail, in fetal lung explants increasing doses of IL-11, induced a biphasic effect in all the morphometric parameters assessed, generally the lowest dose of IL-11 enhanced explant growth, whereas increasingly high doses gradually reduced explant growth when compared to the maximal effect observed.

Regarding the role of CLC in fetal lung growth, in Figure 2A representative examples of lung explants treated with increasing CLC concentrations, after 4 days in culture, are illustrated. CLC appears to have a dose-effect inhibitory action on lung explants growth. In fact, a decrease in the total number of peripheral airway buds (Figure 2B), epithelial perimeter (Figure 2C), area (Figure 2D) and external perimeter (Figure 2E) of lung explants was observed in all concentrations tested, and this effect is most significant in the highest CLC concentration studied, 30nM.

In Figure 3A, representative examples of fetal lung explants treated with increasing CNTF concentrations, after 4 days in culture, are illustrated. CNTF have an inhibitory action on lung explants growth, with the maximal effect induced by the highest CNTF concentration studied, 1000 ng/mL.

Concerning CT-1, CT-1 appears to have a dose-effect inhibitory action on lung explants growth, as illustrated in figure 4A. In fact, a decrease in the total number of peripheral airway buds (Figure 4B), epithelial perimeter (Figure 4C), area (Figure 4D) and external perimeter (Figure 4E) of lung explants was observed in all concentrations tested, this effect is most significant in the highest CT-1 concentration studied, 200 ng/mL.

As illustrated in figure 5A, OSM appears to have an inhibitory effect on lung branching. In fact, OSM at 100 ng/mL induces a significant decrease in the total number of peripheral airway buds (Figure 5B), epithelial perimeter (Figure 5C), area (Figure 5D) and external perimeter (Figure 5E) of lung explants.

Gp130 cytokines supplementation effects on intracellular signaling pathways

The receptors of gp130 cytokines directly control the activities of STAT, MAPK, and PI3K/AKT signaling pathways. In order to further investigate gp130 cytokines effects on fetal lung growth, treated lung explants were evaluated for signaling modulation of these pathways. Pooled samples of lung explants individually treated with recombinant cytokines (selected due to its maximal effect either on stimulation or inhibition of explants growth) were used to assess protein expression levels of non-phosporylated and phosphorylated forms of p38, p44/42 (ERK1/2), JNK, Akt and STAT3 by western blot (Figure 6). On one hand, lung growth stimulation induced by IL-11 significantly increased p38 phosphorylation. On the other hand,

inhibition of lung growth induced by CLC, significantly reduced JNK and AKT phosphorylation levels. Both CNTF and CT-1-induced inhibition of lung growth significantly stimulated STAT3 phosphorylation and decreased JNK phosphorylation. Additionally, CNTF treatment also induced a significant increase of AKT phosphorylation, whereas CT-1 treatment significantly increased p38 phosphorylation. OSM inhibitory effects on lung growth appear to be mediated by significantly increase of p38 and p44/42 phosphorylation, and also of AKT and STAT3 phosphorylation.

Discussion

The gp130 cytokine family collectively exhibits a broad range of physiological functions, including important roles in embryonic development. Recently, some of these cytokines, namely IL-6 and LIF, have been proposed to be mediators in fetal lung development (3-4), but otherwise little is known about the role of additional classical members of this family in the developing lung.

In order to further clarify the role of the gp130 family of cytokines in lung branching morphogenesis, *in vitro* supplementation studies were performed individually. Thus, fetal lung explants were cultured with increasing concentrations of IL-11, CLC, CNTF, CT-1 or OSM, selected according to literature (15-24). Supplementation studies showed that cytokines within the gp130 family can elicit opposite effects in lung explant growth. Such observation suggests that despite their shared use of the common receptor subunit gp130, these cytokines can generate contradictory signals in branching morphogenesis. Additionally, intracellular signaling contribution to the effects of each cytokine on fetal lung growth were investigated by assessing non-phosphorylated and phosphorylated protein expression levels of several intracellular mediators, namely p38, p44/42, JNK, Akt and STAT3. It is well-established that numerous players account for the molecular basis of cytokine action, thus unsurprisingly in fetal lung development each cytokine proved to elicit the activation of either simple or combinatory signals from different signal-transducing pathways.

In this study, it was demonstrated that IL-11 supplementation stimulates lung branching evidenced by increased number of peripheral airways buds, epithelial perimeter, area and external perimeter of fetal lung explants, whereas CLC, CNTF, CT-1 and OSM inhibit lung growth. Together with previously obtained results which demonstrated that IL-6 and LIF have opposite effects in branching morphogenesis, the first stimulated lung explant growth while the latter inhibited, the current study extensively contributes for a thorough comprehension of the role of these cytokines in the complex process of lung development.

Similarly to what was previously describe for IL-6, IL-11 supplementation stimulated fetal lung branching. Interestingly, IL-11 has been demonstrated to stimulate proliferation and differentiation of intestinal cells and prevent apoptosis of epithelial cells (25, 26). Regarding the lung, IL-11 is produced by a variety of structural cells (fibroblasts, epithelial cells, human airway smooth muscle cells) and eosinophils in response to a variety of stimuli (27, 28). Indeed, IL-11 acts as a healing cytokine in the asthmatic airway and in other tissues. Interestingly, IL-11 also provides protective effects against oxidant-mediated injury in fetal and adult lung (29, 30).

On the contrary, it was demonstrated that CLC, CNTF, CT-1 and OSM inhibit lung growth. Several evidences point towards a role of these inflammatory cytokines in varied aspects of lung physiology. Regarding CLC, during development this cytokine is expressed in lung, kidney, brain, tooth, epithelia and skeletal muscles. In the lung, CLC is expressed faintly in distal airway epithelium (31), which suggests important biological roles of CLC in cellular functions. In opposition to lung growth inhibition here demonstrated, during kidney development (an organ that just like the lung develop by branching mechanisms), CLC promotes mesenchymal to epithelial conversion and nephrogenesis (17). Concerning CNTF, it was already described that it is widely expressed in the adult brain, lung, heart, liver, kidney and testis (32). During embryonic development, CNTF is specifically expressed in rat pineal gland and eyes (33). This cytokine has been described to act as a lesion factor, preventing neuronal cell death and facilitating axonal regeneration after nerve injury (21). About CT-1, it was already demonstrated that this cytokine is expressed in adult lung, heart, liver, kidney, skeletal muscle, dorsal root ganglia, ovary, colon, prostate and testis, as well as in fetal lung, heart and kidney (5, 34-36). In opposition to inhibitory effect on fetal lung growth described in this study, CT-1 has been related with hypertrophic and cytoprotective actions (34). In fact, CT-1 has been related with chronic asthma, contributing to airway wall thickening through reduction of apoptosis rates and induction of hypertrophy of airway smooth muscle cells (36). Finally, during fetal life, OSM has been described to be expressed only in hematopoietic tissues, choroid plexus and limb (37). After birth, it is detectable in other organs, such as lymph node, bone, heart, kidney, small intestine, skin, nasal cavity, and lung (38, 39). In the lung, OSM is detected in alveolar and bronchiolar epithelium (38). Interestingly, OSM overexpression is deleterious during mouse development (9). Moreover, OSM is a potent mediator of lung inflammation and extracellular matrix accumulation, and is up-regulated in patients with idiopathic pulmonary fibrosis and scleroderma (40).

Our findings on this dual contribution of gp130 family of cytokines, with inductive and suppressive actions in lung growth, clearly suggest a regulatory role in fetal lung development. Previously stressing a role for gp130 signaling during embryo development are many studies of transgenic and knockout mice for different components of this cytokine family or their receptors, which report defects in bone and neurologic development, disrupted placental architecture, hypoplastic development and a decrease in fetal liver hematopoiesis. The most severe phenotypes are displayed by mice lacking receptor components used by several members of the gp130 family. In fact, gp130 or LIFR knockout mice die during development or shortly after birth (11, 41,

42). Investigation of the lungs, in conditional gp130-mutant mice, revealed pulmonary defects and development of emphysema with increasing age (42). Moreover,

mice with a lung epithelial cell-specific deletion in gp130 or STAT3 were unable to restore cell shape and cell density after pulmonary injury, which highlights an important role for gp130 signaling in normal repair of the bronchiolar epithelium following injury (41, 44).

In the present study, it was revealed that individual gp130-type cytokines can both enhance or inhibit fetal lung growth. Besides, providing specificity for individual cytokines in fetal lung development, these data underlines that cytokines operating through gp130 homodimers may induce different and even opposite biological responses than those operating through gp130 heterodimers. Both IL-6 and IL-11 receptors are gp130 homodimers and stimulate lung growth, whereas all the other receptors for this family of cytokines are gp130 heterodimers and inhibit lung growth. Thus, the main findings of this study stress the composition of these signaling receptor complexes as an important mechanism to acquire signaling specificity from pleiotropic-acting cytokines in lung development. This is in agreement with the well-documented fact that such cytokines with pleiotropic activities can also retain tissue-specific activities. In fact, several mechanisms can be accountable for generating and limiting those responses, specifically: cytokine restricted temporal and spatial release, differential expression of cell surface receptors and different signaling pattern between gp130 homodimers and heterodimers (45). In lung branching morphogenesis, our observations clearly support a specific and contrasting physiological response obtained from cytokines acting on gp130 homomeric receptors versus gp130 heteromeric receptors.

Cytokine signaling on a developing lung cell-specific context triggers diverging and non-overlapping intracellular signaling cascades. For instance, IL-11 stimulating effect on lung growth was associated with an increase in p38 phosphorylation.

Interestingly, the stimulation of lung growth induced by IL-6 was also previously reported to be associated with increased p38 activation (4). In the case of gp130 cytokines that exert an inhibitory effect in lung explant growth, diverse and combinatory intracellular signals are more frequent. Clearly this study emphasizes that also in lung branching morphogenesis, gp130 cytokine receptor activation is a rather complex means of initiation of signal transduction that leads to numerous possible signaling patterns able to elicit a similar biological outcome. CLC induced lung growth inhibition and concomitantly a decrease in the activation of JNK and AKT. Both CNTF and CT-1-induced lung growth inhibition is associated with activation of STAT3 and

decreased JNK phosphorylation. CNTF additionally activates PI3K/AKT cascade whereas CT-1 activates p38. OSM inhibition of lung growth demonstrated to activate PI3K/AKT, different MAPK signaling pathways (p38 and p44/42) and also STAT3. Collectively, these results suggest that integration of the activities transduced by multiple pathways might ultimately provide a balanced biological outcome intended to respond to a particular physiological situation.

In conclusion, in a similar way to IL-6, IL-11 acts in a gp130 homodimer receptor and it was demonstrated that stimulates lung branching. On the other hand, CLC, CNTF, CT-1 and OSM receptors are gp130 heterodimers and it was described that they inhibit lung growth. All these results demonstrated that cytokine signaling through gp130 homodimers stimulate, whereas cytokine signaling through gp130 heterodimers inhibit lung branching. This specificity of gp130-type cytokines might represent a regulatory mechanism of lung morphogenesis, intrinsic to this family of cytokines, in order to achieve the correct lung growth.

Acknowledgments

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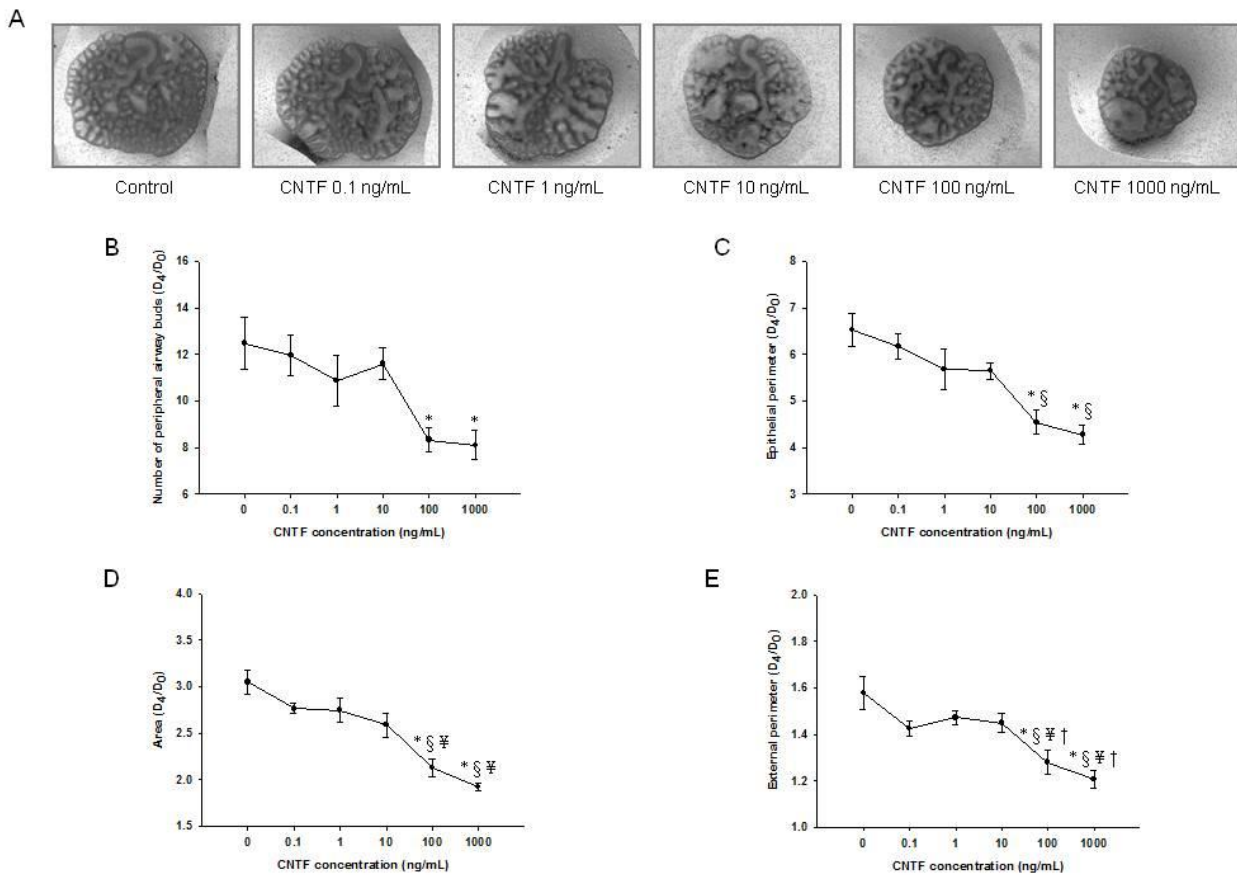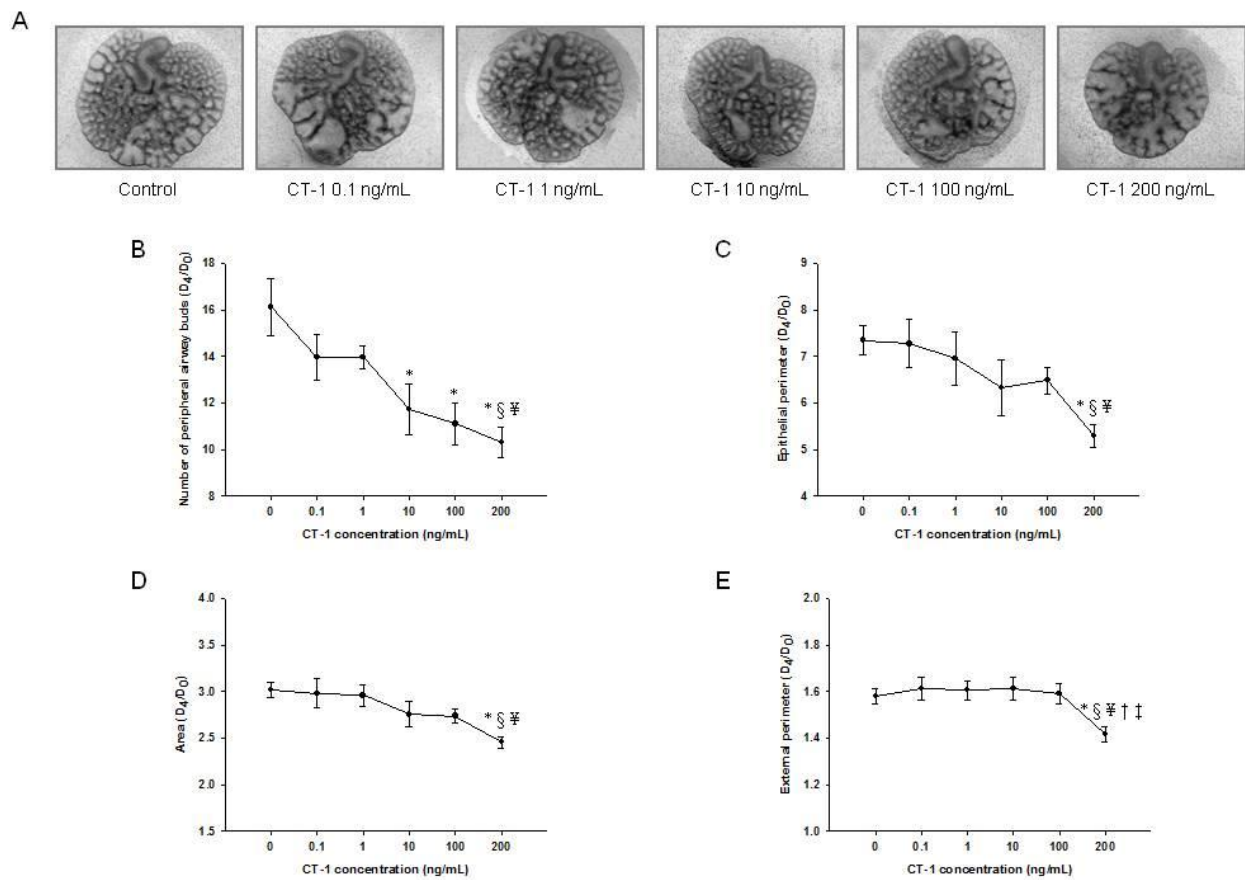
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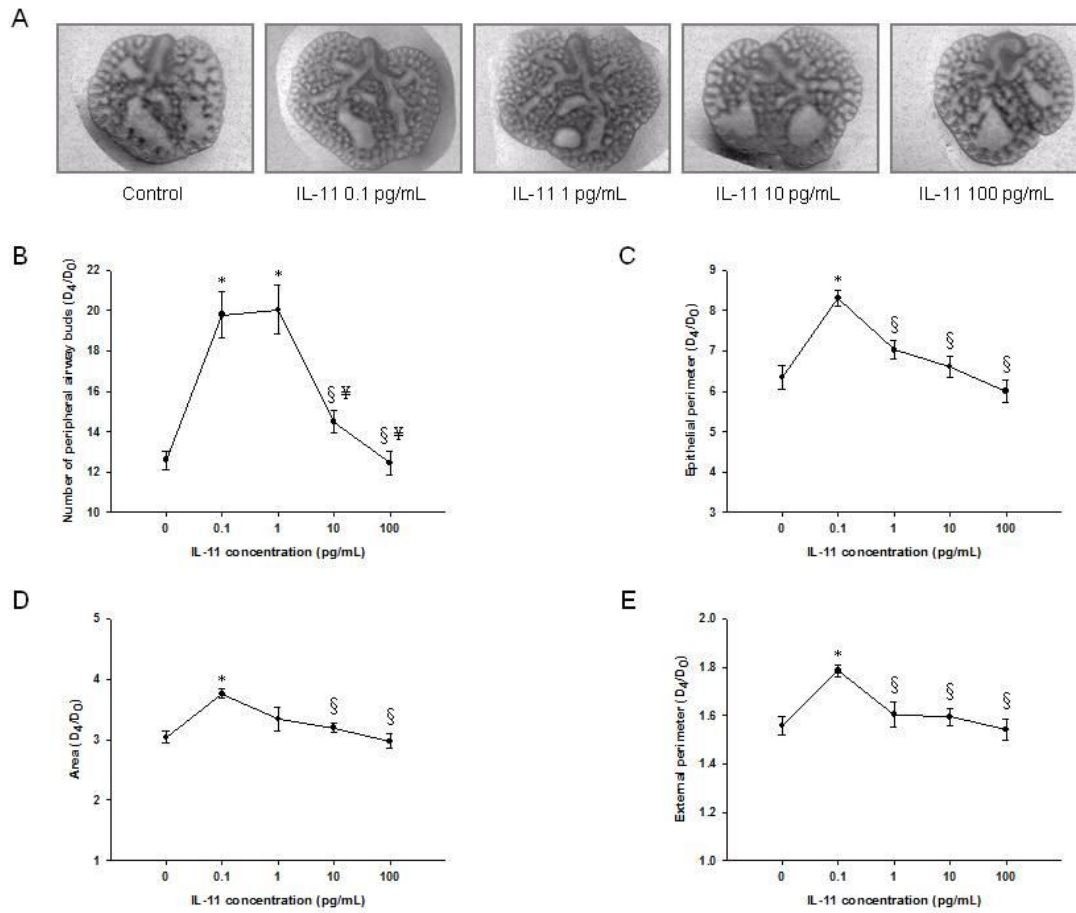
Figure 1. IL-11 supplementation studies in a fetal lung explant culture system. (A) Representative examples of fetal lung explants treated daily with increasing concentrations of recombinant IL-11, after 4 days in culture. Original magnification: x25. (B) Number of total airway buds; (C) Epithelial perimeter; (D) Area; (E) External perimeter of lung explants treated with IL-11. Results are expressed as ratio of day 4 (D4) and day 0 (D0) of culture (D4/D0 ratio). $p < 0.05$: * vs. IL-11 at 0 pg/mL (control), § vs. IL-11 at 0.1 pg/mL, ¥ vs. IL-11 at 1 pg/mL.
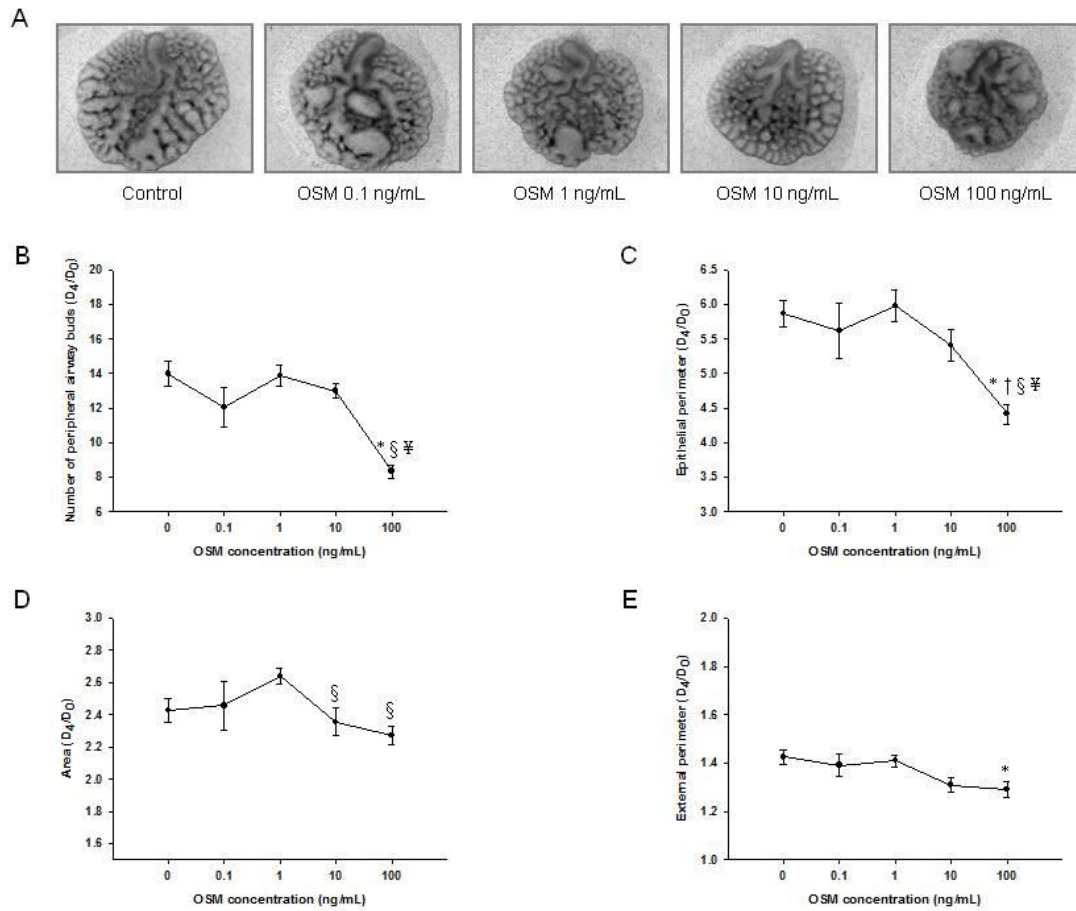
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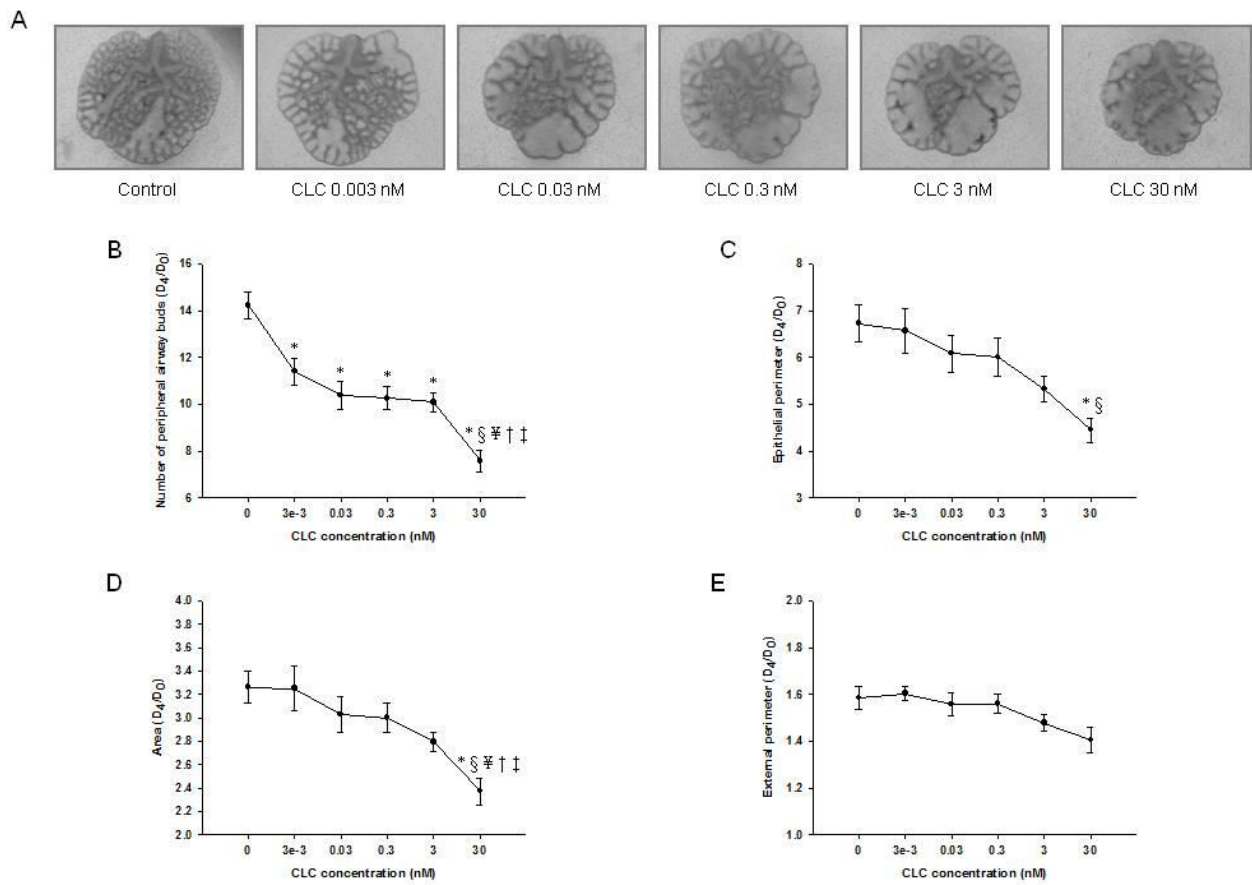
Figure 2. CLC supplementation studies in a fetal lung explant culture system. (A) Representative examples of fetal lung explants treated daily with increasing concentrations of recombinant CLC, after 4 days in culture. Original magnification: x25. (B) Number of total airway buds; (C) Epithelial perimeter; (D) Area; (E) External perimeter of lung explants treated with CLC. Results are expressed as ratio of day 4 (D4) and day 0 (D0) of culture (D4/D0 ratio). $p < 0.05$: * vs. CLC at 0 nM (control plus 4 mM HCL), § vs. CLC at 0.003 nM, ¥ vs. CLC at 0.03 nM, † vs. CLC at 0.3 nM, ‡ vs. CLC at 3 nM.

Figure 3

Figure 3. CNTF supplementation studies in a fetal lung explant culture system. (A) Representative examples of fetal lung explants treated daily with increasing concentrations of recombinant CNTF, after 4 days in culture. Original magnification: x25. (B) Number of total airway buds; (C) Epithelial perimeter; (D) Area; (E) External perimeter of lung explants treated with CNTF. Results are expressed as ratio of day 4 (D4) and day 0 (D0) of culture (D4/D0 ratio). $p<0.05$: * vs. CNTF at 0 nM (control), § vs. CNTF at 0.1 ng/mL, ¥ vs. CNTF at 1 ng/mL, † vs. CNTF at 10 ng/mL.
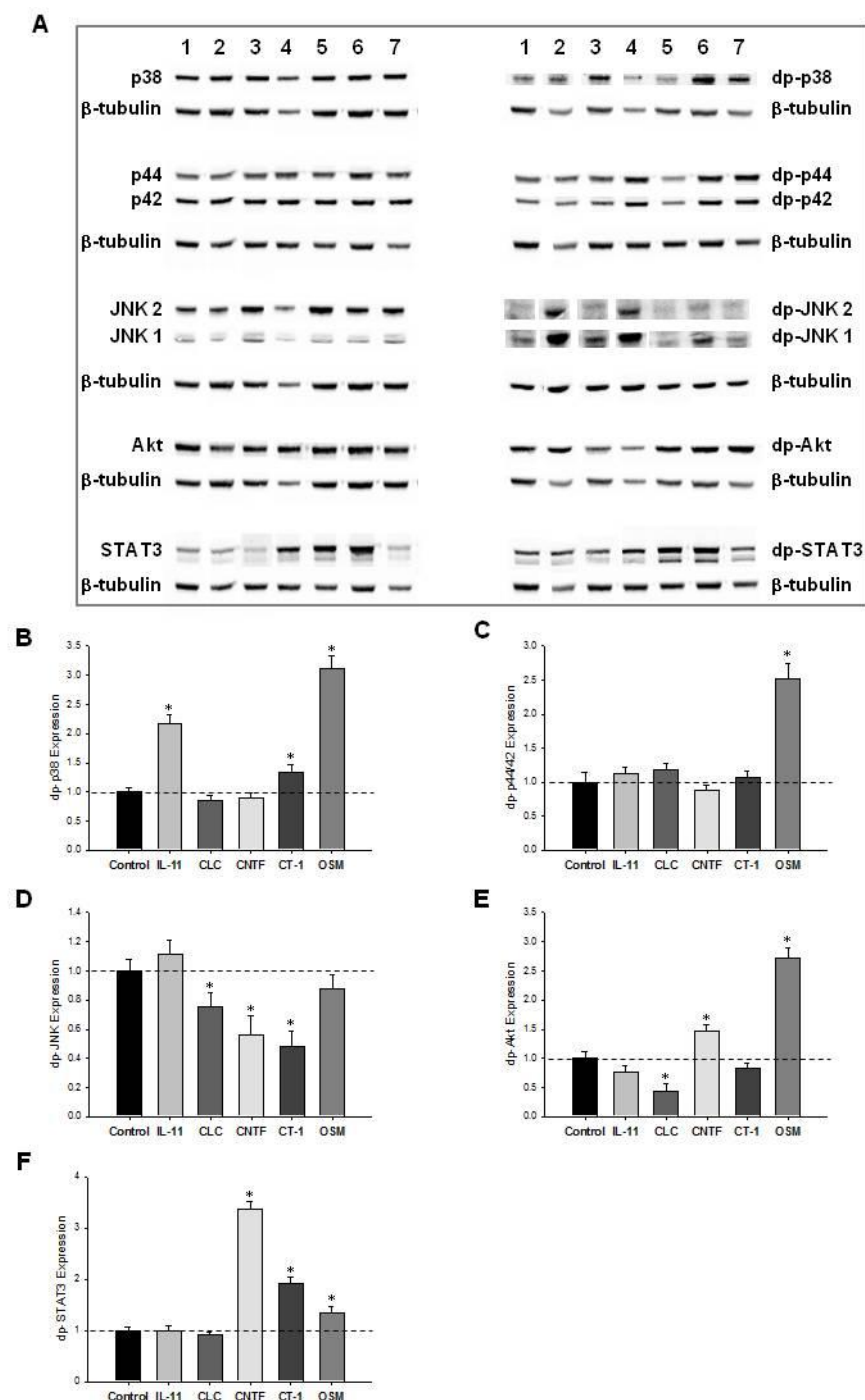
Figure 4

Figure 4. CT-1 supplementation studies in a fetal lung explant culture system. (A) Representative examples of fetal lung explants treated daily with increasing concentrations of recombinant CT-1, after 4 days in culture. Original magnification: x25. (B) Number of total airway buds; (C) Epithelial perimeter; (D) Area; (E) External perimeter of lung explants treated with CT-1. Results are expressed as ratio of day 4 (D4) and day 0 (D0) of culture (D4/D0 ratio). $p < 0.05$: * vs. CT-1 at 0 ng/mL (control plus 4 mM HCL), § vs. CT-1 at 0.1 ng/mL, ¥ vs. CT-1 at 1 ng/mL, † vs. CT-1 at 10 ng/mL, ‡ vs. CT-1 at 100 ng/mL.

Figure 5

Figure 5. OSM supplementation studies in a fetal lung explant culture system. (A) Representative examples of fetal lung explants treated daily with increasing concentrations of recombinant OSM, after 4 days in culture. Original magnification: x25. (B) Number of total airway buds; (C) Epithelial perimeter; (D) Area; (E) External perimeter of lung explants treated with OSM. Results are expressed as ratio of day 4 (D4) and day 0 (D0) of culture (D4/D0 ratio). $p < 0.05$: * vs. OSM at 0 ng/mL (control), † vs. OSM at 0.1 ng/mL, § vs. OSM at 1 ng/mL, ¥ vs. OSM at 10 ng/mL.

Figure 6

Figure 6. Analysis of intracellular signaling pathways that mediates IL-11, CLC, CNTF, CT-1 and OSM actions on lung growth. (A) Western blot analysis of p38, p44/42, JNK1/2, Akt and STAT3, and to diphosphorylated forms of p38 (dp-p38), p44/42 (dp-p44/42), SAPK/JNK (dp-JNK1/2), Akt (dp-Akt) and STAT3 (dp-STAT3) in control (1), control plus 4 mM HCL for CLC and CT-1 lung explants (2) and treated with IL-11 at 0.1 pg/mL (3), CLC at 30 nM (4), CNTF at 1000 ng/mL (5), CT-1 at 200 ng/mL (6), and OSM at 100 ng/mL (7). Control loading was performed using -tubulin (55 kDa). p38 corresponds to 38 kDa. p44/42 correspond to 44 and 42 kDa, respectively. JNK1 and 2 correspond to 46 and 54 kDa, respectively. Akt corresponds to 60 kDa. STAT3 corresponds to two bands, 79 and 86 kDa. Semi-quantitative analysis for dp-p38 (B), dp-p44/42 (C), dp-JNK1/2 (D), dp-Akt (E), and dp-STAT3 (F). Results are presented as arbitrary units normalized for -tubulin and the respective control. $p < 0.05$: * vs. control.
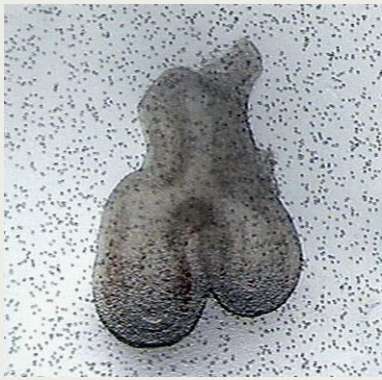
CHAPTER V
IL-6 AND LUNG CATCH-UP GROWTH

Intrinsic Catch-Up growth of Hypoplastic Fetal Lungs is Mediated by Interleukin-6

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Intrinsic Catch-Up Growth of Hypoplastic Fetal Lungs Is Mediated by Interleukin-6

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Summary. Fetal lung hypoplasia is a common finding in several fetal conditions such as congenital diaphragmatic hernia (CDH). Interestingly, previous studies have demonstrated that hypoplastic lungs have the ability to recover to normal size, when relieved from mechanical factors. However, the underlying mechanisms remain largely unknown. Recently, interleukin-6 (IL-6) has been involved in *catch-up growth* phenomenon in children. Thus, we hypothesized that IL-6 could mediate fetal growth recover from hypoplastic lungs. Control and nitrofen-induced hypoplastic lung explants were cultured either in normal conditions or with IL-6 neutralizing antibodies. The total number of peripheral airway buds, epithelial perimeter, and total explant area were analyzed and daily branching rates were calculated. Additionally, IL-6 mRNA and protein expression was assessed both in qualitative (by in situ hybridization and immunohistochemistry) and in quantitative (by real-time PCR and Western blot) approaches, in control and hypoplastic lungs (nitrofen and CDH groups). Nitrofen-induced hypoplastic lungs showed in vitro, out of systemic environment, the ability to recover from hypoplasia and presented daily branching rates significantly higher than controls. Blocking IL-6 activity significantly diminished the intrinsic capacity of hypoplastic fetal lungs to recover from hypoplasia and attenuated their daily branching rates. Although more exacerbated in CDH, both nitrofen-exposed lungs presented significant IL-6 mRNA and protein over-expression throughout all studied gestational ages. The present study suggests, for the first time, that fetal lung is able to recover from growth retardation through a way that resembles the *catch-up growth* phenomenon, and it seems to be, at least partially, orchestrated by intrinsic mechanisms implicating IL-6. **Pediatr Pulmonol. 2008; 43:680−689.** © 2008 Wiley-Liss, Inc.

Key words: fetal lung hypoplasia; lung development; congenital diaphragmatic hernia.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a severe developmental anomaly with a mean incidence of approximately 1/2,500 live-born infants.[1] It is characterized by a congenital diaphragmatic defect that allows passage of the developing viscera into the thoracic cavity.[2] Consequently, lung development is impaired, and at birth CDH infants suffer from severe respiratory distress due mainly to lung hypoplasia, what limits their chance to survive.[1]

The nitrofen-induced CDH rat model is considered one of the best experimental models to study the pathophysiology of pulmonary hypoplasia.[1] In this model, it was previously demonstrated that fetal lung hypoplasia results from a complex dynamic process associating early and late gestational determinants.[3] Early determinants appear to be secondary to molecular disturbances, in a background of genetic and environmental factors, affecting predominantly the pseudoglandular stage of lung development (branching stage). Recently, using proteomics analysis, it was demonstrated that a deficiency in contractile proteins myosin light chain 1a and 2 might have a role amongst the early molecular determinants of lung hypoplasia in nitrofen-induced CDH rat model.[4] On the other hand, late determinants seem to be primarily related to mechanical compression secondary to visceral thoracic herniation and affect essentially canalicular, saccular and alveolar stages of lung development (maturation stages).[3,5,6]

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The *catch-up growth* is a classic infantile phenomenon defined as growth acceleration beyond the normal rate.[7–9] An interesting point about CDH is the ability of hypoplastic lung to recover to normal size, when relieved from mechanical factors. In fact, in rat fetuses exposed to nitrofen that did not develop diaphragmatic defect, fetal lung recovered from lung growth retardation throughout gestation.[5,6] Similarly, clinical observations demonstrated that hypoplastic lungs from surviving CDH infants undergo a tremendous growth after post-natal diaphragmatic repair. Recently, it was also demonstrated that in vitro nitrofen-exposed fetal lungs were able to recover from hypoplasia, suggesting that a phenomenon of *catch-up growth* might be occurring.[10] Although the underlying mechanisms of this phenomenon remain largely unknown, it is possible that their knowledge might be particularly useful envisioning prenatal therapy of lung hypoplasia.

Interleukin-6 (IL-6) is a pleiotropic cytokine with important roles on processes of branching organs, such as fetal lung. Several studies have emphasized the importance of IL-6 signaling in fetal lung maturation by enhancing pulmonary surfactant proteins.[11–14] Recently, it was demonstrated that IL-6 is constitutively expressed in pulmonary primitive epithelium during normal lung morphogenesis, and enhances fetal lung branching.[10]

As IL-6 is commonly involved in repairing mechanisms, we hypothesized that IL-6 could mediate the fetal *catch-up growth* phenomenon of hypoplastic lungs.

MATERIALS AND METHODS

Animal experiments were performed according to the Portuguese law for animal welfare. Animals were housed in an accredited mouse house and treated as specified by the recommendations of the ''Guide for the Care and Use of Laboratory Animals'' published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

Animal Model and Experimental Design

Sprague–Dawley female rats (225 g; Charles-River; Barcelona, Spain) were maintained in appropriate cages under controlled conditions and fed with commercial solid food. The rats were mated and checked daily for vaginal plug. The day of plugging was defined as gestational day 0.5 for time dating purposes. Pregnant rats were exposed on 9.5 dpc (days post-conception) to 100 mg of nitrofen (2,4-dichlorophenyl-*p*-nitrophenylether) dissolved in 1 ml of olive oil administered by gavage or with an equal volume of vehicle.[15] At different time-points (13.5, 15.5, 17.5, 19.5, and 21.5 dpc), fetuses were harvested by caesarean section. Under binocular surgical microscopy (Leica, Wild M651.MS-D, Heerbrugg, Switzerland) and after fetal decapitation, a laparotomy was performed to inspect the diaphragm. Through a median sternotomy, the lungs were excised. Fetuses were assigned to three experimental groups: (i) *Control group*, fetuses exposed to olive oil alone; (ii) *Nitrofen group*, fetuses exposed to nitrofen without CDH; (iii) *CDH group*, fetuses exposed to nitrofen with CDH. Because at 13.5 and 15.5 dpc accurate identification of the diaphragmatic defect was not feasible, only two groups were defined at these gestational ages: *Control* and *Nitrofen* (exposed to nitrofen with or without CDH).

For lung explant cultures, fetuses were harvested at 13.5 dpc from control and nitrofen groups and their lungs were dissected.

For in situ hybridization (ISH), real-time PCR, immunohistochemistry (IHC) and Western blot studies, fetuses were harvested by caesarean section at 15.5, 17.5, 19.5, and 21.5 dpc. Random left lung samples of each experimental group from all time-points were processed and embedded in paraffin for ISH and IHC, whereas other samples were collected for Western blot analysis. Samples from 15.5 dpc were also collected for the quantification of IL-6 expression by real-time PCR. These samples were immersed in RNAlater (Qiagen, Hieden, Germany) and stored at $-80°C$.

Fetal Lung Explant Cultures

Harvesting and dissection of 13.5 dpc lungs was made in DPBS (Cambrex, NJ) under a dissection microscope (Leica MZFLIII, Herbrugg, Switzerland). The lungs were transferred to Nucleopore membranes with an 8 µm pore size (Whatman, NY) and incubated in a 12-well culture plates from Costar (Corning, NY). The membranes were pre-soaked in DMEM (Cambrex) for 1 hr before the explants were placed on them. Floating cultures of the explants were incubated in 200 µl of 50% DMEM, 50% nutrient mixture F-12 (Invitrogen, Carlsbad, CA) supplemented with 100 µg/ml streptomycin, 100 units/ml penicillin (Invitrogen), 0.25 mg/ml ascorbic acid (Sigma Chemical, Gillingham, UK) and 10% FCS (Invitrogen).

Twelve control and 12 nitrofen explants were incubated without any treatment. Thirteen control and 12 nitrofen explants were treated daily with IgG anti-rat IL-6 neutralizing antibody at concentration of 1.0 µg/ml (30 times the concentration previously shown to suppress IL-6 effect; R&D Systems, Minneapolis, MN). Non-specific effects were evaluated by adding identical concentration of normal goat IgG control antibody (control n = 10, nitrofen n = 9) (R&D Systems).

The fetal lung explants were incubated in a 5% CO_2 incubator at 37°C for 96 hr, and the medium was replaced every 48 hr. The branching morphogenesis was monitored daily by photographing the explants. At day 0 (D_0: 0 hr), 1 (D_1: 24 hr); 2 (D_2: 48 hr); 3 (D_3: 72 hr), and 4 (D_4: 96 hr) of culture, the total number of peripheral airway buds

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(branching) in all lung explants was determined, whereas the epithelial perimeter and total explant area were measured using AxionVision Rel. 4.3 (Carl Zeiss, Göttingen, Germany). For all experimental conditions, the results of branching, epithelial perimeter and explant area were expressed as D_4/D_0 ratio, whereas daily branching rate was calculated through the formula D_n/D_{n-1}.

In Situ Hybridization (ISH) Studies

Digoxigenin-labeled IL-6 probe was synthesized from a 901 bp fragment of IL-6 coding sequence, inserted on a pBluescript KS-vector (kindly supplied by Dr. Georg H. Fey, University of Erlangen-Nurnberg, Germany). The plasmid was linearized with *Bam*HI and T3 RNA polymerase was used to synthesize the antisense riboprobe. The in vitro transcription reaction was performed using DIG RNA Labeling Mix (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions.

Tissue sections (10 μm thick) were processed for ISH as previously described by Strahle et al.[16] ISH slides were observed and photographed on light microscope with an Axiocam color video camera (Carl Zeiss, Germany).

Real-Time PCR Studies

RNA Extraction

Total RNA from left lung samples of 18 fetuses (15.5 dpc: control n = 9, nitrofen n = 9) was extracted using the RNeasy Mini Kit Protect (Qiagen). After extraction, total RNA was digested with RNase-free DNase (Promega, Madrid, Spain) to avoid DNA contamination. Total RNA quantification was done by spectrophotometry (Bio-Photometer, Eppendorf, Cologne, Germany). The ratio A260/A280 was used to test protein contamination of the extracted RNA.

Reverse Transcription

For the cDNA synthesis, 100 ng of extracted RNA was used. The reverse transcription was carried out on a T-gradient thermoclycer (Biometra, Goettingen, Germany) using the Superscript II kit (Invitrogen). Briefly, 125 ng random primers (Invitrogen), 4 μl of 5 × buffer, 2 μl of 1 mM DTT, 1 μl of 10 mM dNTP mix (MBI Fermentas, St. Leon-Rot, Germany), 1 μl of recombinant Rnasin (Promega) and 1 μl of Superscript II Reverse Transcriptase

were added in a total volume of 20 μl. The following program was used to carry out the reverse transcriptase reaction: 42°C for 60 min and 70°C for 15 min. In all reactions a negative control, omission of mRNA, was used.

Quantitative Real-Time PCR

According to a 2-Step model, cDNA was used for relative quantification of IL-6 and of reference gene β-actin expression by real-time PCR (LightCycler, Roche Molecular Biochemicals). Each run consisted of 15 min hot-start and 55 cycles (95°C, 15 sec; 58°C, 20 sec; 72°C, 15 sec). The following protocol was used: 10 μl of QuantiTech SYBRgreen PCR mix (Qiagen), 2 μl of cDNA and 10 pmol of each primer were added in a total volume of 20 μl. A negative control was included in all the runs, which consisted in omitting the cDNA and performing a melting curve analysis, thus allowing the detection of putative contamination.

Primer design was based on available sequences in *GenBank* (NCBI-NLM-PubMed-Gene). All the primers are intron-spanning (Table 1). For IL-6 and β-actin primer sets standard amplification curves (ST curves) were made with randomly selected cDNA samples setting r = 0.99. In all samples IL-6 expression was normalized for β-actin.

Immunohistochemistry (IHC) Studies

IL-6 immunostainings were performed on formalin-fixed and paraffin-embedded lung. Sections (5 μm) were placed on SuperFrost®Plus slides (Menzel-Glaser, Braunschweig, Germany). The primary antibody, a polyclonal goat anti-IL-6 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), was used in a 1:50 dilution. After dewaxing in xylene and rehydration in ethanol, antigen retrieval was achieved by boiling in 10 mM citrate buffer followed by cool down at room temperature. Incubation with the goat Immuno Cruz™ Staining System (Santa Cruz Biotechnology, Inc.) was carried according to manufacturer's instructions. Incubation of the primary antibody occurred at 4°C overnight. Negative control reactions included omission of the primary antibody. To visualize the peroxidase activities in sections, diaminobenzidine tetrahydrochloride was used. Sections were counterstained with hematoxylin. The slides were observed and photographed with Olympus BX61 microscope (Olympus, Hamburg, Germany). The percentage of IL-6 stained cells per microscopic field was scored

TABLE 1—Primers Used for Quantitative PCR

Gene	Accession number	Primer set	Product size (bp)
IL-6	NM_012589	5′-CAA GAG ACT TCC AGC CAG-3′, 5′-CTC CGA CTT GTG AAG TGG T-3′	141
β-actin	NM_031144	5′-GAT TTG GCA CCA CAC TTT CTA CA-3′, 5′-ATC TGG GTC ATC TTT TCA CGG TTG G-3′	114

in a single-blinded fashion (magnification $400\times$) in four independent peripheral areas per section (four sections per each experimental group). Scoring was as follows: 1, 0–25% cells/field; 2, 25–50% cells/field; 3, 50–75% cells/field; 4, 75–100% cells/field.

Western Blot Analysis

Proteins were obtained according to Kling et al.[17] Fifty micrograms of protein were loaded onto 12.5% acrylamide minigels, electrophoresed at 100 V at room temperature and then transferred to Hybond™-C Extra (GE Healthcare Life Sciences, Orsay, France). Blots were probed with polyclonal rabbit anti-IL-6 (Abcam, Inc., Cambridge, UK) (1:500) according to the manufacturer's instructions. For loading control, blots were probed with β-tubulin rabbit polyclonal antibody (1:80,000) (Abcam, Inc.). Afterwards blots were incubated with a secondary horseradish peroxidase conjugate (Cell Signaling Technology, Inc., Danvers, MA), developed with Super Signal®West Femto Substrate (Pierce Biotechnology, Rockford, IL) and exposed to Hyperfilm™ ECL™ (GE Healthcare Life Sciences).

Statistical Analysis

All quantitative data are presented as mean $\pm$ SEM. For morphometric analysis of lung explant cultures, statistical analysis was performed, using SigmaStat 3.5, by two-way ANOVA (factor A: groups (control vs. nitrofen); factor B: treatment (no treatment vs. treatment with IgG or anti-IL-6)). The Holm–Sidak test was used for post-test analysis. For real-time PCR studies, Mann–Whitney rank sum test was used. For score of IL-6 stained cells, one-way ANOVA analysis on ranks was used and the Dunn test was performed for post-test analysis. Statistical significance was set at $P < 0.05$.

RESULTS

Fetal Lung Explant Culture

Figure 1 shows representative fetal lung explants of control (Fig. 1A,B) and nitrofen groups (Fig. 1E,F) at D_0 and D_4, respectively. Although nitrofen-induced hypoplastic explants are significantly smaller at D_0 (Fig. 1E), after 4 days in culture, normal and nitrofen hypoplastic lung explants are apparently similar. In fact, morphometric analysis revealed that the number of peripheral airway buds, the epithelial perimeter and the explant area were significantly reduced in nitrofen when compared to normal explants at D_0. However, after 4 days in culture no significant differences between control and nitrofen groups were observed relating to these parameters (Table 2). Figure 1 also illustrates control and nitrofen lung explants treated with normal goat IgG (Fig. 1C—control $+$ IgG; Fig. 1G—nitrofen $+$ IgG) and IgG anti-rat IL-6 antibodies (Fig. 1D—control $+$ anti-IL-6; Fig. 1H—nitrofen $+$ anti-IL-6) at D_4. IL-6 blocking antibodies significantly reduced the number of peripheral airway buds and epithelial perimeter of control explants (Table 2). Inhibition of IL-6 activity also reduced the ability of growth recover of nitrofen lung explants. The number of peripheral airway buds, the epithelial perimeter and the explant area were significantly reduced in
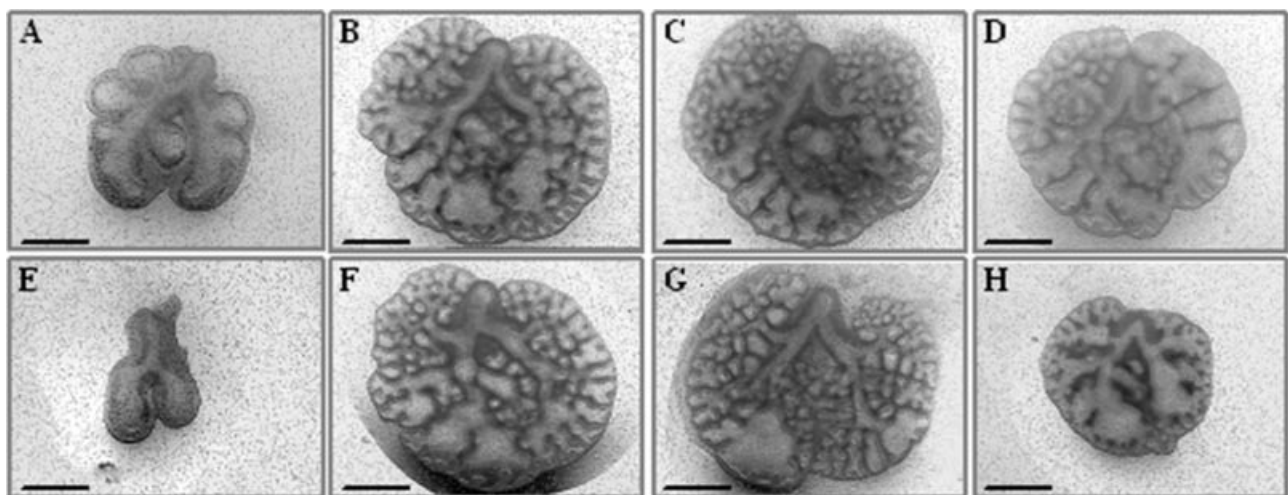

Fig. 1. Branching morphogenesis in rat lung explant system. Representative examples of control lung explants at day 0 (A) and day 4 (B) of culture; control lung explants treated with IgG (C) and IgG anti-IL-6 antibody (D) at day 4 of culture; nitrofen-induced hypoplastic lung explants at day 0 (E) and day 4 (F) of culture; nitrofen-induced hypoplastic lung explants treated with IgG (G) and IgG anti-IL-6 antibody (H) at day 4 of culture. Scale bar $=$ 6349 μm (all images at same magnification).

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TABLE 2—Number of Peripheral Airway Buds, Epithelial Perimeter and Explant Area in Control, Nitrofen, Control and Nitrofen Treated With IgG and Control and Nitrofen Treated With IgG Anti-IL-6 Groups

Group	Number of peripheral airway buds			Epithelial perimeter (mm)			Area (mm^2)		
	D_0	D_4	Ratio D_4/D_0	D_0	D_4	Ratio D_4/D_0	D_0	D_4	Ratio D_4/D_0
Control	6.0 ± 0	60.5 ± 4.1	10.1 ± 0.7	7.3 ± 0.3	35.0 ± 3.9	5.4 ± 0.3	1.2 ± 0.03	3.2 ± 0.4	3.1 ± 0.1
Nitrofen	4.7 ± 0.3^d	62.6 ± 4.9	13.7 ± 1.1^d	5.4 ± 0.4^d	38.1 ± 3.9	7.1 ± 0.6^d	0.9 ± 0.09^d	3.5 ± 0.2	3.6 ± 0.2^d
Control + IgG	6.3 ± 0.2	100.8 ± 5.2^d	16.9 ± 0.7^d	7.7 ± 0.2	60.3 ± 1.8^d	7.8 ± 0.3^d	1.4 ± 0.01	4.7 ± 0.1^d	3.4 ± 0.1
Nitrofen + IgG	4.8 ± 0.4^f	70.0 ± 4.8^f	15.2 ± 1.2	5.5 ± 0.3^f	$50.1\pm2.2^{e,f}$	$9.4\pm0.6^{e,f}$	1.0 ± 0.05^f	3.9 ± 0.1^f	$4.1\pm0.2^{e,f}$
Control + anti-IL-6	5.9 ± 0.4	$50.1\pm4.1^{d,f}$	9.1 ± 1.2^f	7.4 ± 0.3	32.2 ± 1.8^f	4.8 ± 0.4^f	1.2 ± 0.04	3.6 ± 0.3	3.0 ± 0.1
Nitrofen + anti-IL-6	4.9 ± 0.5^h a	$31.8\pm2.1^{e,g,h}$ a,b,c	$7.0\pm0.8^{e,g,h}$ b,c	5.9 ± 0.5^h a	$22.2\pm1.8^{e,g,h}$ a,b,c	$3.7\pm0.2^{e,g,h}$ a,b,c	1.0 ± 0.07^h a	$2.1\pm0.2^{e,g,h}$ a,b,c	$2.1\pm0.1^{e,g,h}$ a,b,c

D_0: day 0; D_4: day 4; $P < 0.05$: ^{a} versus factor A, ^{b} versus factor B, ^{c}: interaction (factor A $\times$ factor B), ^{d} versus control, ^{e} versus nitrofen, ^{f} versus control + IgG, ^{g} versus nitrofen + IgG, ^{h} versus control + anti-IL-6.

nitrofen + anti-IL-6 group when compared to nitrofen, nitrofen + IgG and control + anti-IL-6 groups, at D_4 (Table 2). The significant decrease of morphometric parameters in nitrofen + anti-IL-6 group when compared with control + anti-IL-6 at D_4 demonstrates that anti-IL-6 treatment affected more the nitrofen treated explants than the controls (Table 2).

Figure 2 shows the evolution of total number of peripheral airway buds in control, nitrofen, control + anti-IL-6 and nitrofen + anti-IL-6 groups, throughout all days of culture. At D_0, nitrofen and nitrofen + anti-IL-6 groups present a significant decrease of number of peripheral airway buds when compared to control and control + anti-IL-6 groups. However, the nitrofen group shows the ability to recover from hypoplasia and the number of peripheral airway buds of nitrofen group exceeds the branching of control group, between D_2 and D_3. Interestingly, at D_4 no significant differences between control and nitrofen explants could be detected. Treatment with IL-6 blocking antibodies reduced normal growth
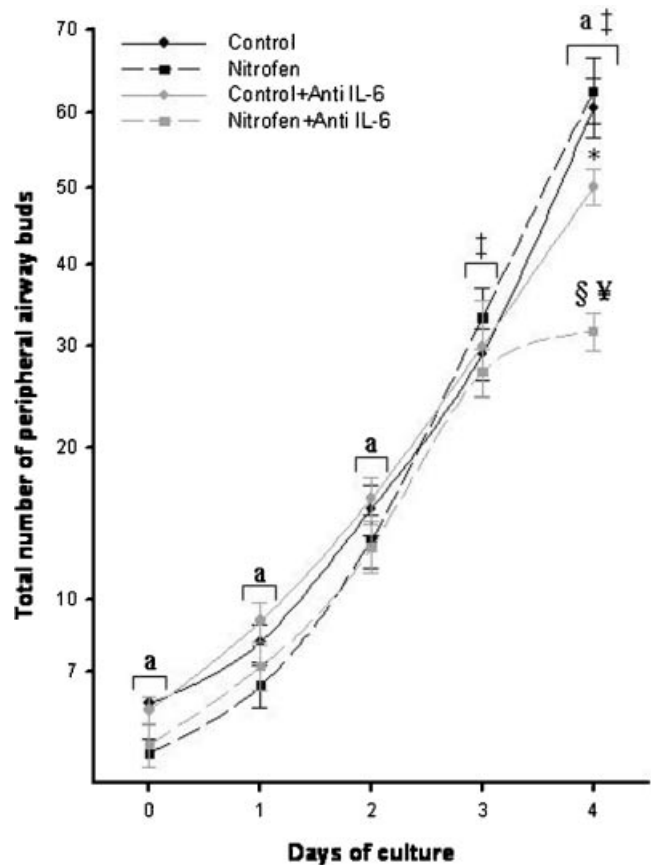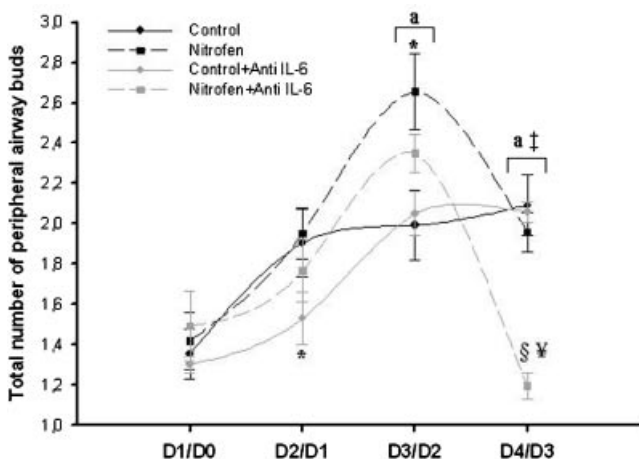

Fig. 2. Analysis of the total number of peripheral airway buds in control, nitrofen, control + anti-IL-6 and nitrofen + anti-IL-6 groups, throughout 4 days of culture. The graph was constructed using a logit scale on *Y*-axis in order to clearly show the differences at D_0. $P < 0.05$: ^{a} versus factor A, * versus control, § versus nitrofen, $^¥$ versus control + anti-IL-6, ‡ interaction (factor A $\times$ factor B).

of control explants and the ability of nitrofen group to recover from hypoplasia. In fact, inhibition of IL-6 action significantly reduced branching of control explants, at D_4. Furthermore, nitrofen + anti-IL-6 explants' growth started to decrease at D_2 and was progressively reduced. At D_4, blocking IL-6 significantly reduced branching of nitrofen + anti-IL-6 lung explants when compared with nitrofen and control + anti-IL-6 explants. At D_3 and D_4 there is a statistically significant interaction between variables: groups × treatment.

In Figure 3 daily growth rates of control, nitrofen, control + anti-IL-6 and nitrofen + anti-IL-6 explants are shown. D_1/D_0 ratio of the number of peripheral airway buds was similar in all groups. However, D_3/D_2 ratio of nitrofen explants was significantly higher than control explants. This ability of nitrofen-induced hypo-plastic explants to grow in culture with higher rates than control explants explains the absence of differences between control and nitrofen explants on the number of peripheral airway buds, the epithelial perimeter and the explant area, at D_4 (Table 2). The representative curves of daily growth rates of nitrofen and nitrofen + anti-IL-6 explants have a similar shape. However, IL-6 inhibition in nitrofen lung explants induced a decrease in later growth rates, mainly at D_4/D_3 ratio. In fact, the D_4/D_3 ratio of the number of peripheral airway buds was significant lower in nitrofen + anti-IL-6 group than in nitrofen and control + anti-IL-6 groups. Moreover, in D_4/D_3 ratio there was a statistically significant interaction between variables: groups × treatment ($P < 0.001$).

Relatively to control groups, the representative curves of daily growth rates of control and control + anti-IL-6 explants have also a similar shape. However, in contrast to the results observed in nitrofen groups, IL-6 blocking antibodies induced a decrease in earlier growth rates,

Fig. 3. Daily growth rates of the number of peripheral airway buds in control, nitrofen, control + anti-IL-6 and nitrofen + anti-IL-6 groups. $P < 0.05$: [a] versus factor A, * versus control, [§] versus nitrofen, [¥] versus control + anti-IL-6, [‡] interaction (factor A × factor B).

mainly D_2/D_1. Indeed, the D_2/D_1 ratio was significant lower in control + anti-IL-6 explants than in control explants.

IL-6 Expression Pattern During Hypoplastic Pulmonary Development

The IL-6 mRNA and protein expression was assessed both in qualitative (ISH and IHC) and in quantitative (real-time PCR and Western blot) approaches. ISH studies revealed that primitive pulmonary epithelium expresses IL-6 mRNA throughout all studied gestational ages in control, nitrofen and CDH groups (Fig. 4A) mainly during pseudoglandular stage. As depicted in Figure 4A, CDH lungs (Fig. 4A,b''−d'') seem to express higher IL-6 mRNA levels than control lungs at any gestational age (Fig. 4A,a−d). At 17.5 dpc, nitrofen lungs seem express similar IL-6 mRNA to CDH lungs. However, at 19.5 and 21.5 dpc, nitrofen lungs seem express lower IL-6 mRNA than CDH lungs and similar to control lungs. These data were corroborated by real-time PCR studies (Fig. 4B), which demonstrated a significant increase of IL-6 mRNA expression in nitrofen group when compared with control group at pseudoglandular stage (15.5 dpc), the stage for maximal pulmonary IL-6 expression which corresponds to fetal lung cultures timing.

IL-6 protein expression pattern during normal and hypoplastic lung development is presented in Figure 5. Likewise IL-6 mRNA expression, IL-6 protein is expressed in pulmonary epithelium throughout all studied gestational ages in control, nitrofen and CDH groups. However, whereas in normal lung IL-6 expression appeared to be restricted to the epithelium, in nitrofen (at 17.5 dpc) and CDH lungs (at all gestational ages) mesenchymal tissue also displayed scattered IL-6 positive cells. Additionally, CDH lungs (Fig. 5A,b''−d'') seem to express higher IL-6 protein levels than control lungs (Fig. 5A,a−d). At 17.5 dpc, nitrofen lungs seem express similar IL-6 protein as CDH lungs. However, at 19.5 and 21.5 dpc, nitrofen lungs seem express lower IL-6 protein than CDH lungs and similar to control lungs. At 15.5, nitrofen lungs presented a statistically significant increase of score of IL-6 stained cells when compared to control lungs. At 17.5 dpc, both nitrofen-exposed groups (nitrofen and CDH) presented a significant increase of score of IL-6 stained cells when compared to control group (Fig. 5B). However, as gestation progressed (19.5 and 21.5 dpc), in the nitrofen group the score of IL-6 stained cells decreased, whereas in the CDH group score was maintained high. In fact, at 21.5 dpc, CDH group presented a statistically significant increase of score of IL-6 stained cells when compared to control and nitrofen lungs (Fig. 5B). Moreover, this IL-6 protein over-expression in CDH group was confirmed by Western blot analysis (Fig. 5C).
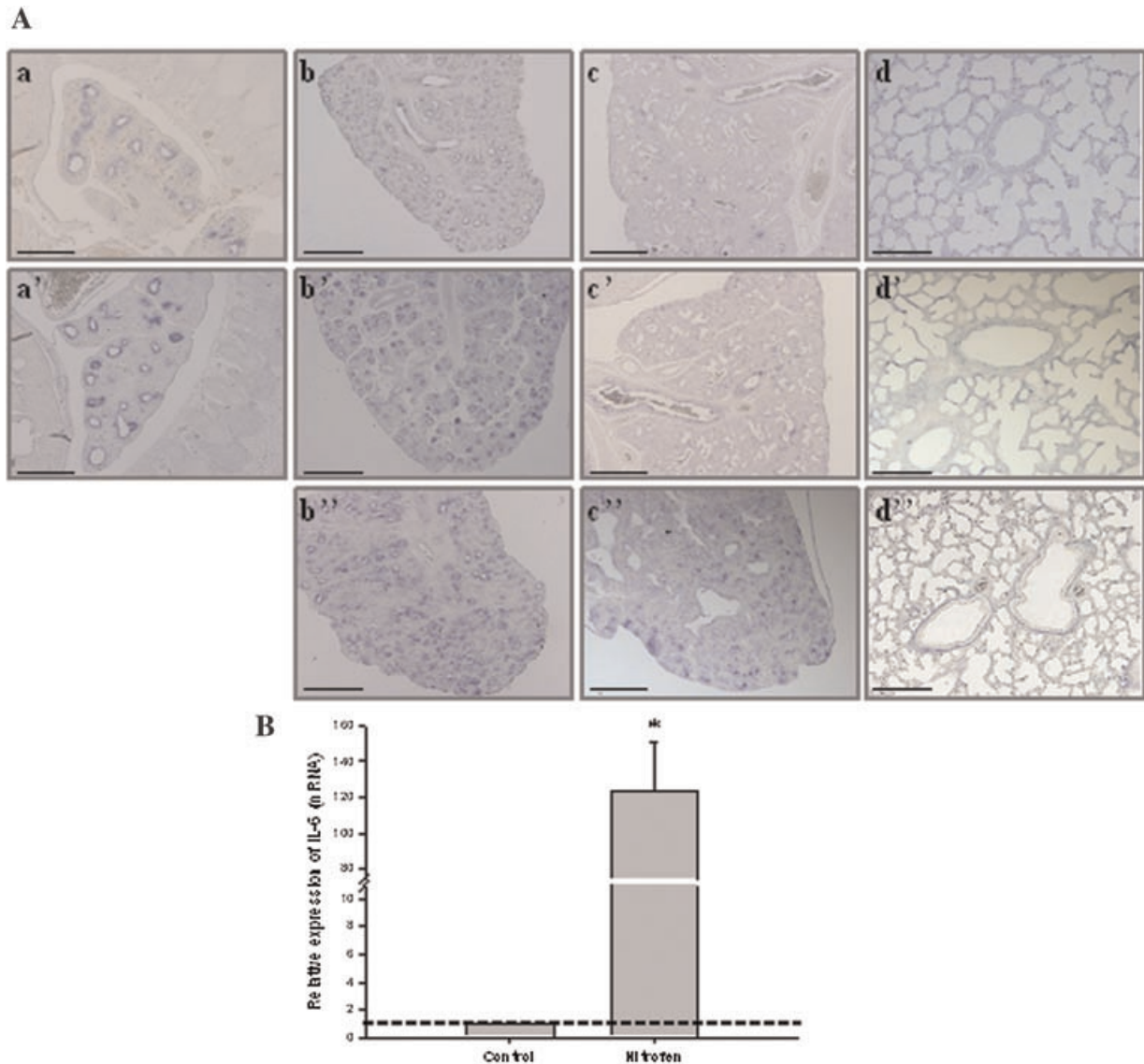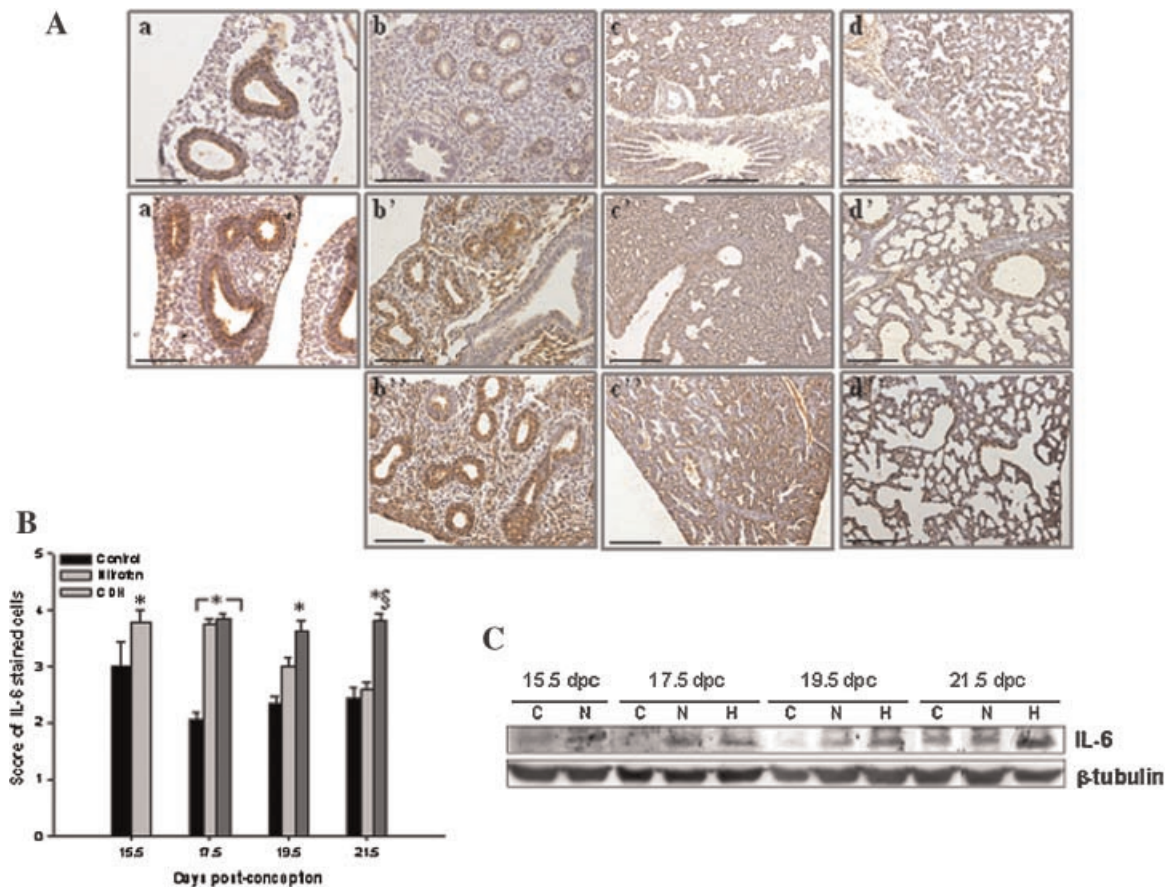
Fig. 4. IL-6 mRNA expression during rat lung development. A: ISH studies in control (a, 15.5 dpc; b, 17.5 dpc; c, 19.5 dpc; d, 21.5 dpc), nitrofen (a′, 15.5 dpc; b′, 17.5 dpc; c′, 19.5 dpc; d′, 21.5 dpc) and CDH (b″, 17.5 dpc; c″, 19.5 dpc; d″, 21.5 dpc) lungs. B: IL-6 mRNA levels in control and nitrofen-induced hypoplastic rat lungs at 15.5 dpc, expressed in arbitrary units normalized for β-actin. Scale bar: 15.5, 17.5, and 19.5 dpc = 250 μm, 21.5 dpc = 100 μm. $P < 0.001$: * versus control group. [Color figure can be viewed in the online issue, which is available at www.interscience. wiley.com.]

DISCUSSION

The current study clearly demonstrated that nitrofen-induced hypoplastic explants have the ability to grow in vitro at higher rates than control explants and consequently recover from early hypoplasia. Moreover, this work suggests that IL-6 might be involved in the mechanisms underlying this phenomenon, since IL-6 mRNA and protein were over-expressed in fetal hypoplastic lungs and blocking of IL-6 activity significantly reduced the ability of nitrofen lung explants to recover from growth retardation.

The *catch-up growth* phenomenon was defined in infants as growth acceleration beyond the normal rate for age after a transient period of growth inhibition.[7−9] In humans, this phenomenon has been described following a wide variety of growth-retarding illnesses, such as growth hormone deficiency, intrauterine growth retardation,[7] celiac disease,[18] hypothyroidism[19] and malnutrition.[7]

In clinical and experimental studies about CDH pathophysiology, it was already recognized that hypoplastic lungs have the ability to recover to normal size when relieved from mechanical factors. In fact, hypoplastic lungs from surviving CDH infants undergo a tremendous

Fig. 5. IL-6 protein expression during lung development. **A:** IHC studies in control (a, 15.5 dpc; b, 17.5 dpc; c, 19.5 dpc; d, 21.5 dpc), nitrofen (a′, 15.5 dpc; b′, 17.5 dpc; c′, 19.5 dpc; d′, 21.5 dpc) and CDH (b″, 17.5 dpc; c″, 19.5 dpc; d″, 21.5 dpc) lungs. **B:** Graph showing results of the scores of IL-6 stained cells, in control, nitrofen and CDH lungs. **C:** IL-6 (21 kDa) Western blot analysis in control (C), nitrofen (N) and CDH (H) groups. β-tubulin (55 kDa) was employed as loading control. Scale bar: 15.5 and 17.5 dpc = 50 μm, 19.5 and 21.5 dpc = 100 μm. $P < 0.05$: * versus control, § versus nitrofen. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

growth after post-natal diaphragmatic repair. Furthermore, in nitrofen-induced CDH rat model was also demonstrated that lung hypoplasia observed in fetuses exposed to nitrofen (that did not develop diaphragmatic hernia) decreased as gestational age progresses. Such studies suggested that when mechanical factors are removed, fetal lung shows an ability to recover from lung growth retardation throughout gestation.[5,6] This study demonstrated that nitrofen-induced hypoplastic explants have ability to growth recover by increasing significantly their daily branching rates. In fact, although nitrofen explants are significantly smaller at D_0, no significant differences between control and nitrofen groups were observed in the number of peripheral airway buds, the epithelial perimeter and the explant area, after 4 days in culture. Moreover, in this study daily branching rates were calculated and it was demonstrated that D_3/D_2 ratio of nitrofen explants was significantly higher than control ones. This phenomenon of lung growth recover with acceleration beyond the normal rate, after a transient

period of growth inhibition (molecular disturbances due to nitrofen), clearly resembles the phenomenon of height *catch-up growth* well-known in infants.

An interesting point about lung ability to recover to normal growth relates to the fact that it occurs either in vivo or in vitro scenarios. This lung ability to growth recover in vitro, out of systemic environment, such as stimulation/inhibition of neuroendocrine axis, supports a novel idea that hypoplastic lungs have local and intrinsic mechanisms orchestrating *catch-up growth* phenomenon. Interestingly, similar intrinsic mechanisms have also been attributed to growth plate to explain the body size *catch-up growth* phenomenon.[20,21] In fact, the *catch-up growth* was primarily attributed to a central nervous system mechanism that compared the individual's actual body size with an age-appropriated set-point and then adjusted the growth rate accordingly.[22] However, this systemic hypothesis has not been subject to a definitive experimental test.[21] More recently, it was demonstrated that transient growth inhibition within a single growth

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plate is followed by local catch-up growth, a finding not readily explained by the systemic model.[20,21] Additional research is needed to determine whether the mechanisms governing catch-up growth are local, systemic, or both. Although these mechanisms remain largely unknown, IL-6 has been involved. Recently, it was demonstrated that small for gestational age children who showed rapid post-natal weight gain and catch-up growth had an associated proinflammatory state with increased IL-6 serum levels.[23] Additionally, growth hormone therapy was found to induce a proinflammatory shift, as judged by a rise of neutrophil count and circulating IL-6.[23,24] Such observations clearly suggest involvement of inflammatory mediators, namely IL-6, in the *catch-up growth* phenomenon.

Also in liver regeneration process, in which liver is able to undergo a compensatory hyperplasia in order to reestablish the optimal mass set in relationship to its body size, it was shown that IL-6 is involved. In fact, in liver regeneration serum levels of IL-6 rise within the first few hours after partial hepatectomy, whereas in IL-6 knockout mice liver regeneration is significantly impaired. These studies clearly indicate that IL-6 is of fundamental importance for liver regeneration.[25–27]

Recently, it was demonstrated that IL-6 is constitutively expressed in pulmonary primitive epithelium during lung morphogenesis, and regulates fetal lung branching most likely implicating p38 phosphorylation.[10] Considering all evidences that involve IL-6 in growth plate *catch-up growth*, in liver regeneration and lung morphogenesis, we hypothesized that IL-6 might also have a role on intrinsic fetal lung *catch-up growth*. To test this hypothesis control and nitrofen-induced hypoplastic lung explants were cultured with IL-6 neutralizing antibodies. According to previously described,[10] IL-6 blocking antibodies significantly reduced the number of peripheral airway buds and epithelial perimeter of control explants (Table 2). This study also demonstrated that the inhibition of IL-6 activity induced a decrease in growth rates. Furthermore, IL-6 neutralizing antibodies clearly arrested the intrinsic capability of hypoplastic fetal lungs to recover from hypoplasia (Figs. 1 and 2, Table 2) and significantly attenuated daily branching rates (Fig. 3) exhibited by nitrofen fetal lungs. Interestingly, IL-6 blocking antibodies decreased earlier growth rates of control explants, whereas it reduced later growth rates of nitrofen-induced hypoplastic explants. Whereas the reduction of earlier growth rates of control lungs support the physiological role for IL-6 on pulmonary branching mechanisms (as previously suggested), the decrease of later growth rates corroborate the role of IL-6 on *catch-up* growth. It is also relevant that anti-IL-6 treatment influenced more the nitrofen treated explants than the controls, and this fact supports the role of IL-6 in the *catch-up* growth phenomenon.

Since these results clearly suggest that intrinsic *catch-up growth* of hypoplastic fetal lungs is mediated by IL-6, the IL-6 mRNA and protein expression during hypoplastic lung development was studied. In hypoplastic lungs (nitrofen and CDH groups), IL-6 mRNA expression was localized in the primitive lung epithelium, as it was previously documented for normal lung morphogenesis.[10] However, whereas in normal lungs IL-6 protein expression appeared to be restricted to the epithelium, in nitrofen (at 17.5 dpc) and CDH lungs (at all gestational ages) mesenchymal tissue also displayed scattered IL-6 positive cells. Moreover, nitrofen-induced hypoplastic lungs presented higher IL-6 mRNA levels than control lungs as confirmed by real-time PCR studies at pseudoglandular stage, which corresponds to fetal lung cultures timing. IL-6 protein levels were also over-expressed in fetal hypoplastic lungs (nitrofen group at 15.5 and 17.5 dpc; and CDH groups during all studied gestational ages) when compared to control lungs, as corroborated by score of IL-6 stained cells and Western blot analysis. Interestingly, nitrofen and CDH groups presented IL-6 mRNA and protein over-expression at the gestational ages, where the lungs were hypoplastic. In fact, it was already described that at 17.5 dpc both nitrofen-exposed groups present similar and significant lung hypoplasia. With gestation progression (19.5 and 21.5 dpc), lung hypoplasia decrease in the nitrofen group, whereas in the CDH group lung hypoplasia is exacerbated relative to control lungs.[5,6] Thus, the present results demonstrate that IL-6 over-expression accompanies the severity of lung hypoplasia. This fact supports the role of IL-6 in the *catch-up* growth phenomenon.

Although all these observations clearly suggest an intrinsic mechanism, one cannot rule out an involvement of systemic or local neuroendocrine loops. In fact, some genes such as EGF[28] and neuroendocrine products (bombesin, ghrelin)[29,30] are also over-expressed in CDH lungs and might be involved in this growth recover phenomenon. However, it remains unknown if their over-expression is induced by local or systemic factors. Moreover, although IL-6 is certainly an important factor on *catch-up* growth mechanisms, several growth factors might be involved and more studies are necessary to identify those factors.

In conclusion, this study suggests for the first time that fetal lung is able to recover from growth retardation through a way that resembles the *catch-up growth* phenomenon, which seems to be, at least partially, orchestrated by intrinsic mechanisms implicating IL-6.

ACKNOWLEDGMENTS

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CHAPTER VI
LUNG RENIN-ANGIOTESIN SYSTEM AND CONGENITAL DIAPHRAGMATIC HERNIA

Local Fetal Lung Renin-Angiotensin System as a Target to Treat Congenital Diaphragmatic Hernia

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Local Fetal Lung Renin-Angiotensin System as a Target to Treat Congenital Diaphragmatic Hernia

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Antenatal stimulation of lung growth is a reasonable approach to treat congenital diaphragmatic hernia (CDH), a disease characterized by pulmonary hypoplasia and hypertension. Several evidences from the literature demonstrated a possible involvement of renin-angiotensin system (RAS) during fetal lung development. Thus, the expression pattern of renin, angiotensin-converting enzyme, angiotensinogen, type 1 (AT_1) and type 2 (AT_2) receptors of angiotensin II (ANGII) was assessed by immunohistochemistry throughout gestation, whereas the function of RAS in the fetal lung was evaluated using fetal rat lung explants. These were morphometrically analyzed and intracellular pathway alterations assessed by Western blot. In nitrofen-induced CDH model, pregnant rats were treated with saline or PD-123319. In pups, lung growth, protein/DNA ratio, radial saccular count, epithelial differentiation and lung maturation, vascular morphometry, right ventricular hypertrophy and overload molecular markers, gasometry and survival time were evaluated. Results demonstrated that all RAS components were constitutively expressed in the lung during gestation and that ANGII had a stimulatory effect on lung branching, mediated by AT_1 receptor, through p44/42 and Akt phosphorylation. This stimulatory effect on lung growth was mimicked by AT_2-antagonist (PD-123319) treatment. *In vivo* antenatal PD-123319 treatment increased lung growth, ameliorated indirect parameters of pulmonary hypertension, improved lung function and survival time in nonventilated CDH pups, without maternal or fetal deleterious effects. Therefore, this study demonstrated a local and physiologically active RAS during lung morphogenesis. Moreover, selective inhibition of AT_2 receptor is presented as a putative antenatal therapy for CDH.
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INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a severe developmental anomaly, with a mean incidence of 1:2,500 live births, in which etiology remains poorly understood (1,2). This congenital anomaly is characterized by a diaphragmatic defect that allows intrathoracic herniation of abdominal organs, and consequently, maldevelopment of the alveoli and pulmonary vessels. For

many years, this malformation was thought to be a surgical emergence, solely related to a diaphragmatic defect, and potentially curable by surgical closure of this defect after birth, which allowed lung expansion. However, during 90 years, CDH pathophysiology progressed for a physiological emergence (3,4). It is now clear that lung hypoplasia and consecutive persistent pulmonary hypertension (PH) associated

with this disorder are the key determinants of mortality (1–4). Despite the improvements in understanding CDH pathophysiology and advances in neonatal care, such as the use of extra corporeal membrane oxygenation and inhaled nitric oxide, the mortality (50%) and morbidity rate in CDH newborns remains exceedingly high (1–4). In humans, CDH can be accurately diagnosed at second trimester during routine ultrasound examination. Therefore it is amenable to antenatal therapies. Hence, antenatal therapies that promote fetal lung growth remain an appealing approach to improve survival of CDH fetuses. However, fetal surgical interventions, such as fetal tracheal occlusion, are invasive, technically demanding, limited by the maternal and fetal risks, and their efficacy is still not determined, with controversial results in survival

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and morbidity rates (5,6). Therefore, less invasive approaches such as antenatal pharmacological treatment to stimulate lung growth before birth and to treat PH are also under investigation (3,7–10).

The renin–angiotensin system (RAS) is a classical endocrine system regulating blood pressure, electrolyte and fluid homeostasis, involving several key components, namely angiotensinogen (the hepatic-derived precursor), two critical enzymes, renin (secreted from the juxtaglomerular apparatus of the kidney) and angiotensin-converting enzyme (ACE, pulmonary-bound metalloproteinase), whose sequential actions produce angiotensin I and the physiologically active, angiotensin II (ANGII), respectively (11). ANGII operates on two G protein-coupled receptors, the ANGII type 1 (AT_1) and type 2 (AT_2) receptors (11). During the last two decades, in addition to this classic endocrine system, evidence has demonstrated the presence of a local RAS with autocrine/paracrine actions in several developing organs, such as fetal kidney, heart, vasculature and adrenal development (12).

Regarding lung morphogenesis, there is some evidence that lung expresses ACE as well as AT_1 and AT_2 receptors during fetal development (13–15). However, RAS components expression pattern, as well as effects during lung morphogenesis, is largely unknown.

In this study, we assessed the expression pattern and function of RAS during fetal lung development. Moreover, we assessed the role of RAS as a putative target for treatment of fetal lung hypoplasia in CDH.

MATERIALS AND METHODS

Animal experiments were performed according to the Portuguese law for animal welfare (16; *Diário da República*, Portaria 1005/92). Animals were housed in an accredited mouse house and treated as specified by the recommendations of the Helsinki convention (17) and the *Guide for the Care and Use of Laboratory Animals*, published by the National Academy Press (18).

Experimental Design and Animal Model

In vitro studies were carried out to assess expression and function of RAS in fetal lung, whereas *in vivo* studies were performed to explore RAS as a target to treat fetal lung hypoplasia using the nitrofen-induced CDH rat model (19).

Sprague Dawley female rats (225 g; Charles River; Barcelona, Spain) were maintained in appropriate cages under controlled conditions and fed with commercial solid food. The rats were mated and checked daily for vaginal plug. The day of plugging was defined as gestational d 0.5 for time-dating purposes. According to the nitrofen-induced CDH rat model (19), at 9.5 d postconception (dpc), randomly selected pregnant rats were exposed to 100 mg nitrofen (2,4-dichlorophenyl-p-nitrophenylether). At different time points, fetuses were harvested by cesarean section. After fetal decapitation, a toraco-laparotomy was performed under a binocular surgical microscope (Leica, Wild M651.MSD, Heerbrugg, Switzerland) to inspect the diaphragm and harvest the organs. Fetuses were assigned to three experimental groups: (i) Control group (C), fetuses not exposed to nitrofen; (ii) Nitrofen group (N), fetuses exposed to nitrofen without CDH; (iii) CDH group, fetuses exposed to nitrofen with CDH.

In Vitro Studies

Normal fetuses removed by cesarean section at 13.5, 15.5, 17.5, 19.5 and 21.5 dpc were killed by decapitation, and lungs dissected and processed for immunohistochemistry (IHC). Lungs of fetuses with 13.5 dpc were also dissected to perform fetal lung explants cultures and posterior western blot analysis.

IHC

IHC was performed on formalin-fixed and paraffin-embedded lungs, as previously described by Nogueira-Silva *et al.* (7). Renin antibody (sc-27320; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used in a 1:25 dilution, ACE antibody (sc-20791; Santa Cruz Biotechnology Inc.) in a

1:75 dilution, angiotensinogen antibody (Abbiotec LLC, San Diego, CA, USA) in a 1:100 dilution, AT_1 receptor antibody (sc-1173; Santa Cruz Biotechnology Inc.) in a 1:50 dilution and AT_2 receptor antibody (sc-9040; Santa Cruz Biotechnology Inc.) in a 1:25 dilution. Incubation with the UltraVision detection system anti-polyvalent horseradish peroxidase (Lab Vision Corporation, Fremont, CA, USA) or with the goat ImmunoCruz™ Staining System (Santa Cruz Biotechnology Inc.) was carried out according to the manufacturer's instructions.

Fetal Lung Explant Cultures

Recombinant ANGII (Sigma, St Louis, MO, USA), AT_1-antagonist (ZD-7155; Tocris Cookson, Bristol, UK) and AT_2-antagonist (PD-123319; kindly supplied by Pfizer, Groton, CT, USA) were added daily to lung explants to achieve increasing concentrations of ANGII (from 10^{-9} to 10^{-4} mol/L) or ZD-7155 or PD-123319 at 10^{-5} mol/L. These doses were selected according to the literature (20,21). This set of experiments created the following groups: control ($n = 30$), ANGII 10^{-9} ($n = 10$), ANGII 10^{-8} ($n = 11$), ANGII 10^{-7} ($n = 11$), ANGII 10^{-6} ($n = 12$), ANGII 10^{-5} ($n = 12$), ANGII 10^{-4} ($n = 12$), ZD-7155 ($n = 12$), and PD-123319 ($n = 15$). Furthermore, explants were treated with ANGII at 10^{-9} mol/L in the presence of ZD-7155 or PD-123319 at 10^{-5} mol/L, creating the additional groups: ANG 10^{-9} + ZD-7155 ($n = 12$) and ANG 10^{-9} + PD-123319 ($n = 15$). Cultures were photographed daily and branching morphogenesis was assessed according to Nogueira-Silva *et al.* (22).

Western Blot Analysis

After 4 d in culture, lung explants treated with ANGII at 10^{-9} mol/L, ZD-7155 or PD-123319 were processed for western blot analysis of nonphosporylated and phosporylated forms of mitogen-activated protein kinases (MAPKs) [p44/42, p38, JNK (mAbs; Cell Signaling Technology, Danvers, MA, USA)] and phosphatidylinositol-3-kinase (PI3K/Akt) (Cell Signaling Technology) according to Nogueira-Silva *et al.* (22). For loading

control, blots were probed with β-tubulin mAb (Abcam plc, Cambridge, UK) ($n = 3$). Quantitative analysis was performed with Quantity One 4.6.5 1-D Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA).

In Vivo Studies

Pregnant rats, control or exposed to nitrofen, were anesthetized at 12.5 dpc with a mix of ketamine (75 mg/kg) and medetomidine (0.5 mg/kg) for subcutaneous implantation of an osmotic micropump filled either with saline or PD-123319 (randomly), on mid scapular region (Alzet osmotic pump Model 2ML1; Durect Corporation, Cupertino, CA, USA). Saline or PD-123319 solution was infused with a rate of 10 μL/h (20 mg/kg/d for PD-123319). For the reversal of the sedative effect, atipamezole (0.25 mg/kg) was used and butorfanole (1 mg/kg) was administered immediately after the surgery. Fetuses were assigned to six experimental groups: Control rats treated with saline (C + S); Control rats treated with PD-123319 (C + PD); Nitrofen rats treated with saline (N + S); Nitrofen rats treated with PD-123319 (N + PD); CDH rats treated with saline (CDH + S); and CDH rats treated with PD-123319 (CDH + PD).

Pregnant rats were randomly assigned for cesarean section at 21.5 dpc or for spontaneous delivery at term.

The pregnant rats killed at 21.5 dpc were laparotomized, and after longitudinal hysterotomy, each fetus (C + S, $n = 14$; C + PD, $n = 15$; N + S, $n = 15$; N + PD, $n = 12$; CDH + S, $n = 15$; CDH + PD, $n = 24$) was extracted, weighed and decapitated for organ harvesting (lungs, heart and kidneys). Organs were also weighed independently, organs-to-body weight ratios were assessed and lungs were either snap frozen in liquid nitrogen for biochemical analyses or fixed in 4% paraformaldehyde (PAF) for histological analyses. In this set of experiments, the level of lung hypoplasia was calculated according to Baptista *et al.* (9).

The pups that were delivered spontaneously at term were placed immediately

Table 1. APGAR-like score.[a]

Sign	Score 0	Score 1	Score 2
Skin color	Marked cyanosis	Mild cyanosis/ pale	Pink
Breathing	Apnea	Irregular or weak breathing	Regular breathing
Spontaneous motor activity	No movements	Weak movements	Vigorous movements
Reactivity to stimulus	No response	Gasping movements	Active movements

[a]This score is obtained by assigning a value (0, 1 or 2) to 4 characteristics: skin color, breathing, spontaneous motor activity and reactivity to stimulus. The scores vary between 0 and 8.

after birth in a light-heated box and randomly killed 5 min after birth or allowed to survive, without any care, support strategies or ventilatory support. Those killed by decapitation at min 5 (C + S, $n = 15$; C + PD, $n = 9$; N + S, $n = 11$; N + PD, $n = 6$; CDH + S, $n = 12$; CDH + PD, $n = 14$) were used for blood collection (neck bleeding) and gasometric evaluation (i-Stat1 analyser; Abbott, Chicago, IL, USA). These were then dissected for identification of CDH. Moreover, hearts were dissected for right ventricular hypertrophy evaluation and myocardial samples of right ventricular free wall were harvested and processed for q-PCR studies. The remaining fetuses were allowed to survive (C + S, $n = 10$; C + PD, $n = 9$; N + S, $n = 14$; N + PD, $n = 21$; CDH + S, $n = 14$; CDH + PD, $n = 28$), evaluated by two independent–blind observers (MJ Baptista and S Nunes) and scored at 1, 3, 5 and subsequently at each 5 min, using an APGAR-like score (Table 1, adapted from Dauger *et al.* [23]). The moment of death was registered and defined by the moment in which the two observers attributed an APGAR 0 (marked cyanosis, apnea, no spontaneous movements, no response to stimulus). All experiments were recorded in video to clarify any doubt. These pups were opened postmortem for diaphragm inspection.

Lungs, heart and kidneys from all pregnant rats were weighed for maternal organ-to-body weight ratio analysis.

Biochemical Studies for Protein/DNA Ratio Assessment

Total lungs (left and right) were processed to determine protein and DNA contents. Protein content was assessed

by Bradford method (24). DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany).

Histological Studies

The trachea was cannulated and the lungs were fixed with PAF under a constant pressure of 20 cmH$_2$O. Lungs were embedded in paraffin and 4-μm sections were used to determine radial saccular count [RSC; using hematoxilin-eosin stain (H&E)], epithelial differentiation [IHC for clara cell secretory protein (CCSP) and prosurfactant protein C (SP-C) and determination of glycogen-content using periodic acid-Schiff stain (PAS)] and medial arterial thickness (using Weigert stain).

RSC was estimated according to Emery and Mithal adapted method (25), at 100× magnification (Olympus BX61 microscope; Olympus, Tokyo, Japan), in 5 animals per group, 6 slides per animal (200 μm apart each other), and 10 segments per slide, by a blinded examiner (E Carvalho-Dias).

Regarding epithelial differentiation, IHC was performed as previously described by Nogueira-Silva *et al.* (7), briefly described above. CCSP antibody [07-623; Upstate (Millipore), Billerica, MA, USA] was used in a 1:800 dilution and SP-C antibody (AB3428; Chemicon International, Temecula, CA, USA) in a 1:400 dilution. These slides and PAS stained slides were observed and photographed (Olympus BX61 microscope). The percentage of CCSP, SP-C and PAS stained cells per microscopic field was scored in a single-blinded fashion (100× and 400× magnification, respectively) in 6 independent peripheral and 4 central

areas per section (6 slides per animal, 200 μm apart each other, and 5 animals per each experimental group). Scoring was as follows: 1, 0–20% cells/field; 2, 20–40% cells/field; 3, 40–60% cells/field; 4, 60–80% cells/field; 5, 80–100%.

Weigert resorcin fuchsin solution stains elastic fibbers, and it was used for morphometric assessment of pulmonary arteries (26). Pulmonary arteries were distinguished from pulmonary veins on the basis of the structure and position. Arteries that were approximately round [that is, the longest external diameter (ED, distance between the external elastic laminae), did not exceed the minimal ED by more than 50%] and had both clearly visible external and internal elastic laminae were analyzed. As the structural changes and pharmacological effects on pulmonary arteries might be vessel size dependent, we selected for further analysis arteries subcategorized into 3 sizes: ED less than 30 μm, ED 30 μm to 50 μm, and ED greater than 50 μm. Then, using AxionVision Rel. 4.3 (Carl Zeiss, Göttingen, Germany), internal area (IA, defined by internal elastic lamina), external area (EA, defined by external elastic lamina) and total area (TA, defined by edge of the vascular sheath) were measured. The percentage of medial (MA) and adventitial arterial area (AA) were calculated according to the following formulas: MA $(\%) = [(EA - IA)/EA] \times 100$; AA $(\%) = [(TA - EA)/EA] \times 100$. At least 10 arteries for each section were evaluated, 6 sections per animal (200 μm apart each other), and 3 animals per each experimental group (at 400× magnification).

Right Ventricular Hypertrophy Evaluation

Hearts (C + S, $n = 8$; CDH + S, $n = 6$; CDH + PD, $n = 8$) were used for right ventricle (RV) and left ventricle (LV) dissection (LV contains septum). RV and LV were weighed separately. The ratio RV/LV was determined and used as an index of right ventricular hypertrophy.

Other hearts (C + S, $n = 7$; CDH + S, $n = 6$; CDH + PD, $n = 6$) were dissected, transversally cut and orientated accord-

ing to short-axis view of the heart, before fixed in PAF and embedded in paraffin. Four micrometer sections were stained with H&E and photographed at 40× magnification (Olympus BX61 microscope). The right ventricular wall thickness was determined in the short-axis view of the heart, in the maximum distance between the right side of the interventricular septum to the right ventricular free wall, using AxionVision Rel. 4.3 (Carl Zeiss) (5 measurements per animal, 20 μm apart each other).

q-PCR Studies

Right ventricular mRNA expression of angiotensinogen, B-type natriuretic peptide (BNP) and endothelin-1 (ET-1), genes previously defined as ventricular overload markers, was evaluated according to Baptista *et al.* (27).

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed using the statistical software SigmaStat (version 3.5; Systat Software Inc., Chicago, IL, USA). Multiple group comparisons were made by analysis of variance. For morphometric explants studies, Western blot analysis, biochemical studies, histological studies, right ventricular hypertrophy evaluation and q-PCR studies, one-way ANOVA on ranks was used. Two-way ANOVA on ranks was used for organ-to-body weight ratio analysis and gasometric studies. Student-Newman-Keuls test was used for posttest analysis. Statistical significance was confirmed at $P < 0.05$.

All supplementary materials are available online at www.molmed.org.

RESULTS

RAS Components Expression Pattern during Fetal Lung Development

The IHC studies revealed that all RAS components, renin, ACE, angiotensinogen, AT_1 and AT_2 receptors, were expressed throughout all studied gestational ages in the fetal lung (Figure 1).

Renin was expressed in bronchiolar and also in alveolar epithelium throughout gestation (Figure 1A) since 13.5 dpc and appears to be maximal in the most immature buds (see Supplemental Figures 1A-D). Endothelial ACE expression started in larger proximal vessels, early in the gestation, and spreads distally to involve progressively smaller vessels (Figure 1B). Interestingly, ACE protein was also detectable in epithelial cells since 13.5 dpc (see Supplemental Figure 1E). Regarding angiotensinogen, protein expression was clearly observed in epithelial cells since 15.5 dpc (Figure 1C). Mesenchymal tissue also displayed scattered angiotensinogen positive cells, first in endothelial cells and in the later gestational ages (19.5 and 21.5 dpc) clearly in vascular smooth muscle cells (Figure 1C). AT_1 receptor protein was first mainly expressed by undifferentiated mesenchyme at 15.5 dpc and, throughout the gestation, it was predominantly expressed by vascular smooth muscle cells and scattered in the mesenchyme (Figure 1D). AT_2 receptor was expressed in bronchial epithelial cells and since 19.5 dpc muscle cells of large blood vessels also expressed it (Figure 1E). Moreover, the epithelial AT_2 expression was also demonstrated at 13.5 dpc (see Supplemental Figure 1F).

Role of ANGII in Fetal Lung Development

The presence of renin, ACE, angiotensinogen and AT_1 and AT_2 protein on lung during fetal development prompted us to evaluate the role of ANGII, the physiologically active peptide of RAS, on lung morphogenesis. For this purpose, fetal lung explants were treated with increasing concentrations of recombinant ANGII (Figure 2A). ANGII significantly increased the number of peripheral airway buds, mainly with concentration of 10^{-9} mol/L, the minimal concentration studied (Figure 2). On the other hand, treatment of lung explants with an AT_1-antagonist (ZD-7155) significantly inhibited, whereas AT_2-antagonist (PD-123319) treatment significantly
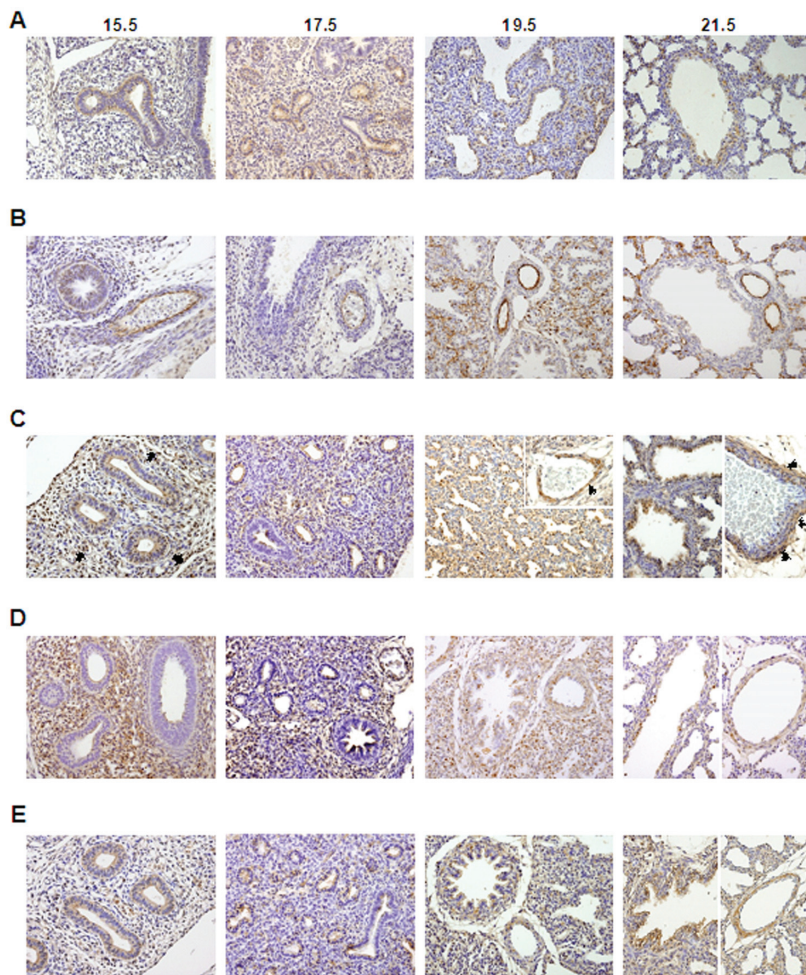
Figure 1. Protein expression pattern of RAS components during fetal lung development (from 15.5 until 21.5 dpc). (A) Renin was predominantly expressed in epithelium. (B) ACE expression. (C) Angiotensinogen expression in epithelial, endothelial (arrow) and vascular smooth muscle cells (arrowhead). (D) AT_1 receptor immunostaining. (E) AT_2 receptor immunostaining. Original magnification 200×.

enhanced lung branching in a similar way to the dose of ANGII-inducing maximal effect (Figure 2C). Moreover, the stimulatory effect on lung branching induced by ANGII 10^{-9} mol/L was completely abolished by AT_1-antagonist treatment, and the simultaneous lung treatment with ANG 10^{-9} mol/L and PD-123319 did not accomplish additional stimulatory effect on explants growth when compared with ANG 10^{-9} mol/L treatment (Figure 2C). Thus, these re-

sults demonstrated that ANGII had a stimulatory effect on lung branching, mediated by AT_1 receptor. Interestingly, this stimulatory effect was mimicked by treatment with AT_2-antagonist alone.

To clarify the intracellular signaling pathways that mediate ANGII actions on lung growth, lung explants treated with ANGII at 10^{-9} mol/L (selected due to its maximal effect in explant growth), ZD-7155 or PD-123319 were evaluated for MAPK and Akt pathways activation

(Figure 3A). AT_1 receptor blockage induced a significant decrease of p-38 and JNK phosphorylation when compared with control explants (Figures 3B, D, respectively). On the other hand, the increase on lung branching, induced by ANGII at 10^{-9} mol/L and AT_2 receptor antagonist, significantly stimulated p44/42 and Akt phosphorylation (Figures 3C, E, respectively).

In Vivo Antenatal PD-123319 Treatment Improves Fetal Lung Growth

Left, right and total lung-to-body weight ratio (LW/BW) were analyzed for different experimental groups. According to the nitrofen-induced CDH experimental model, pups of nitrofen-treated dams presented left and right lung hypoplasia, which was maximal in CDH + S group. Maternal PD-123319 treatment induced significant growth of both left and right lungs in control, nitrofen and CDH groups. In fact, LW/BW was significantly higher in the control, nitrofen and also CDH rats treated with PD-123319 when compared with the respective saline-treated groups (Figure 4A). Indeed, PD-123319 treatment stimulated partial recovery of lung hypoplasia in CDH neonates, inducing an increase of 11.4% in total lung weight (Figure 4B). Considering these results and to assess the potential of PD-123319 as a useful treatment for severe lung hypoplasia associated with CDH, we pursued our study focused in comparing C + S, CDH + S and CDH + PD groups.

Biochemical analysis of lung protein and DNA content demonstrated that there was no significant difference in the protein/DNA ratio between C + S, CDH + S and CDH + PD groups (C + S 0.024 ± 0.005; CDH + S 0.030 ± 0.004; CDH + PD 0.026 ± 0.002).

The histological analysis of lung architecture showed that CDH + S lungs appeared to have a thickened septal and saccular walls and an increased amount of interstitial tissue when compared with C + S. However, CDH + PD had a significantly greater development of saccules
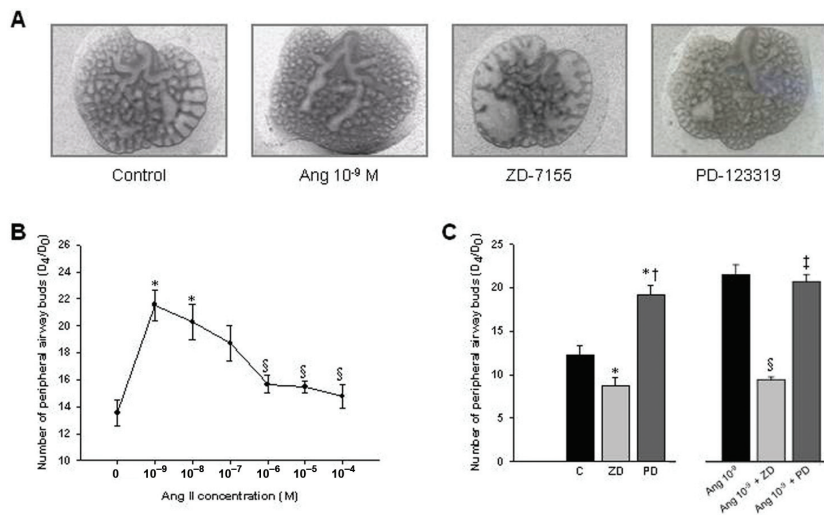
Figure 2. Branching morphogenesis in a rat lung explant system. (A) Representative examples of control and fetal lung explants treated with ANGII at 10^{-9} mol/L, ZD-7155 (an AT_1-antagonist) or PD-123319 (an AT_2-antagonist). Original magnification 25×. (B) Number of total airway buds of lung explants treated with increasing ANGII concentrations or (C) with ZD-7155 (ZD), PD-123319 (PD), ANG 10^{-9} + ZD or ANG 10^{-9} + PD. Results are expressed as ratio of d 4 (D_4) and d 0 (D_0) of culture (D_4/D_0 ratio). $P < 0.05$: *versus ANGII at 0 mol/L (control, C), §versus ANGII at 10^{-9} mol/L, †versus ZD-7155 at 10^{-5} mol/L, ‡versus ANG 10^{-9} + ZD.

and airspaces when compared with CDH + S lungs (Figure 5A). In fact, RSC of CDH + S pups was significantly lower when compared with C + S group (Figure 5B). However, PD-123319 treatment induced a significant increase on RSC of CDH pups, that is, promoted distal lung development (Figure 5B).

Regarding epithelial differentiation and lung maturity, CCSP (proximal epithelial cell differentiation marker), SP-C (distal epithelial cell differentiation marker) and PAS+ glycogen stores (an indirect signal of immaturity) were evaluated (Figure 6). No significant differences in score of CCSP (Figure 6A), SP-C (Figure 6B) and PAS-stained cells (Figure 6C) between C + S and CDH groups were observed.

Concerning other organs than lungs, nitrofen treatment (nitrofen and CDH groups) induced decrease of heart-to-body weight (HW/BW) and kidney-to-body weight (KW/BW) ratios. However, antenatal PD-123319 treatment did not significantly change either HW/BW or KW/BW (see Supplemental Figure 2).

Antenatal PD-123319 Treatment Partially Reversed Arterial Structural Abnormality and Decreased Molecular Markers of PH on CDH Model

MA percentage was significantly increased in all arteries (independently of its size) in CDH + S group when compared with C + S and CDH + PD groups (Figure 7). CDH + PD neonates presented a significant decrease in MA percentage when compared with CDH + S for all arterial sizes, with maximal effect on smaller arteries. The effect was so evident that CDH + PD arteries had no significant difference in MA percentage when compared with C + S group (Figure 7B). Regarding adventitial layer, saline or PD-treated CDH groups presented a significant increase in AA percentage when compared with C + S for all arterial sizes (Figure 7C). Thus, PD-123319 treatment induced decrease of MA percentage and did not induce any change on AA percentage.

Regarding right ventricular hypertrophy index and right ventricle thickness,

markers of right ventricular hypertrophy secondary to chronic PH, CDH neonates did not present differences when compared with control group. Moreover, PD-123319 treatment did not change these parameters (Table 2).

Additionally, expression levels of right ventricular molecular markers of PH (angiotensinogen, BNP and ET-1) were assessed in pups allowed to be delivered at 5 min after birth. In Figure 7D, mRNA levels of these markers normalized to β-actin are shown. CDH + S group presented a significant increase of angiotensinogen and an increase (although not statistical significant) of BNP and ET-1 compared with C + S group. On its turn, PD-123319 treatment significantly decreased these overload markers on CDH pups.

Antenatal PD-123319 Treatment Improves Lung Function and Survival

The adaptation of fetuses to extrauterine life was monitored within the first 5 min after delivery by determining an APGAR-like score (0-8). CDH + S neonates presented an APGAR score of 2.35 ± 0.17, 1.82 ± 0.12 and 2.28 ± 0.18, at 1, 3 and 5 min after birth, respectively. On the other hand, antenatal PD-123319 treatment improved APGAR score to 3.23 ± 0.26, 2.97 ± 0.09 and 2.71 ± 0.13, at 1, 3 and 5 min after birth ($P < 0.05$), respectively.

Five minutes after birth, a moment in which all neonates were alive, neonates of C + S, N + S, CDH + S, N + PD and CDH + PD groups were used for blood collection (collected after decapitation) and gasometric evaluation. When compared with control pups, neonates of nitrofen-treated dams (N + S and CDH + S) presented significant acidosis, decrease of PO_2 and $SatO_2$, and increase of PCO_2 and lactate, which was maximal in CDH + S neonates (pups with maximal degree of lung hypoplasia) (Table 3). On its turn, antenatal PD-123319 treatment allowed a statistical significant increase of pH, PO_2 and $SatO_2$, and decrease of PCO_2 and lactate concentration. Indeed, there was no difference in gaso-
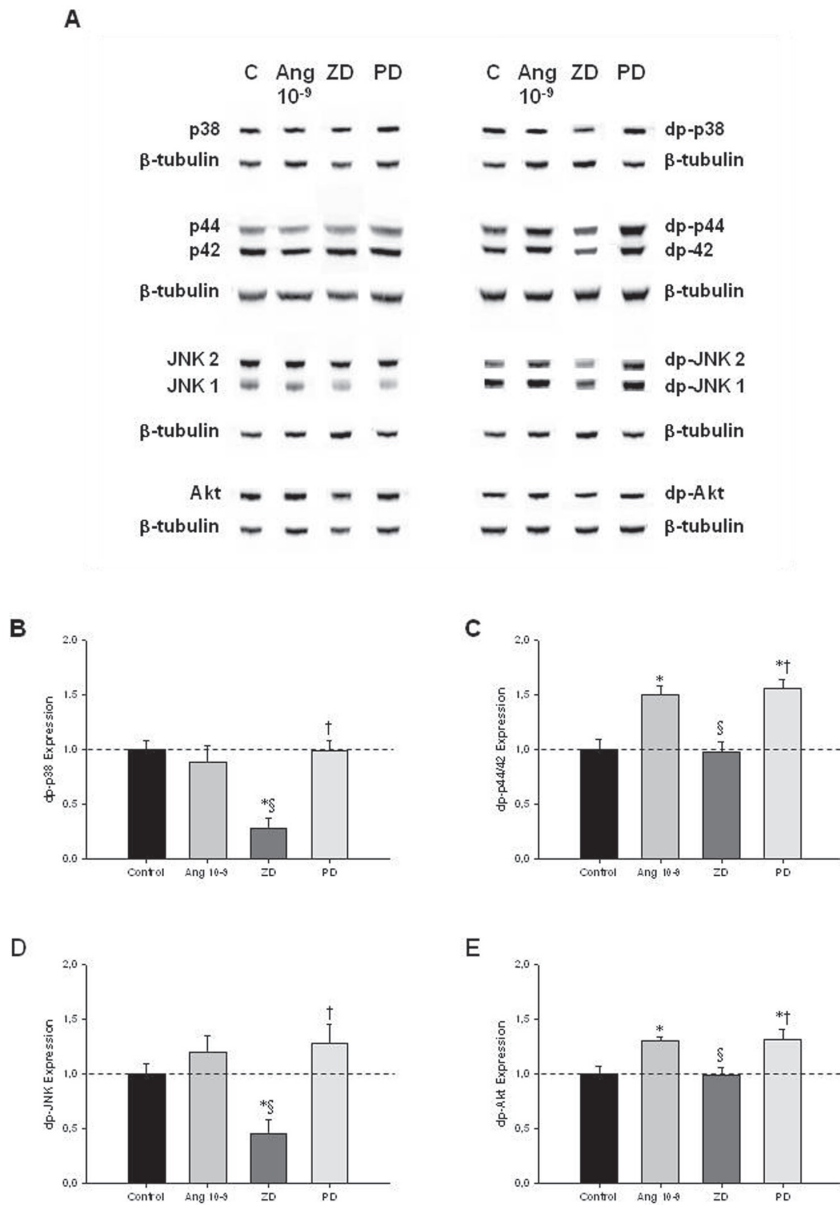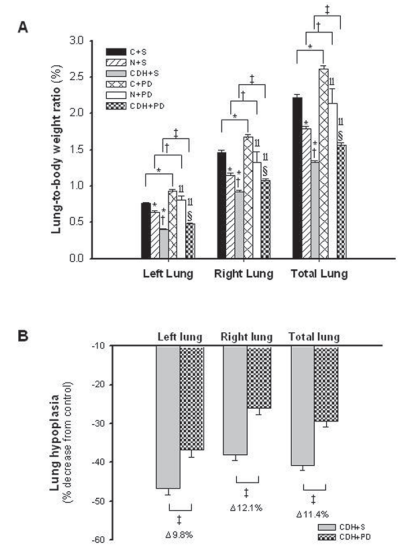
Figure 4. *In vivo* antenatal PD-123319 treatment effects on lung growth. (A) Ratio of left, right and total lung-to-body weight in control (C), nitrofen (N) and CDH groups treated with saline (C + S, N + S, CDH + S) or PD-123319 (C + PD, N + PD, CDH + PD). Antenatal administration of PD-123319 enhanced lung growth in all studied groups. (B) Effect of PD-123319 treatment on left, right and total lung hypoplasia. Prenatal administration of PD-123319 ameliorated both left and right lung hypoplasia. Results are expressed as %. $P < 0.001$: *versus C + S, †versus N + S, ‡versus CDH + S, ‖versus C + PD, §versus N + PD. For left lung, there is a statistically significant interaction between variables: disease–treatment ($P = 0.009$).

metric parameters between control, nitrofen, and CDH groups treated with PD-123319 (Table 3).

Concerning neonatal survival, the average survival time was significantly longer in PD-123319-treated pups than in CDH + S pups (30.3 ± 3.2 min for CDH + S versus 42.4 ± 1.3 min for CDH + PD, $P < 0.001$). As a result, all CDH + PD pups, but only 57% of the CDH + S pups, survived for up to 30 min. At 45 min after birth, only 7% of CDH + S pups were alive, as opposed to over a third of CDH + PD pups. In fact, PD-123319 treatment increased survival rates of CDH neonates for all time points evaluated (Figure 8).

Figure 3. MAPK and Akt kinase activities in control (C) lung explants and treated with ANGII at 10^{-9} mol/L (Ang 10^{-9}), ZD-7155 (ZD) or PD-123319 (PD). (A) Western blot analysis of p38, p44/42, JNK1/2 and Akt and to diphosphorylated forms of p38 (dp-p38), p44/42 (dp-p44/42), SAPK/JNK (dp-JNK1/2) and Akt (dp-Akt). Control loading was performed using β-tubulin (55 kDa). p38 corresponds to 38 kDa. p44/42 correspond to 44 and 42 kDa, respectively. JNK1 and 2 correspond to 46 and 54 kDa, respectively. Akt corresponds to 56 kDa. Semiquantitative analysis for dp-p38 (B), dp-p44/42 (C), dp-JNK1/2 (D), and dp-Akt (E). Results are presented as arbitrary units normalized for β-tubulin. $P < 0.05$: *versus ANGII at 0 mol/L (control, C), §versus ANGII at 10^{-9} mol/L, †versus ZD-7155 at 10^{-5} mol/L.
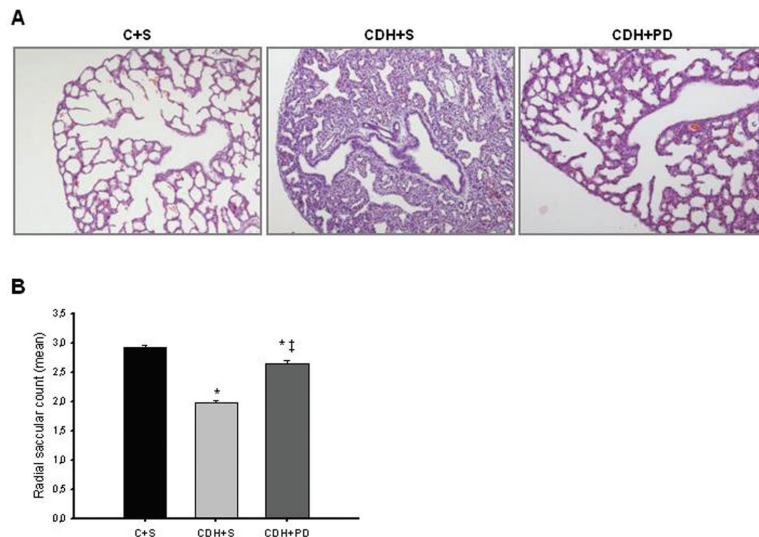
Figure 5. Antenatal PD-123319 treatment increased RSC. (A) Representative H&E stained sections of C + S, CDH + S and CDH + PD lungs, used to RSC analyzes. Note greater saccular development and increase of lung aeration in CDH + PD compared with CDH + S. Original magnification 100×. (B) Mean radial saccular count in C + S, CDH + S and CDH + PD groups. PD-123319 treatment induced increase of RSC in CDH pups. $P < 0.001$: *versus C + S, ‡versus CDH + S.

Regarding potential secondary effects of PD-123319 on maternal organs, their lungs, heart and kidneys were weighed. PD-123319 treatment did not significantly change maternal HW/BW, KW/BW or LW/BW ratios (see Supplemental Figure 3).

DISCUSSION

This study demonstrated that all components of RAS (renin, ACE, angiotensinogen, AT_1 and AT_2 receptors) were constitutively expressed in the lung during all studied gestational ages and that ANGII had a stimulatory effect on lung branching, mediated by AT_1 receptor, through p44/42 and Akt phosphorylation. This stimulatory effect on lung growth was mimicked by treatment with AT_2-antagonist. Therefore, AT_2 receptor antagonist was evaluated as a putative antenatal treatment for diseases characterized by fetal lung hypoplasia such as CDH. In an animal model of CDH, antenatal PD-123319 treatment increased neonatal lung growth, ameliorated indirect parameters of PH, improved lung func-

tion and survival, without maternal or fetal deleterious effects.

In past years, it has been demonstrated that local ANGII formation and its tissue-specific effects on growth and differentiation are thought to be extremely important for embryonic and fetal development (12). Regarding fetal lung, this study corroborated previous evidences concerning ACE, AT_1 and AT_2 expression (13–15,28). For the first time, the present study showed that renin and angiotensinogen are also expressed during lung development. Interestingly, renin, ACE and AT_2 were expressed at very early stages (since 13.5 dpc), suggesting an important role for a local RAS since early stages of lung development.

The results of RAS components expression prompted us to hypothesize that a local RAS is active in the developing lung. Therefore, the role of ANGII on lung morphogenesis was evaluated. ANGII supplementation induced an increase in lung explants growth, and it is necessary to stress that this enhancing effect of ANGII on number of peripheral

airways buds of lung reached about 37%, whereas the stimulatory effect induced by fibroblast growth factor-10 (FGF-10), a classical and very important lung growth factor, in a similar model of murine lung explants, was around 20% (29). The possible mechanism by which RAS interferes with the airway branching or pulmonary vascular development is still unclear, and further investigation is required. However, it was already substantially demonstrated that reciprocal interactions between airways and blood vessels are critical for normal lung development. For instance, it was demonstrated that ablation of lung epithelium impair lung vascular cells development (30). Moreover, VEGF inhibition in neonatal rats leads to arrested alveolar development, suggesting that inhibition of vascular growth itself may directly impair lung development (31–33). Thus, given that, in the present study, it was demonstrated that some components of RAS are expressed on epithelium and others on mesenchyme/vascular cells, it is possible that RAS is involved in both processes: airway and vasculature branching.

Interestingly, AT_1 receptor inhibitor decreased, whereas AT_2-antagonist significantly increased lung growth in explants. This opposite effect of AT_1 and AT_2 receptors, namely a stimulatory effect of AT_1 and inhibitory effect of AT_2, is also described on other tissues (34–39).

Many of the effectors that modulate fetal lung branching seem to activate MAPK and/or PI3K/Akt cascades (40). Thus, MAPK and PI3K/Akt pathway activation by ANGII and AT_1 and AT_2 antagonists in fetal lung development was investigated. ANGII and AT_2 receptor antagonist treatment induced an increase in lung branching by the stimulation of p44/42 and Akt phosphorylation. These intracellular mediators are also involved in AT_1 effects on proliferation and survival of cells in other tissues (34,36). Regarding lung growth inhibition induced by AT_1 antagonist, it was mediated by a decrease of p-38 and JNK phosphorylation. These MAPK families were already
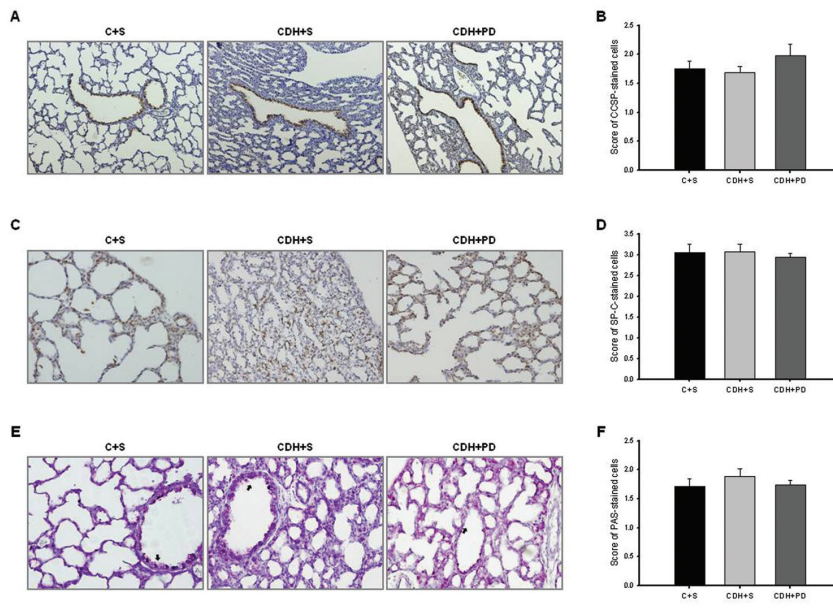
Figure 6. Antenatal PD-123319 treatment did not interfere with epithelial differentiation and lung maturation. (A) Representative CCSP-stained sections of C + S, CDH + S and CDH + PD lungs. Original magnification 100×. (B) Score of CCSP-stained cells in C + S, CDH + S and CDH + PD groups. (C) Representative SP-C stained sections in different groups. Original magnification 200×. (D) Score of SP-C-stained cells. (E) Representative PAS stained sections of C + S, CDH + S and CDH + PD lungs. Original magnification 400×. Arrows: PAS+ (glycogen-rich) cells. (F) Score of PAS-stained cells. No significant difference between experimental groups was observed in CCSP, SP-C and PAS.

demonstrated to be involved on induction of lung branching (22).

The *in vitro* studies demonstrated that a local RAS is functional at early stages of lung morphogenesis. Moreover, the significant stimulatory effect on lung growth mediated by AT$_2$ receptor antagonist led us to hypothesize that AT$_2$ could be a new target for treatment of diseases characterized by fetal lung hypoplasia, such as CDH. In fact, AT$_2$ receptor is described to be expressed virtually only during fetal life (11,12,41), which would annul potential maternal adverse effects. Thus, treatment with AT$_2$ antagonist (PD-123319) was selected for the *in vivo* study, in which the nitrofen-induced CDH rat model (19,42) was used. The gestational age selected for maternal PD-123319 administration was based on the effect observed on lung explants that were harvested at 13.5 dpc.

The nitrofen-induced CDH model is an experimental model of severe lung hypoplasia, which reasonably replicates the major abnormalities and the pathophysiology described in human CDH (42–44). Although the mechanism by which nitrofen induces the diaphragmatic defect and lung hypoplasia is not fully understood, recent evidences suggest the involvement of abnormalities linked with the retinoid signaling pathway in this model and also in human CDH etiology (42–45). Indeed, one clinical study demonstrated the presence of decreased levels of retinol and retinol-binding protein in human CDH, suggesting a possible deterioration of retinol transport across the placenta (46). Regarding the nitrofen-induced CDH model, it was already demonstrated that nitrofen inhibits retinal dehydrogenase 2 (RALDH2), a key enzyme responsible for the conver-

sion of retinal to retinoic acid (47). Moreover, the co-administration of retinoids (Vitamin A or retinoic acid) in nitrofen-induced CDH induces lung growth and reduces the incidence of CDH (9,10,48). Interestingly, some studies have described an inhibitory interaction between retinoid acid pathway and RAS, namely in adult cardiac remodeling (49,50). Thus, a possible interaction between retinoid acid pathway and RAS might be present during lung morphogenesis, but the underlying mechanisms remain unclear and further investigation is required.

Maternal PD-123319 subcutaneous administration significantly increased lung growth in control, nitrofen and CDH groups. In fact, in CDH neonates, despite the presence of mechanical forces that compress lungs, PD-123319 induced partial recovery of lung hypoplasia as assessed by LW/BW (an increase of 11.4%). In face of these results and to assess if the treated-lung is structural and functionally ameliorated, we focused further studies in comparing C + S, CDH + S and CDH + PD groups. In human and experimental CDH there is a reduction in peripheral lung development (1,10). PD-123319 treatment stimulated lung growth by promoting distal lung development as measured by the enhanced RSC. The potential clinical relevance of this effect should be emphasized, since such an increase in lung parenchyma can be determinant in providing a better adaptation of CDH fetus to extrauterine life.

Regarding epithelial differentiation and lung maturity, CCSP (a Clara cells marker, marker for proximal lung), SP-C (a type II pneumocytes marker, marker for distal lung) and PAS glycogen stores (a signal of immaturity) were assessed. No differences between groups were detected in CCSP, SP-C and PAS-stained cells score. Contradictory results about lung maturity and surfactant status in CDH animal models have been published, with studies demonstrating that the CDH lung is surfactant deficient (51–53) and others indicating no change in alveolar surfactant composition, un-
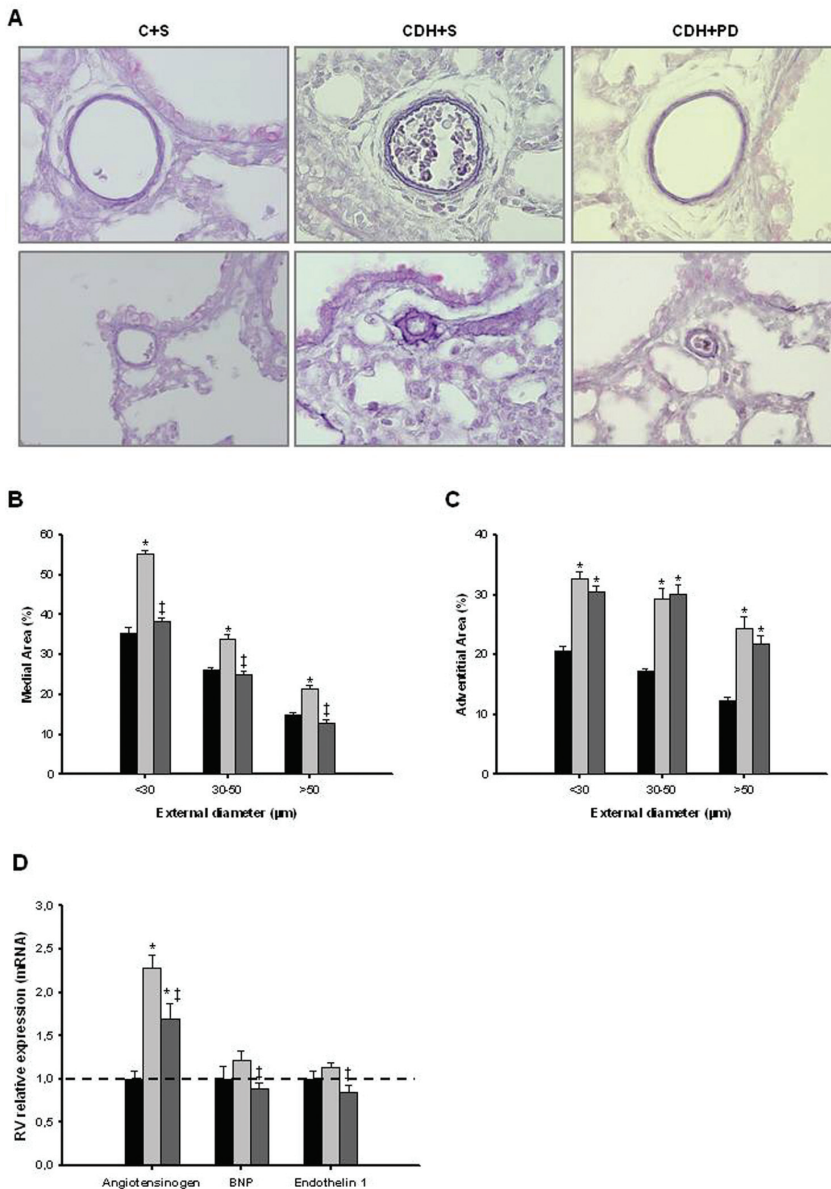
Figure 7. Indirect pulmonary hypertension assessment. (A) In upper panel are presented representative examples of pulmonary arteries > 50 μm stained with Weigert resorcin fuchsin solution of C + S, CDH + S and CDH + PD groups; in lower panel, representative examples of small pulmonary vessels (<30 μm) for each group are presented. Original magnification 400×. (B) Percentage medial area of pulmonary arteries of different external diameters. (C) Percentage adventitial area of pulmonary arteries of different external diameters. (D) Right ventricular levels of angiotensinogen, BNP and ET-1 mRNA in C + S, CDH + S and CDH + PD neonates, expressed in arbitrary units normalized for β-actin. PD-123319 treatment significantly decreased arterial medial area and these overload markers on CDH pups. $P < 0.001$: *versus C + S, ‡versus CDH + S. ■, C + S; ▨, CDH + S; ■, CDH + PD.

changed or even increased surfactant protein expression (54–57). However, one of the most recent studies performed with human CDH fetuses demonstrated that surfactant maturation is not delayed (54). The present study corroborates the idea that CDH does not appear to interfere or delay epithelial differentiation and surfactant accumulation (54). Moreover, PD-123319 treatment did not impair fetal lung maturation in CDH neonates.

Persistent PH accounts for significant mortality and morbidity in CDH (1,2,58). In severe human CDH and also in nitrofen-induced model of CDH, it was already demonstrated that PH results from decreased number of arteries, increased thickness of media and adventitia of pulmonary arterial walls and distal muscular extension to the nonmuscular intraacinar arteries (58,59). In this study, the effect of PD-123319 treatment on PH was indirectly assessed by morphometric pulmonary vascular analysis (59) and its cardiac repercussion by determination of right ventricular hypertrophy index and right ventricle thickness, and quantification of right ventricular overload molecular markers (Angiotensinogen, BNP and ET-1) (27). To better analyze the vessel morphology, areas were measured instead of thickness since this approach avoids the bias introduced by irregular shape of elastic laminae. Thus, PD-123319 treatment induced decrease of MA percentage for all arterial sizes, with maximal effect on smaller arteries. Moreover, CDH + S neonates did not present differences in right ventricular hypertrophy index and right ventricular wall thickness (morphological markers) when compared with C + S group, but they presented an increase of molecular markers of right ventricular overload. Thus, 5 min after birth, CDH + S neonates only presented molecular changes secondary to PH, but not yet morphological alterations (secondary to chronic PH). PD-123319-treated CDH pups presented a significantly decrease of right ventricular overload molecular

Table 2. Right ventricular hypertrophy evaluation 5 min after birth.[a]

	Ratio of right/left ventricular weight	Right ventricular wall thickness (μm)
C + S	0.49 ± 0.08	214.02 ± 9.29
CDH + S	0.44 ± 0.05	200.59 ± 4.27
CDH + PD	0.52 ± 0.05	220.31 ± 12.26

C, control; CDH, congenital diaphragmatic hernia; PD, PD-123319; S, saline.
[a]Values represent the mean ± SEM of measurements. $P < .05$: No significant difference between experimental groups was observed.

Table 3. Neonatal blood gasometric evaluation 5 min after birth.[a]

	pH	PCO_2 (mmHg)	PO_2 (mmHg)	$SatO_2$ (%)	Lactate (mmol/L)
C + S	7.30 ± 0.03	22.69 ± 2.33	102.80 ± 7.78	93.60 ± 2.50	6.83 ± 0.39
N + S	6.94 ± 0.03[b]	38.49 ± 3.21[b]	62.50 ± 5.36[b]	67.25 ± 5.58[b]	8.50 ± 0.51[b]
CDH + S	6.92 ± 0.02[b]	51.05 ± 1.71[bc]	52.00 ± 6.06[b]	58.50 ± 7.06[b]	9.56 ± 0.57[b]
N + PD	7.18 ± 0.08[c]	23.62 ± 3.39[c]	95.67 ± 6.49[c]	90.83 ± 2.44[c]	6.88 ± 0.82[c]
CDH + PD	7.03 ± 0.04[de]	24.63 ± 5.83[d]	95.00 ± 8.20[d]	87.80 ± 3.65[d]	6.73 ± 0.70[d]

C, control; CDH, congenital diaphragmatic hernia; N, nitrofen; PD, PD-123319; PCO_2, CO_2 partial pressure; PO_2, O_2 partial pressure; S, saline; $SatO_2$, O_2 saturation.
[a]Values represent the mean ± SEM of measurements.
[b]$P < 0.05$ versus C + S; [c]versus N + S; [d]versus CDH + S; [e]versus N + PD. There is a statistically significant interaction between variables: disease–treatment for pH, PCO_2, PO_2 and $SatO_2$.

Figure 8. Survival analysis. (A) Representative examples of cyanotic color of CDH + S versus pink coloration of CDH + PD neonates at 5 min of life. (B) Survival rates (expressed as percentage of pups surviving at each 5 min) of CDH + S and CDH + PD neonates. Antenatal PD-123319 treatment improved survival time in CDH pups (mean survival time: CDH + S 30.3 ± 3.2 min; CDH + PD 42.4 ± 1.3 min, $P < 0.001$). ▫, CDH + S; ▪, CDH + PD.

markers and did not influence morphological markers. Therefore, PD-123319 maternal administration partially reversed pulmonary arterial structural abnormality that characterizes CDH and decreased molecular markers of PH, which suggest that PD-123319 might reduce pulmonary vascular reactivity, and the risk of postnatal persistent PH observed in CDH neonates.

Regarding other antenatal pharmacological strategies to decrease PH in CDH, Luong *et al*. demonstrated recently that antenatal sildenafil treatment (from 11.5 to 20.5 dpc, daily subcutaneous injection) attenuates PH in experimental CDH (3). In fact, that study demonstrated that antenatal sildenafil treatment improved lung structure, increased pulmonary vessel density and reduced right ventricular hypertrophy in CDH (3). However, in opposition to PD-123319 treatment, sildenafil had not promoted lung growth, as demonstrated by LW/BW, and consequently it did not induce a recover of lung hypoplasia (3). Moreover, it is necessary to stress that the idea that there is PH in CDH, since

fetal period, is underlying to Luong's study (3). Nonetheless, it was already demonstrated that there is vascular hypoplasia in nitrofen-induced CDH and neonatal PH, but it is not yet proven the presence of PH in CDH fetus (27,58,59). Indeed, immediately after the birth, Luong *et al*. demonstrated the presence of right ventricular hypertrophy (an indirect signal of PH) in CDH neonates (3). In the present study, CDH neonates at 5 min after the birth did not present increase of right ventricular hypertrophy index or right ventricle thickness. However, CDH + S neonates presented an increase of right ventricular overload markers which reveals a molecular cardiac repercussion. According to the literature, it was already demonstrated that CDH vascular pulmonary alterations only affect neonatal and not fetal hemodynamics. Baptista *et al*. demonstrated that CDH is associated with significant molecular alterations secondary to PH, but only in the right ventricle and after birth (27). Moreover, also in fetal lamb CDH model, it was demonstrated that newborn CDH lambs had no differences
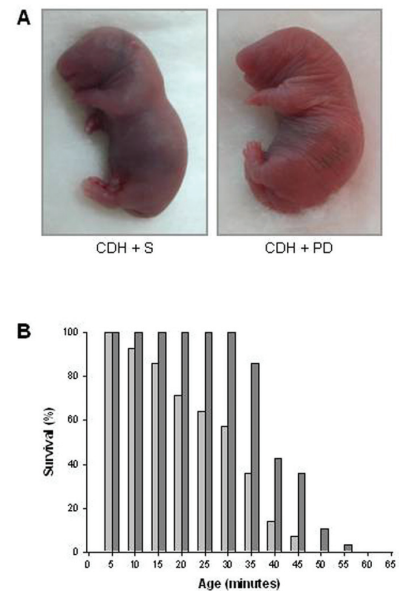
in right ventricular weight or right ventricular wall thicknesses compared with control lambs (60). Thus, despite the occurrence of pulmonary vascular modifications from early stages of prenatal development, the present study also shows that PH is only present after birth, likely occurring secondary to vascular hypoplasia that characterizes CDH, and also consequently to pulmonary vasoconstriction, which is secondary to alveolar hypoplasia, hypoxia and acidosis.

So, antenatal PD-123319 treatment interfered and improved the key determinants of mortality associated with CDH, namely lung hypoplasia and PH. Furthermore, maternal PD-123319 administration improved lung function, namely pulmonary gas exchange, as demonstrated by APGAR score, gasometric

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and survival evaluation. Regarding gasometry it is necessary to stress that due to low fetal blood volume, all blood possibly collected by decapitation was used for gasometric evaluation. So, gasometry evaluated a mix of arterial and venous blood. Nonetheless, antenatal PD-123319 treatment allowed a statistical significant improvement of acidosis, hypercapnia, hypoxia and lactate concentration that characterizes CDH fetuses. The results of these direct indicators of ventilation/perfusion matching quality suggest an obvious improvement of pulmonary gas exchange and peripheral O_2 delivery. Furthermore, this enhancement on lung function had important consequences on neonatal survival, namely PD-123319 treatment induced significantly longer average survival time. However, it is necessary to stress that the survival evaluation was performed without neonatal care or ventilatory support. This fact might be the explanation for the death of all neonates, despite the increase on lung function and survival time induced by antenatal PD-123319 treatment.

Regarding potential fetal adverse effects, the nitrofen-exposed pups presented decrease of HW/BW and KW/BW ratios as previously documented (10,61–63). On the other hand, PD-123319 beneficial effect seemed lung-specific, since HW/BW and KW/BW ratios of the pups were not altered. Concerning potential maternal secondary effects induced by PD-123319, no differences on heart, kidneys and lungs were observed. The absence of maternal deleterious effects could be due to the fact of AT_2 receptor expression is dramatically decreased after birth, being restricted to a few organs (11,12,41). Indeed, an increase of AT_2 receptor expression during adult life has been only observed under pathological conditions (41).

CONCLUSION

In conclusion, this study demonstrated the existence of a functional local RAS in fetal lung. Moreover, it establishes AT_2 receptor antagonist (PD-123319) as a pu-

tative antenatal therapy for pathologies characterized by fetal lung hypoplasia, such as CDH.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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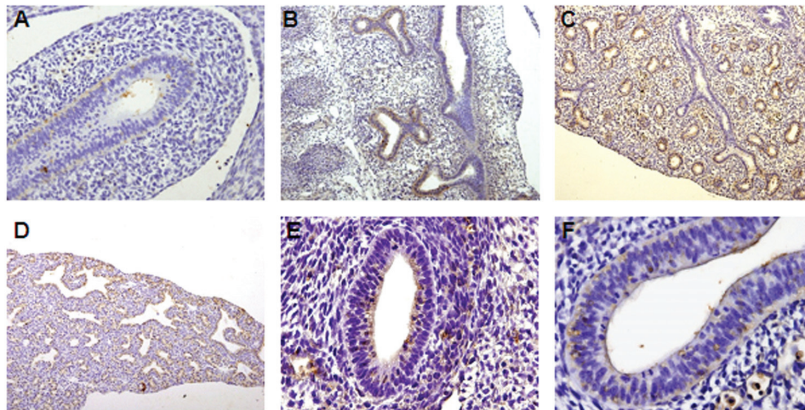
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Supplemental Data

Local Fetal Lung Renin-Angiotensin System as a Target to Treat Congenital Diaphragmatic Hernia

Cristina Nogueira-Silva,[1,2,3] Emanuel Carvalho-Dias,[1,2,4] Paulina Piairo,[1,2] Susana Nunes,[5] Maria J Baptista,[1,2,6] Rute S Moura,[1,2] and Jorge Correia-Pinto[1,2,7]

Supplemental Figure 1. Protein expression pattern of renin-angiotensin system components during early stages of fetal lung development. (A-D) Renin expression at 13.5, 15.5, 17.5 and 19.5 d post-conception (dpc), respectively. Renin protein expression appears to be maximal in the branching buds. (E) ACE and (F) AT_2 receptor expression in epithelial cells at 13.5 dpc. Original magnification: x100 (B-D), x200 (A), x400 (E, F).

PD-123319 AND CONGENITAL DIAPHRAGMATIC HERNIA
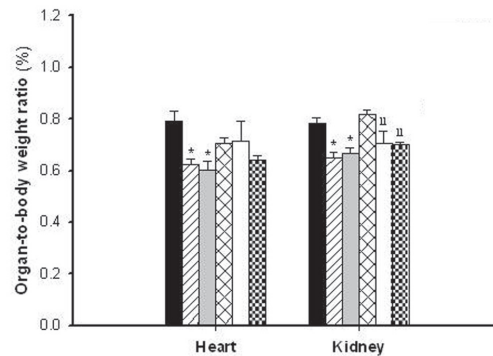
Supplemental Figure 2. Antenatal PD-123319 treatment effects on fetal heart and kidney. Heart-to-body weight and kidney-to-body weight ratios in control (C), nitrofen (N) and CDH groups treated with saline (■, C+S; ▨, N+S; ▥, CDH+S) or PD-123319 (▧, C+PD; □, N+PD; ▩, CDH+PD).The nitrofen treatment (nitrofen and CDH groups) induced decrease of heart and kidney-to-body weight ratios. Antenatal PD-123319 administration did not significantly change either heart or kidney growth. Results are expressed as %. $p < 0.05$: [*] vs. C+S, [‖] vs. C+PD.
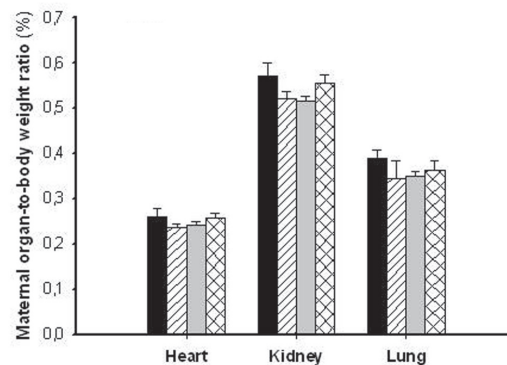
Supplemental Figure 3. Heart, kidney and lung-to-body weight ratios in control and exposed to nitrofen pregnant rats, treated with (■, C+S; ▨, N+S) or PD-123319 (▥, C+PD; ▧,N+PD). PD-123319 treatment did not significantly change heart, kidney or lung-to-body weight ratios. Results are expressed as %.

Supplemental Table 1. Morphometric assessment of pulmonary arteries stained with Weigert's resorcin fuchsin solution.

	Arteries examined per animal (n)	ED (μm)	ID (μm)	MA(%)	AA (%)
C+S	71.33±11.62	49.86±1.65	44.43±1.63	23.71±0.62	16.04±0.41
CDH+S	62.2±12.99	35.01±1.51[*]	27.75±1.49[*]	43.67±1.27[*]	30.27±0.87[*]
CDH+PD	70.33±9.60	34.69±1.34[*]	29.96±1.33[*‡]	30.07±0.84[*‡]	29.14±0.79[*]

AA: adventitial area; C: control; CDH: congenital diaphragmatic hernia; ED: external diameter; ID: internal diameter; MA: medial area; PD: PD-123319; S: saline. Values represent the mean ±SEM of measurements. $p < 0.001$: [*]vs. C+S; [‡]vs. CDH+S.

CHAPTER VII
DISCUSSION
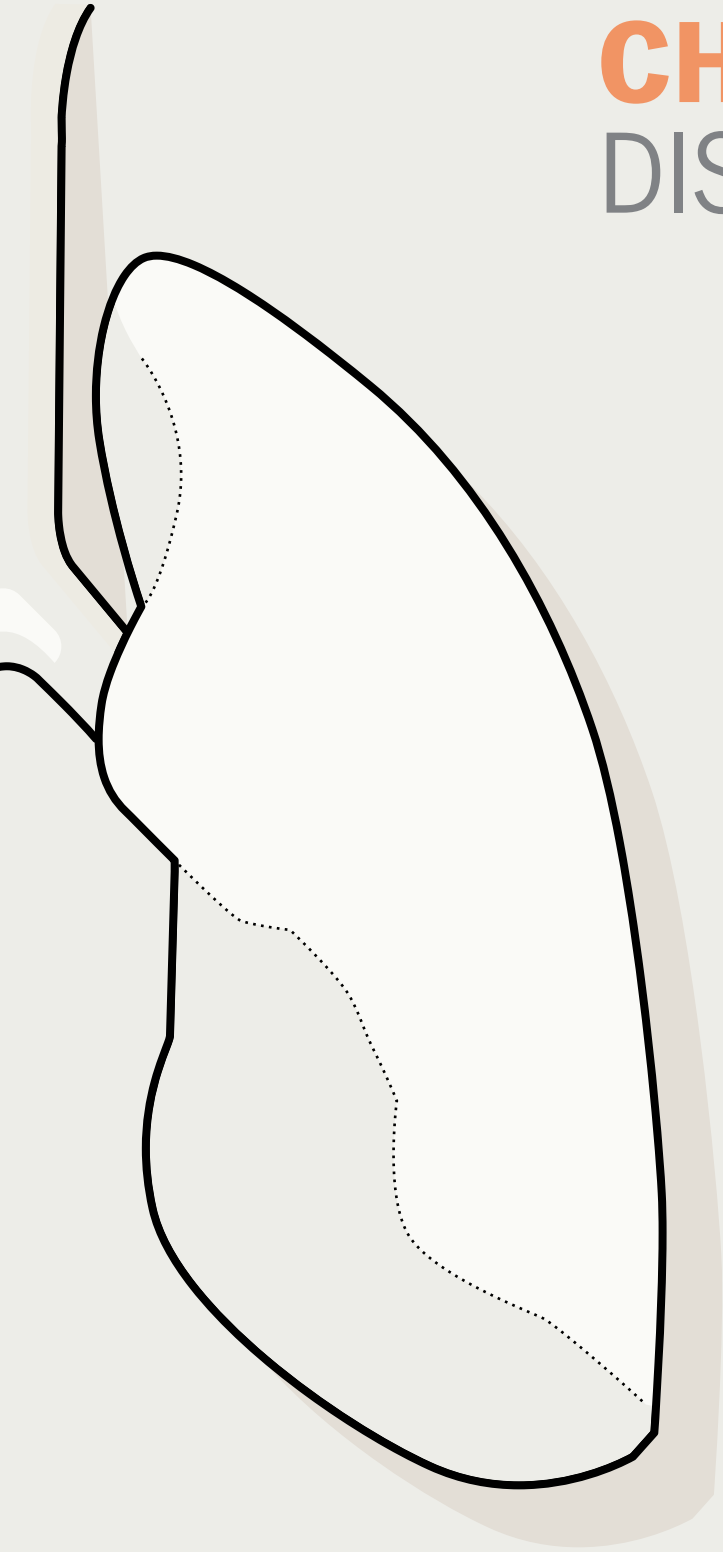
Discussion

Pulmonary development involves two distinctively regulated complex processes consisting of different cellular phenomena: early branching morphogenesis and late maturation processes [Warburton *et al.*, 2005; McMurtry, 2002]. The branching morphogenesis or lung growth process occurs through fundamental cross-talk interactions between epithelial and mesenchymal tissues, involving a multitude of effectors [Warburton *et al.*, 2005]. The knowledge of all effectors and regulators of lung development might be particularly useful envisioning therapy of lung diseases characterized by fetal lung hypoplasia as well as in modulation of lung repair. It is in this scientific context that this project comes out.

In this thesis, two new physiological regulators of fetal lung growth are proposed: inflammatory mediators and RAS.

Preterm birth is associated with both acute and chronic intrauterine infection and inflammation. Indeed, local uterine inflammation has been demonstrated in association with preterm delivery, and both chorioamnionitis and high amniotic fluid concentrations of pro-inflammatory cytokines are found among infants born before 30 weeks' gestation [Murthy & Kennea, 2007; Kallapur & Jobe, 2006; Demayo *et al.*, 2002]. Lung hypoplasia and immaturity and, consequently, RDS and BPD, are one of the most important complications associated to prematurity [Murthy & Kennea, 2007]. Since inflammation is common in the lungs of the preterm newborns, complex interactions between inflammation and lung development are been explored.

The fetal lung inflammation has been associated with decreased alveolar septation and microvascular development [Kallapur *et al.*, 2004]. At molecular level, it has been demonstrated that TNF-α, which is abundantly expressed in the lungs of preterm neonates, negatively regulates TTF-1 gene expression [Demayo *et al.*, 2002; Ohmori *et al.*, 1999]. Since TTF-1 controls lung development by regulating target genes, the latter interactions may inevitably inhibit lung development,

cellular differentiation, and production of pulmonary surfactant. Furthermore, members of the TGF-β family of growth factors play vital roles in early embryogenesis. A significant correlation exists between lung TGF-β levels immediately following premature birth and adverse pulmonary outcome [Demayo *et al.*, 2002; Lecart *et al.*, 2000]. TGF-β inhibits epithelial cell proliferation and differentiation, and lung morphogenesis in general. Moreover, inflammation activates the ubiquitous transcription factor NF-κβ, which mediates many of its biological effects. Induced perturbations in NF-κβ gene expression disrupt epithelial-mesenchymal interactions, repress BMP-4 and FGF-10 expression and, consequently, induce abnormal lung morphogenesis [Demayo *et al.*, 2002; Muraoka *et al.*, 2000]. Thus, a tight operational and functional linkage between inflammation and lung development exist [Demayo *et al.*, 2002]. Interestingly, injection of lipopolysaccharide into the amniotic fluid of fetal mice (at 15 dpc) induces toll-like receptor 4 (TLR4)-mediated inflammatory responses that alter airway fibronectin expression, potentially inhibiting distal-airway branching and alveolarization [Prince *et al.*, 2005; Levy, 2007]. In addition, activation of TLR2 or TLR4 in the developing mouse lung inhibits the production of FGF-10 [Benjamin *et al.*, 2007; Levy, 2007]. Overall, these observations provide examples of potential interactions between inflammatory mediators and normal morphogenetic pathways.

Regarding lung maturation, in opposition to deleterious effects on lung growth, several clinical and animal-based studies have suggested that antenatal exposure to inflammatory mediators may improve lung volume and compliance as well as accelerate fetal lung maturation [Jobe & Ikegami, 2001; Jobe *et al.*, 2000]. In fact, using different animal models, such as the rat, the rabbit or the sheep, it was shown that intra-amniotic injection of endotoxin or continuous administration of IL-1α, IL-6 or IL-8 improved lung function by increasing expression of SPs [Moss *et al.*, 2002; Kramer *et al.*, 2002; Willet *et al.*, 2002; Jobe & Ikegami, 2001; Bachurski *et al.*, 2001; Jobe *et al.*, 2000; Ikegami *et al.*, 2000; Willet *et al.*, 2000]. In human premature newborns, it was already proved that an increase of cytokines, namely IL-6, promotes lung maturation and decreases the incidence of RDS [Kallapur & Ikegami 2006; Jobe & Ikegami, 2001; Shimoya *et al.*, 2000]. Moreover, the presence of funisitis has been associated with a decreased risk for the development of neonatal RDS in preterm gestations. This observation also suggests that the fetal involvement of placental inflammation may be beneficial to the fetal lung maturation [Lee *et al.*, 2011]. Thus, several clinical and experimental studies validated the concept that fetal exposure to inflammation can have profound effects on the lung and fetal responses vary depending on the severity of the inflammation [Kallapur & Jobe, 2006]. Nonetheless, several controversies involve this issue.

IL-6, a multifunctional inflammatory cytokine, presented contradictory roles according to adult or fetal perspectives. Several adult pulmonary diseases, namely asthma, chronic obstructive pulmonary disease and non-small-cell lung cancer, implicate IL-6 in their pathophysiology [Doganci *et al.*, 2005; Bhatia & Moochhala, 2004; Chung, 2001]. Regarding fetal lung development, previous reports focused IL-6 in maturation processes and in a pathological context [Willet *et al.*, 2002; Kramer *et al.*, 2002; Moss *et al.*, 2002; Bachurski *et al.*, 2001; Shimoya *et al.*, 2000; Ikegami *et al.*, 2000; Willet *et al.*, 2000]. However, several studies have demonstrated that IL-6 can also modulate lung growth. Indeed, it was demonstrated that IL6Rα is widely distributed in developing human fetuses including in bronchial epithelial cells, which suggest an IL-6 nonhematopoietic role in the developing fetus [Dame & Juul, 2000]. Moreover, McClintock & Wagner described that IL-6 plays an important modulatory role in lung angiogenesis, a fundamental process in fetal lung morphogenesis [McClintock & Wagner, 2005]. Furthermore, several studies have emphasized the importance of IL-6 signaling in several processes of branching organs, such as embryonic submandibular gland development, mammary gland remodeling, and benign and malign prostate growth [Culig *et al.*, 2005; Zhao *et al.*, 2004; Jobe & Ikegami, 2001; Melnick *et al.*, 2001; Jaskoll & Melnick, 1999]. In this context, we evaluated the role of IL-6 during fetal lung development (**Chapter II**).

Our study demonstrated that IL-6 was constitutively expressed in primitive lung epithelium throughout gestation, displaying highest mRNA levels of expression during pseudoglandular stage. While previous studies on IL-6 and lung development emphasized maturational process, the highest expression of IL-6 on the early fetal lung development stages suggests that the physiological role for IL-6 is most likely linked to branching. Thus, hypothesizing a role for endogenous epithelial expression of IL-6 during lung growth, functional studies were performed. Blocking IL-6 significantly reduced branching and cellular proliferation rate in normal fetal lung explants, whereas IL-6 supplementation induced a biphasic effect on branching and cellular proliferation rate, with increased effect at lower doses, while no additional effect was obtained with the highest dose. These IL-6's effects were most likely mediated through p38 signaling pathway. Regarding the biphasic pattern of IL-6 effects on branching rate, it is interesting to stress that these results mimic the effect of intrauterine infection, which seems to protect very premature infants against RDS, while high quantities of pro-inflammatory cytokines cause serious disease [Hallman *et al.*, 2001]. In conclusion, this study suggested a physiological role for endogenous IL-6 on pulmonary branching mechanisms. Interestingly, chorioamnionitis induces the production of IL-6, possi-

bly providing a mechanism by which the innate immune system can hasten the development of respiratory function in the context of infection-induced preterm birth [Levy, 2007].

IL-6 belongs to the family of the gp130-type cytokines. This family comprises IL-6, LIF, IL-11, OSM, CNTF, CT-1 and CLC [Heinrich *et al.*, 2003; White & Stephens, 2011]. Recently, IL-27 and NP had been also added to this cytokines family [White & Stephens, 2011]. These cytokines share the membrane glycoprotein gp130 as a common signal transducer which explains the fact that these show some functional redundancy, even though they also exhibit specific biological activities [Carbia-Nagashima & Arzt, 2004; Heinrich *et al.*, 2003]. According to our results demonstrating that IL-6 is an important regulator of normal lung growth, it is possible that other inflammatory mediators, namely the other members of gp130 family of cytokines, are involved on normal lung development. Thus, we decided to clarify if the other gp130-type cytokines are important physiological regulators of fetal lung growth (**Chapters III** and **IV**).

LIF is a pleiotropic cytokine, that exists in both soluble and matrix-bound forms, and that binds to a heterodimer LIFRα/gp130 [Carbia-Nagashima & Arzt, 2004; Heinrich *et al.*, 2003; Metcalf, 2003; Auernhammer & Melmed, 2000]. LIF was already largely studied and it was demonstrated that it regulates many cellular processes and displays several biological activities [De Breuck *et al.*, 2006; McColm *et al.*, 2006; Park *et al.*, 2005; Kurdi *et al.*, 2005; Metcalf, 2003; Kritikou *et al.*, 2003; Wang *et al.*, 2003; Sariola, 2002; Plisov *et al.*, 2001; Auernhammer & Melmed, 2000]. Regarding the lung, numerous lung structural cell types express LIF and the LIFRα, suggesting previously unrecognized roles for this cytokine in lung biology [Knight, 2001; Knight *et al.*, 1997; Knight *et al.*, 1999]. It was already described that LIF, with IGF-I, regulates lung maturation. In fact, LIF/IGF-I double deficient embryos present lung hypoplasia and defective differentiation of the alveolar epithelium and vasculogenesis [Moreno-Barriuso *et al.*, 2006; Pichel *et al.*, 2003]. Our study demonstrated that LIF and its subtype receptor is constitutively expressed during lung development and that, in opposition to IL-6, LIF inhibits lung branching (**Chapter III**). These findings suggest that although cytokines of the gp130 family share a common signal transducer, there are specific biological activities for each cytokine on lung development. In fact, specific characteristics are emerging for each member of this family, brought about mainly through restricted temporal and spatial release, differential expression of cell surface receptors and different signaling patterns between gp130 homodimers and heterodimers [Knight, 2001]. Interestingly, LIF receptor is a gp130 heterodimer, whereas IL-6 receptor is a gp130 homodimer. Thus, this raises the hypothesis that the effect, on lung development, of cytokine signaling through gp130 heterodimers might be different and even opposite to gp130 homodimers.

In this sequence, the other members of the gp130-type cytokines (IL-11, OSM) were also assessed (**Chapter IV**). In a similar way to IL-6, IL-11 acts in a gp130 homodimer receptor and it was demonstrated that stimulates lung branching. On the other hand, OSM receptors are gp130 heterodimers and it was described that inhibit lung growth. All these results demonstrated that cytokine signaling through gp130 homodimers stimulate, whereas cytokine signaling through gp130 heterodimers inhibit lung branching. This specificity of gp130-type cytokines might represent a regulatory mechanism of lung morphogenesis, intrinsic to this family of cytokines, in order to achieve the correct lung growth. Moreover, regarding previous studies relating inflammation, lung maturation and incidence of RDS, it is well known that although intrauterine infection seems to protect very premature infants against RDS, high quantities of pro-inflammatory cytokines cause serious disease [Hallman *et al.*, 2001]. Therefore, our results arouse a new explanation for inflammation effects on lung growth and maturation. In this way, the beneficial effect of a mild inflammatory context and the deleterious effect of a severe inflammatory context on these processes might not rely on the dose-effect of each cytokine, but instead on the balance between agonists of homodimers *vs.* heterodimers receptors. These results bring a new field for further studies not only in normal lung development, but also in lung diseases and regeneration.

As well known, lung is composed by two branched tree-like systems, the airways and the vessels, which development occur in a reciprocal coordinated way [Morrisey & Hogan, 2010; Shi *et al.*, 2009]. In fact, developing pulmonary vasculature may not be passively accompanying the development of airways, as was previously believed, but it has an important regulatory role in airway branching morphogenesis in mammals [Tuder *et al.*, 2009; Chinoy, 2002]. It was demonstrated that an anti-angiogenic factor, endothelial monocyte activating peptide II, also inhibits lung airway morphogenesis [Schwarz *et al.*, 2000]. In a heart–lung culture model, the enhancement of pulmonary vasculature development was associated with improved airway development [Zgleszewski *et al.*, 1997]. Moreover, VEGF inhibition in neonatal rats leads to arrested alveolar development, suggesting that inhibition of vascular growth itself may directly impair lung development [Thébaud *et al.*, 2005; van Tuyl *et al.*, 2005; Compernolle *et al.*, 2002; Jakkula *et al.*, 2000; Healy *et al.*, 2000; Gerber HP *et al.*, 1999]. On the other hand, lung explants supplementation with VEGF stimulated epithelial and vascular morphogenesis [Del Moral *et al.*, 2006; Compernolle *et al.*, 2002; Corne *et al.*, 2002]. It was also demonstrated that ablation of lung epithelium impairs lung vascular cells development [Sarah *et al.*, 2000]. Therefore, reciprocal
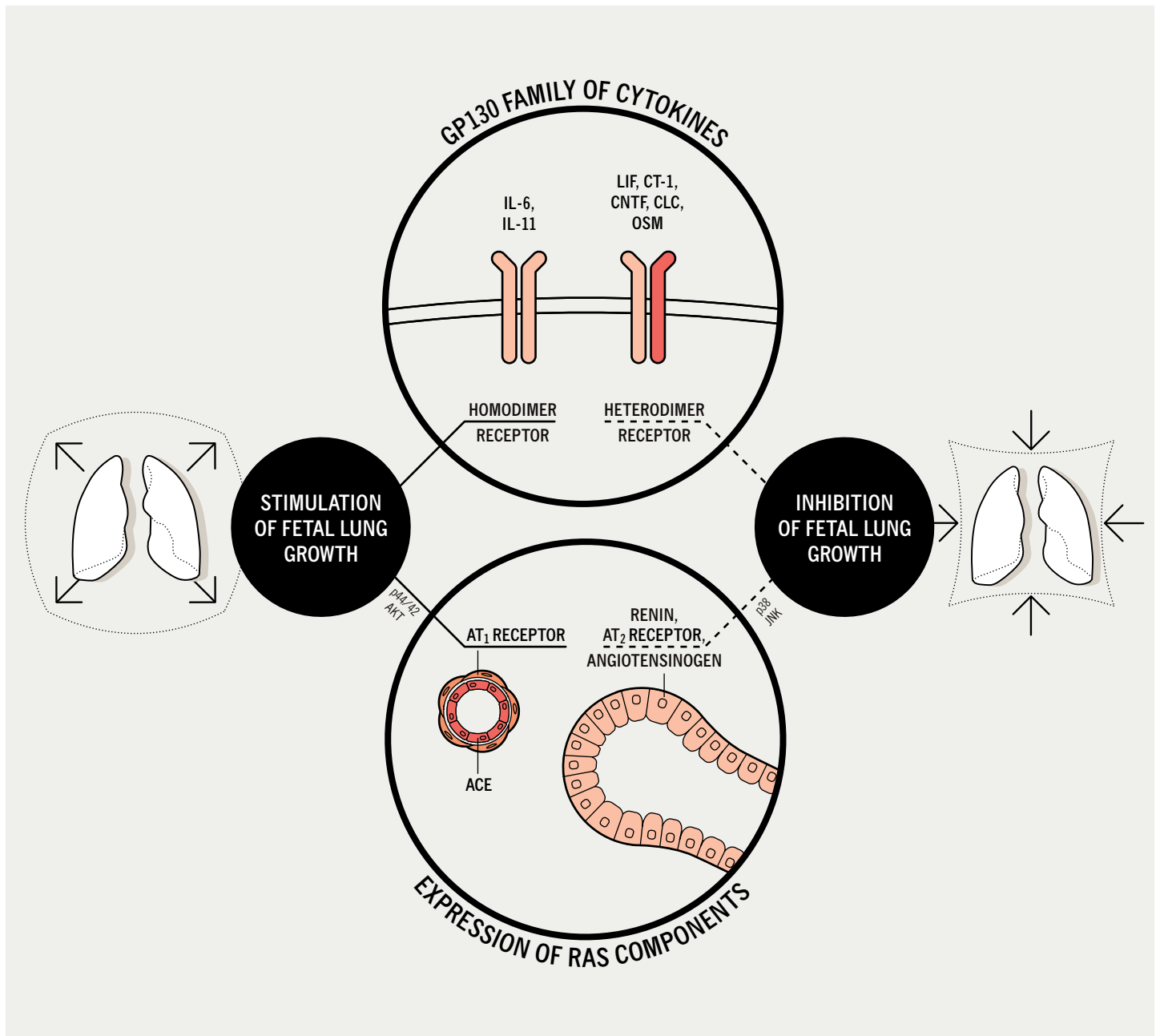
Figure 5. Overview of the two new physiological regulators of fetal lung development identified in the present dissertation: gp130 family of cytokines and lung RAS. Regarding the role of gp130 family of cytokines during fetal lung development, cytokines signaling through gp130 homodimers (IL-6 and IL-11) stimulate fetal lung growth, whereas cytokines acting in a gp130 heterodimer receptor (LIF, CT-1, CNTF, CLC and OSM) inhibit lung growth. Furthermore, a local and physiologically active RAS was described during fetal lung morphogenesis. Renin, angiotensinogen and AT2 receptor are predominantly expressed on lung epithelium, whereas ACE is expressed in endothelial cells and AT1 receptor in vascular smooth muscle cells. ANG II induces a stimulatory effect on lung branching, mediated by AT_1 receptor, through p44/42 and Akt phosphorylation. In opposition, ANG II acting in AT_2 receptor inhibit lung branching, through decrease of p38 and JNK phosphorylation.

interactions between airways and blood vessels are critical for normal lung development [Hislop, 2002; Warburton *et al.*, 2000; Kasahara *et al.*, 2000).

Having in the mind the major aim of this thesis, the discovery of new physiological regulators of lung development, and after the elucidation of the role of gp130-type cytokines, epithelial media-tors, looking for new vascular mediators emerged as the logical approach.

The RAS has been largely studied and it has important physiological roles. Classically, it is a major regulator of blood pressure, electrolyte and fluid homeostasis [Lavoie & Sigmund, 2003]. During the last decades, evidences have demonstrated the presence of a functional local RAS with autocrine/paracrine actions in a lot of organs or systems and with functions on develop-ment of fetal kidney, heart, vasculature and adrenal development. In fact, local ANGII formation and its tissue-specific effects on growth and differentiation are thought to be extremely important for embryonic and fetal development [Paul *et al.*, 2006]. Regarding lung morphogenesis, there is some evidence that lung expresses ACE as well as AT_1 and AT_2 receptors during fetal develop-ment [Morrell *et al.*, 1996; Shanmugam *et al.*, 1996; Shanmugam *et al.*, 1994]. Moreover, it is known that the maternal administration of ACE-I or ARA against hypertension, during gestation, induces several congenital anomalies, such as lung hypoplasia [Walfisch *et al.*, 2011; Quan, 2006; Cooper *et al.*, 2006]. Motivated by these evidences, a possible role of RAS during fetal lung development was studied (**Chapter VI**)

Our study demonstrated that all RAS components (renin, ACE, angiotensinogen, AT_1 and AT_2 receptors) are constitutively expressed in the lung during gestation and that ANG II has an important stimulatory effect on lung branching, mediated by AT_1 receptor, through p44/42 and Akt phosphorylation. These results allowed the description of a local and physiologically active RAS during fetal lung morphogenesis. Interestingly, an opposite effect of AT_1 and AT_2 receptors on lung growth, namely a stimulatory effect of AT_1 and inhibitory effect of AT_2, were described. This functional antagonism between AT_1 and AT_2 receptors, with respect to tissue growth, could be physiological interpreted as a basis of a RAS homeostasis at the tissue level.

The possible mechanism by which RAS interferes with the airway branching or pulmonary vascular development is still unclear and further investigation is required. However, given that in our study it was demonstrated that some components of RAS are expressed on epithelium and others on mesenchyme/vascular cells, it is possible that RAS is involved in both processes: airway and vasculature branching. Indeed, renin and its substrate angiotensinogen are mainly expressed in epithelial cells, while ACE that will produce ANG II is expressed in endothelial cells.

AT_1 receptor is predominantly expressed by vascular smooth muscle cells, while AT_2 receptor is expressed in bronchial epithelial cells. Thus, ANG II, the physiologically active peptide, could act on the airway and vasculature. Interestingly, excluding renin, all the other RAS components are expressed in vascular cells in the later gestational ages. This fact might represent a functional change of RAS near of the term.

Regarding the literature there are reports describing lung hypoplasia as a deleterious effect of ACE-I or ARA in pregnant women. Until this moment, the mechanism by which pulmonary hypoplasia develops is unknown, but may be related to compression of the fetal chest wall in the oligohydramniotic milieu with a restriction of fetal breathing movements [Quan, 2006]. Our study provides a new possible molecular explanation for fetal lung hypoplasia induced by ACE-I or ARA drugs, independent of amniotic fluid volume. ACE-I induce complete inhibition of ANG II synthesis and, as demonstrated by our results, ANG II increases lung growth. Regarding ARA drugs, these are AT_1 receptor specific, and AT_1 receptor mediates stimulation of lung growth. So, in this way, the fetal lung hypoplasia induced by ACE-I and ARA could be explained.

In figure 5 is schematically depicted the two new physiological regulators of lung development described in the present work.

We are conscious of the apparent absence of pulmonary defects in KO mice for gp130-type cytokines [Fasnacht & Müller, 2008; Carbia-Nagashima & Arzt, 2004; Nakashima, 1999; Betz *et al.*, 1998] and RAS components [Paul *et al.*, 2006; Stoll & Unger, 2001; Akishita *et al.*, 1999; Kakinuma Y *et al.*, 1997; Hein *et al.*, 1995]. Despite, the KO approach bears intrinsic problems, since the ensuing phenotype may consist of compensatory events during development and later life [Stoll & Unger, 2001], we believe that gp130 family of cytokines and RAS contributes to branching morphogenesis, but most likely as a modulator. Thus, we propose that in lung growth there are morphogens, factors that plays a critical role in patterning the early branching events in lung development, such as FGF-10, TTF-1, SHH and BMP-4. Null mutant mice for these morphogens present a dramatic inhibition of bronchial branching [DeFelice *et al.*, 2003; Sekine *et al.*, 1999; Motoyama *et al.*, 1998; Pepicelli *et al.*, 1998; Bellusci *et al.*, 1996; Peters *et al.*, 1994]. The new physiological regulators now described are more modulator systems of the lung growth, but they are not the only modulators and other systems could have the same modulator function on lung growth.

In last decades, the knowledge in science and medicine has evolved exceedingly. In clinical practice, health indicators significantly improved. Nonetheless, pediatric and adult diseases characterized by lung hypoplasia or dysplasia are yet an epidemiological relevant issue, involving

important morbidity and mortality rates. For someone that has a medical and physiological formation and everyday performs functions as resident in Obstetrics and Gynecology, the development of basic studies in fetal lung development, looking for its new regulators, has obviously a clinical rationale and aim: try to contribute for the development of new therapeutic strategies for lung diseases. In this sequence, since long, the lab where this thesis was developed has focused research on the pathophysiology and development of potential prenatal therapeutics for CDH.

CDH is still a mysterious disease and a clinical huge challenge. Despite the extensive experimental and clinical investigation in this disease as well as advances in neonatal care, in the most severely affected subset of CDH newborns, the prognosis remains poor, with a morbidity and mortality rates that remains exceedingly high [Luong *et al.*, 2011; Keller *el al.*, 2010; van den Hout *et al.*, 2009; Colvin *et al.*, 2005; Gallot *et al.*, 2005; Greer *et al.*, 2003]. Thus, the actual consensus for the high-risk fetuses is that something needs to be done before birth.

Lung hypoplasia and persistent PH associated with this disorder are the key determinants of mortality [Luong *et al.*, 2011; Keller *el al.*, 2010; van den Hout *et al.*, 2009]. Since prenatal therapy directed to cope with late mechanical determinants of lung hypoplasia (surgical approach) is apparently unsuccessful, antenatal therapies aiming to promote fetal lung growth and/or modulation of PH remain an appealing approach. In this context, antenatal pharmacological treatment to stimulate lung parenchyma and to treat PH are under investigation [Luong *et al.*, 2011; Santos *et al.*, 2006; Baptista *et al.*, 2005; Thébaud *et al.*, 1999]. In fact, it should be emphasized that even a small gain in total lung parenchyma might be determinant in providing a better adaptation of CDH fetuses to extra-uterine life. However, until the moment, none of the above strategies had resulted in significant clinical impact. Therefore, new therapeutic strategies are required and we believe that it is necessary to go and fro from the bed to bench side and to investigate the disease and new regulators of normal lung growth. Furthermore, it is necessary to stress that fetal lung hypoplasia and PH are common findings in other several conditions (such as oligoamnios, congenital cystic adenomatoid malformation, congenital heart disease, idiopathic pulmonary arterial hypertension, pulmonary capillary hemangiomatosis), which increase the relevance to develop new strategies that promote lung growth and treat PH.

In this context, we assessed the role of the new physiological regulators of lung development described by us, gp130 family of cytokines and local lung RAS, in CDH (**Chapters V** and **VI**).

For that we used the nitrofen-induced CDH model that is one of the best to investigate the etiology, pathogenesis and therapeutic options in CDH. In fact, the nitrofen model reasonably replicates
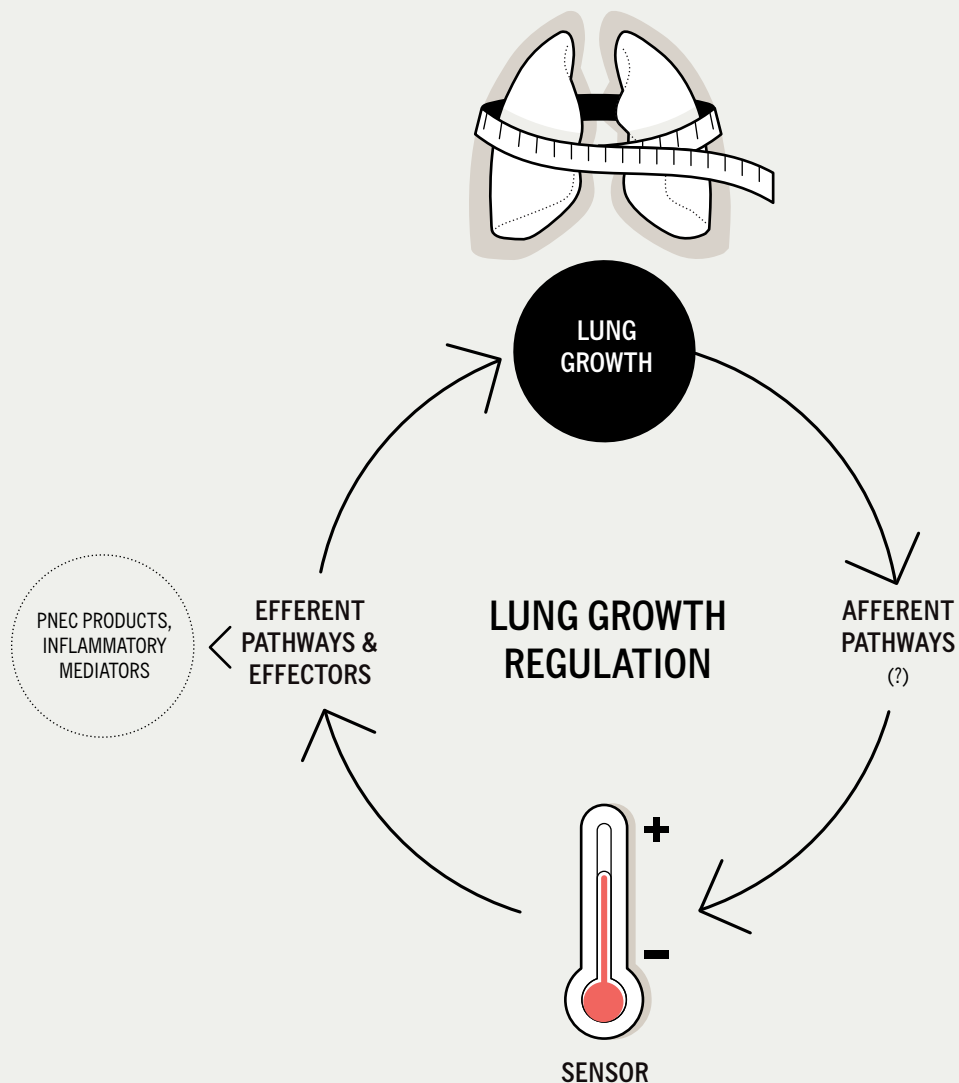
Figure 6. Proposed model for regulation of lung growth. This dissertation demonstrated that hypoplastic fetal lung has the intrinsic ability to regulate its growth in order to achieve the expected size. These observations suggest an intrinsic mechanism of lung growth regulation. We proposed that afferent pathways detect the lung size and send this information to lung size sensor. This sensor is able to modulate lung growth, stimulating or inhibiting efferent effectors that induce or inhibit lung growth. PNEC products and inflammatory mediators, namely IL-6, are upregulated in fetal hypoplastic lungs and we proposed them as possible effectors, responsible for inducing fetal lung compensatory response to growth retardation. The knowledge of mediators (afferent arms, sensor and efferent arms) involved on this hypothetic feedback mechanism during lung development is relevant, since this would open the possibility to modulate lung growth.

the major abnormalities (diaphragmatic defect, lung hypoplasia, PH, and cardiovascular and skeletal defects) and the pathophysiology described in human CDH [van Loenhout *et al.*, 2009; Kling & Schnitzer, 2007; Migliazza *et al.*, 1999a; Migliazza *et al.*, 1999b; Tenbrinck *et al.*, 1990].

An interesting point about CDH is the ability of hypoplastic lung to recover to normal size, when relieved from mechanical factors. In fact, clinical observations demonstrated that hypoplastic lungs from surviving CDH infants undergo a tremendous growth after post-natal diaphragmatic repair. Similarly, in the nitrofen-induced CDH rat model, it was already described that in fetuses exposed to nitrofen that did not develop diaphragmatic defect, fetal lung recovered from lung growth retardation throughout gestation [Correia-Pinto *et al.*, 2003; Baptista *et al.*, 2005]. Moreover, we also demonstrated that *in vitro* nitrofen-exposed fetal lungs are able to recover from hypoplasia (Chapter II). On the other hand, the *catch-up growth* is a classic infantile phenomenon defined as growth acceleration beyond the normal rate [Kay's & Hindmarsh, 2006; Gafni & Baron, 2000]. Based on this classical concept, we analyzed the daily branching rates of nitrofen-induced hypoplastic explants and we demonstrated that hypoplastic explants have the ability to grow *in vitro* at higher rates than control explants and, consequently, recover from early hypoplasia. Thus, this phenomenon of lung growth recovery with acceleration beyond the normal rate, after a transient period of growth inhibition, clearly resembles the phenomenon of height *catch-up growth* well-known in infants. Therefore, we demonstrated, for the first time, that fetal lung is able to recover from growth retardation through a way that resembles the *catch-up growth* phenomenon (**Chapter V**).

Envisioning prenatal therapeutic strategies that promote lung growth in CDH, the knowledge of underlying mechanisms of this phenomenon might be particularly useful. Then, since we described that IL-6 is involved in lung morphogenesis and that this inflammatory mediator had already been involved in growth plate *catch-up growth* and in liver regeneration, we hypothesized that IL-6 might also have a role on intrinsic fetal lung *catch-up growth*. In fact, we showed that IL-6 might be involved in the mechanisms underlying this phenomenon, since blocking of IL-6 activity significantly reduced the ability of nitrofen lung explants to recover from growth retardation.

An interesting point about lung ability to recover to normal growth relates to the fact that it also takes place in the *in vitro* scenario, out of systemic environment. This clearly suggests that lung growth is regulated by a local and intrinsic feedback mechanism. Interestingly, similar intrinsic mechanisms have also been attributed to growth plate to explain the body size *catch-up growth*
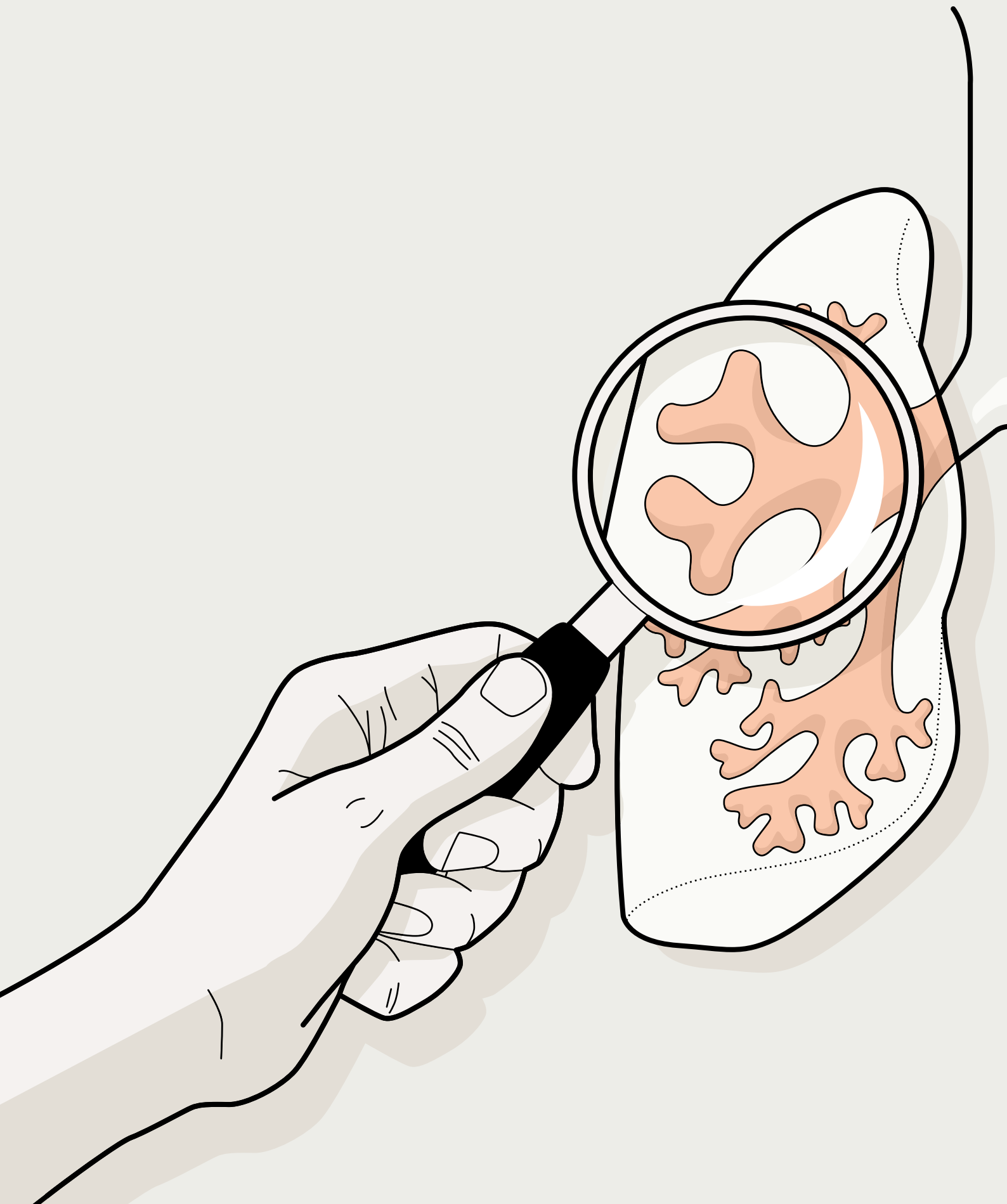
phenomenon [Gafni *et al.*, 2001]. The knowledge of mediators (afferent arms, sensors, efferent arms) involved on this hypothetic feedback mechanism during lung development is relevant, since this would open the possibility to modulate lung growth. In this context, another remarkable observation of our studies is the fact that IL-6 mRNA and protein were over-expressed in fetal hypoplastic lungs. Interestingly, IL-6 over-expression accompanies the severity of lung hypoplasia. Also PNEC and its products, calcitonin gene-related peptide (CGRP), GRP, and ghrelin, have been reported to be increased in CDH [Santos *et al.*, 2006; Dakhama *et al.*, 2004; Emanuel *et al.*, 1999; Ijsselstijn *et al.*, 1997; IJsselstijn *et al.*, 1995]. These PNEC products as well as IL-6 have mitogenic properties and promote lung growth. Thus, we propose that these upregulated molecules in hypoplastic lungs are biologically responsible for inducing fetal lung compensatory response to growth retardation. Therefore, these IL-6 overexpression and PNEC hyperfunction might be interpreted as the efferent actions, aiming to increase the mitogenic potential of CDH hypoplastic fetal lungs. Other questions remain to be clarified namely the afferent mediators and the sensors of lung hypoplasia as well as other efferent growth factors. In addition to these observations that clearly suggest an intrinsic mechanism of lung growth regulation, one can not rule out also an involvement of systemic loops. In fact, it remains unknown if IL-6 and neuroendocrine products over-expression is induced by local and/or systemic factors.

In figure 6 is depicted our proposed model for regulation of correct lung size and growth. Future studies aiming to clarify this regulatory loop and its mediators have clinical relevance for pathologies characterized either by lung hypoplasia or dysplasia.

Concerning new therapeutic targets and strategies that promote lung growth is necessary to remind that, in CDH fetuses, lung growth recovery of the affected side is limited by mechanical compression. On the other hand, the contra-lateral lung develops almost normally, it is not being compressed and, therefore, theoretically it has potential to develop compensatory overgrowth if therapeutically stimulated. In this context, regarding the existence of a functional local RAS in fetal lung morphogenesis, our results demonstrated a significant stimulatory effect on lung growth mediated by ANG II and mimicked by the treatment with an AT_2-antagonist (PD-123319). These results led us to hypothesize that AT_2 could be an excellent new target (in opposition to maternal treatment with ANG II) for treatment of diseases characterized by fetal lung hypoplasia, such as CDH (**Chapter VI**). In fact, AT_2 receptor is described to be expressed virtually only during fetal life [Paul *et al.*, 2006; Lavoie & Sigmund, 2003; Kaschina & Unger, 2003], which would annul potential maternal adverse effects. Indeed, an increase of AT_2 receptor expression during adult life has been only

observed under pathological conditions [Kaschina & Unger, 2003]. Interestingly, activation of RAS, namely activation of AT_1 receptor, was been related with development of pre-eclampsia [Zhou *et al.*, 2008]. So, hypothetically, the use of an AT_2 receptor antagonist during pregnancy could activate preferably AT_1 receptor and induce pre-eclampsia. However, it was recently described that angiotensin receptor binding was undetectable in normal and pre-eclamptic pregnant uterine placental bed. Thus, placental bed does not express AT_2 receptor, which annuls a possible development of pre-eclampsia induced by AT_2 receptor antagonist.

In the nitrofen-induced CDH model, *in vivo* antenatal AT_2-antagonist (PD-123319) treatment improved the key determinants of mortality associated with CDH, namely lung hypoplasia and PH. In CDH neonates, despite the presence of mechanical forces that compress lungs, AT_2-antagonist increased lung growth by promoting distal lung development and, consequently, induced partial recovery of lung hypoplasia (a recover of 11.4%). The potential clinical relevance of this effect should be emphasized, since such an increase in lung parenchyma can be determinant in providing a better adaptation of CDH fetus to extra-uterine life. Furthermore, maternal PD-123319 administration ameliorated indirect parameters of PH (pulmonary arterial structural abnormalities and molecular markers of PH), which suggest that PD-123319 might reduce pulmonary vascular reactivity, and the risk of postnatal persistent PH observed in CDH neonates. Moreover, maternal PD-123319 administration improved lung function and survival time of CDH pups. Actually, the increase of lung growth enhanced pulmonary gas exchange and peripheral O_2 delivery, as demonstrated by significant improvement of acidosis, hypercapnia, hypoxia and lactate concentration that characterizes CDH fetuses. Furthermore, this enhancement on lung function had important consequences on neonatal survival. Thus, the discovery of RAS as a new regulator of fetal lung growth allowed the establishment of AT_2 receptor antagonist (PD-123319) as a putative antenatal therapy for pathologies characterized by fetal lung hypoplasia, such as CDH. These results open the perspectives for future pre-clinical studies.

CHAPTER VIII
CONCLUSIONS
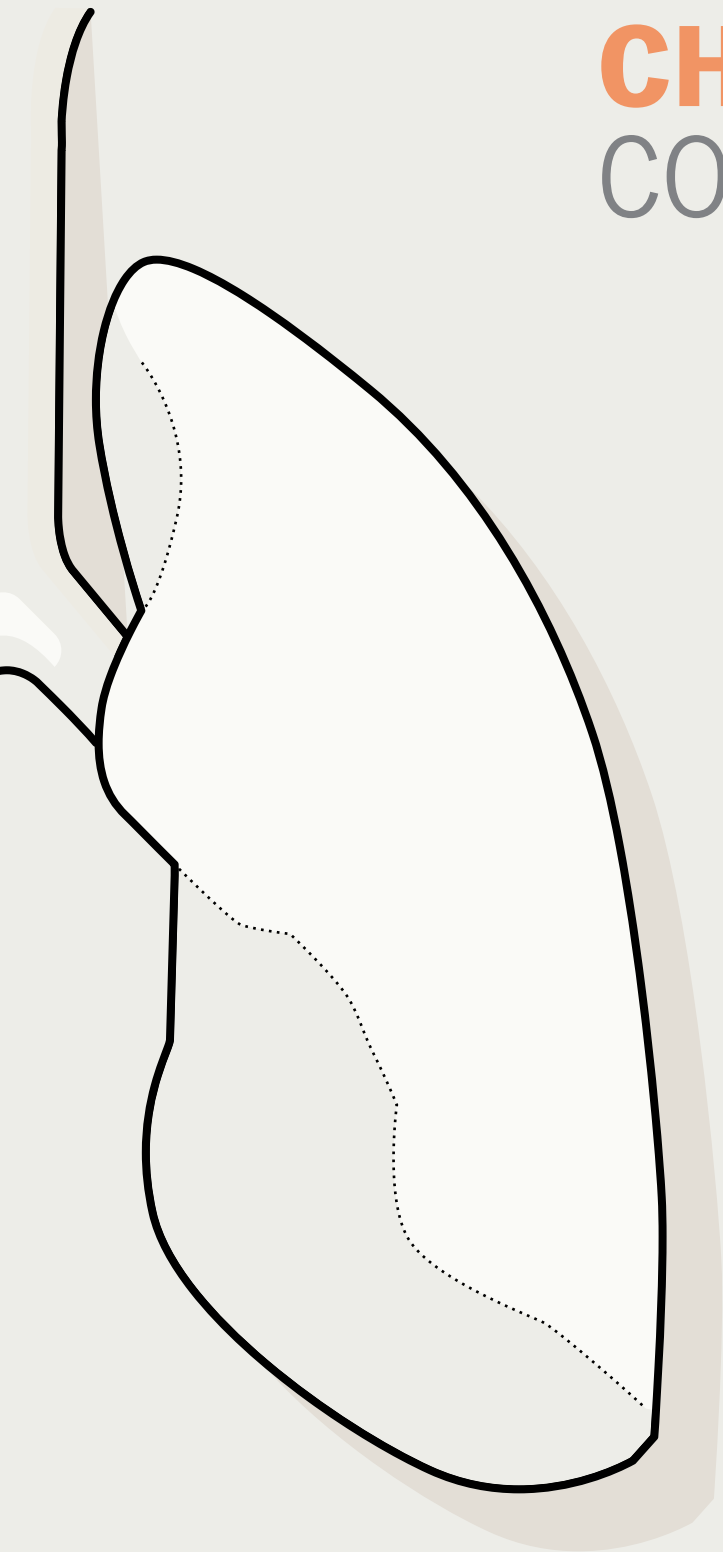
Conclusions

Having in the mind the aims of the present dissertation, namely the discovery of novel physiological regulators of lung development and their use as therapeutic targets for CDH, main achievements derived from this dissertation are listed below.

i. IL-6 is constitutively expressed in pulmonary epithelium throughout fetal lung development, presenting highest levels of expression during pseudoglandular stage. Moreover, IL-6 enhances fetal lung explants branching, most likely involving p38-MAPK intracellular pathway. Thus, endogenous IL-6 has a physiological role on pulmonary branching mechanisms.

ii. LIF and its subunit receptor LIFRα are constitutively expressed during fetal lung development and they have an inhibitory physiological role on fetal lung branching.

iii. The effect, on lung development, of cytokines signaling through gp130 homodimers is opposite to gp130 heterodimers. Indeed, cytokines signaling through gp130 homodimers (IL-6 and IL-11) stimulate lung branching, whereas cytokines acting in a gp130 heterodimer receptor (LIF and OSM) inhibit lung growth. This specificity of gp130-type cytokines might represent a regulatory mechanism of lung morphogenesis, intrinsic to this family of cytokines, in order to achieve the correct lung growth.

iv. We demonstrated that nitrofen-induced hypoplastic explants have the ability to grow *in vitro* at higher rates than control explants. Thus, hypoplastic fetal lungs are able to recover from growth retardation through a way that resembles the *catch-up growth* phenomenon, which suggests the presence of an intrinsic mechanism of lung growth regulation. Moreover, we showed that IL-6 might be involved in the mechanisms underlying this phenomenon.

v. There is a local and physiologically active RAS during lung morphogenesis. In fact, all components of RAS (renin, ACE, angiotensinogen, AT1 and AT2 receptors) are constitutively expressed in the lung throughout gestation. Moreover, ANG II induces a stimulatory effect on lung branching, mediated by AT_1 receptor, through p44/42 and Akt phosphorylation.

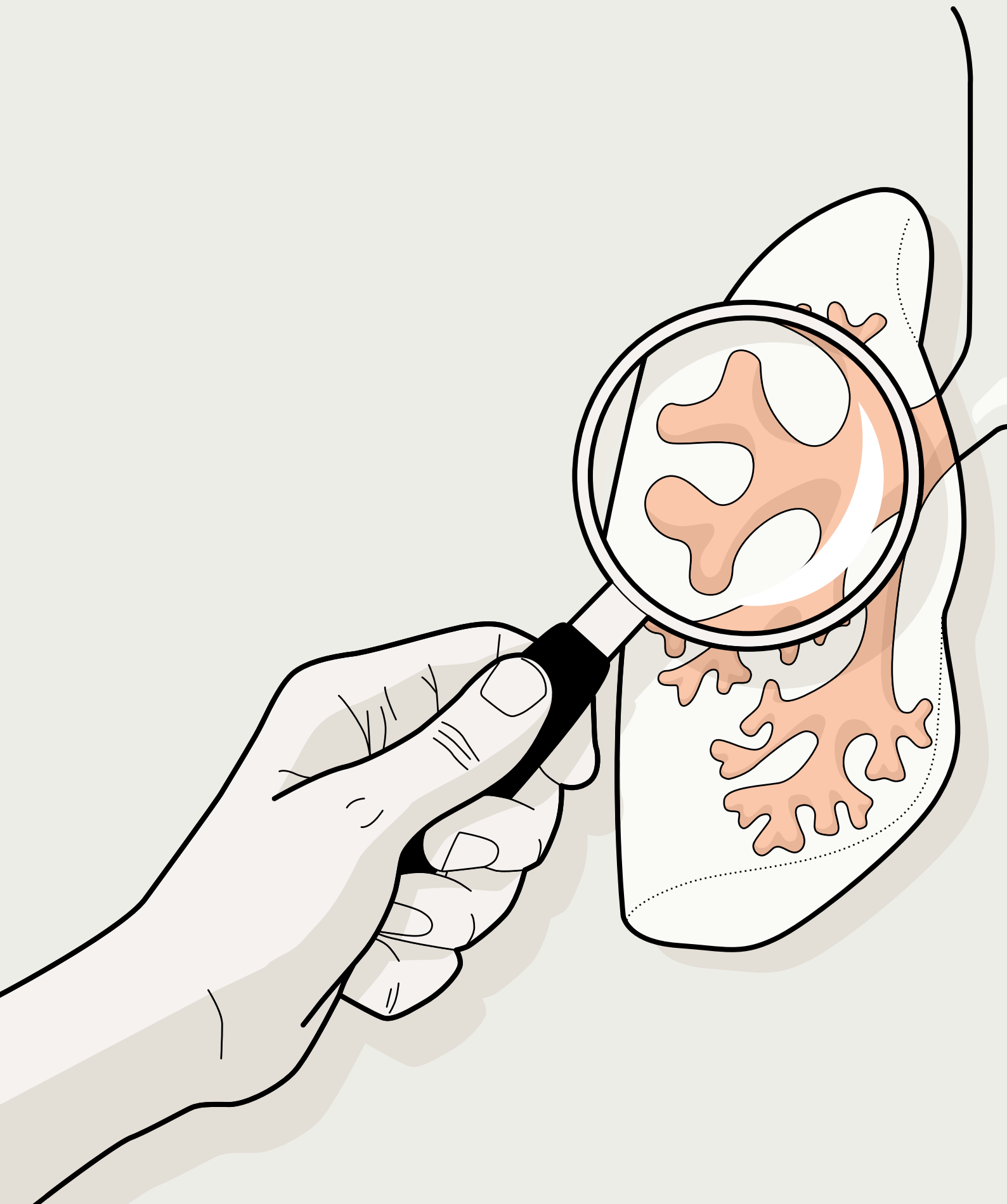vi. The selective inhibition of AT_2 receptor is a putative antenatal treatment for pathologies characterized by fetal lung hypoplasia, such as CDH. In fact, in nitrofen-induced CDH model, antenatal PD-123319 (an AT_2 receptor antagonist) treatment increased neonatal lung growth, ameliorated indirect parameters of PH, improved lung function and survival, without maternal or fetal deleterious effects.

In summary, in this PhD thesis, according to the proposed aims, we have identified two novel physiological regulators of fetal lung development: gp130 family of cytokines and local lung RAS. Moreover, we demonstrated that fetal lung has the intrinsic ability to regulate its growth in order to achieve the expected size. Finally, we showed that selective inhibition of AT_2 receptor is a putative antenatal therapy for pathologies characterized by lung hypoplasia, such as CDH.

Regarding **future perspectives**, it is our perception that this thesis naturally leads to three fields of research:

i. The description of the role of gp130 family of cytokines on lung development, namely an opposite effect induced by homodimers and heterodimers receptors, arouse a new hypothetical explanation for inflammation effects on lung growth and maturation. The beneficial effect of a mild inflammatory context and the deleterious effect of a severe inflammatory context on these processes might not rely on the dose-effect of each cytokine, but instead on the balance between agonists of homodimers vs. heterodimers receptors. Thus, this hypothesis should be clarified not only in context of prematurity, but also in lung diseases, repair and fibrosis. The role of the most recently gp130 cytokines described, IL-27 and neuropoietin, needs also to be investigated. Moreover, a possible crosstalk between gp130 dependent family of cytokines and other cytokines with relevant roles on preterm birth (IL-1a, IL-8, TNF-a) should be elucidated. Furthermore, the study of the role of these other inflammatory mediators as lung growth regulators (and not only maturation regulators) might be important. Indeed, increasingly, to the classic inflammatory mediators have been assigned non-immune roles, namely developmental and morphogenetic roles.

ii. As we demonstrated that fetal lung has the intrinsic ability to regulate its growth, we proposed a model for regulation of correct lung size and growth. Nonetheless, future studies are necessary to clarify this regulatory loop and its mediators, namely the sensors of lung size, afferent mediators, other effectors (efferent mediators) and signaling pathways involved in compensatory growth. Moreover, the possible involvement of systemic loops needs to be investigated.

iii. The establishment of AT_2 receptor antagonist (PD-123319) as a putative antenatal therapy for pathologies characterized by fetal lung hypoplasia, such as CDH, open the perspectives for future pre-clinical studies.

At this time, for someone who believes that basic research is the keystone for clinical research and progression, which has technical laboratorial abilities and concern about congenital lung diseases, these fields of research would be the key of future investigation.

CHAPTER IX
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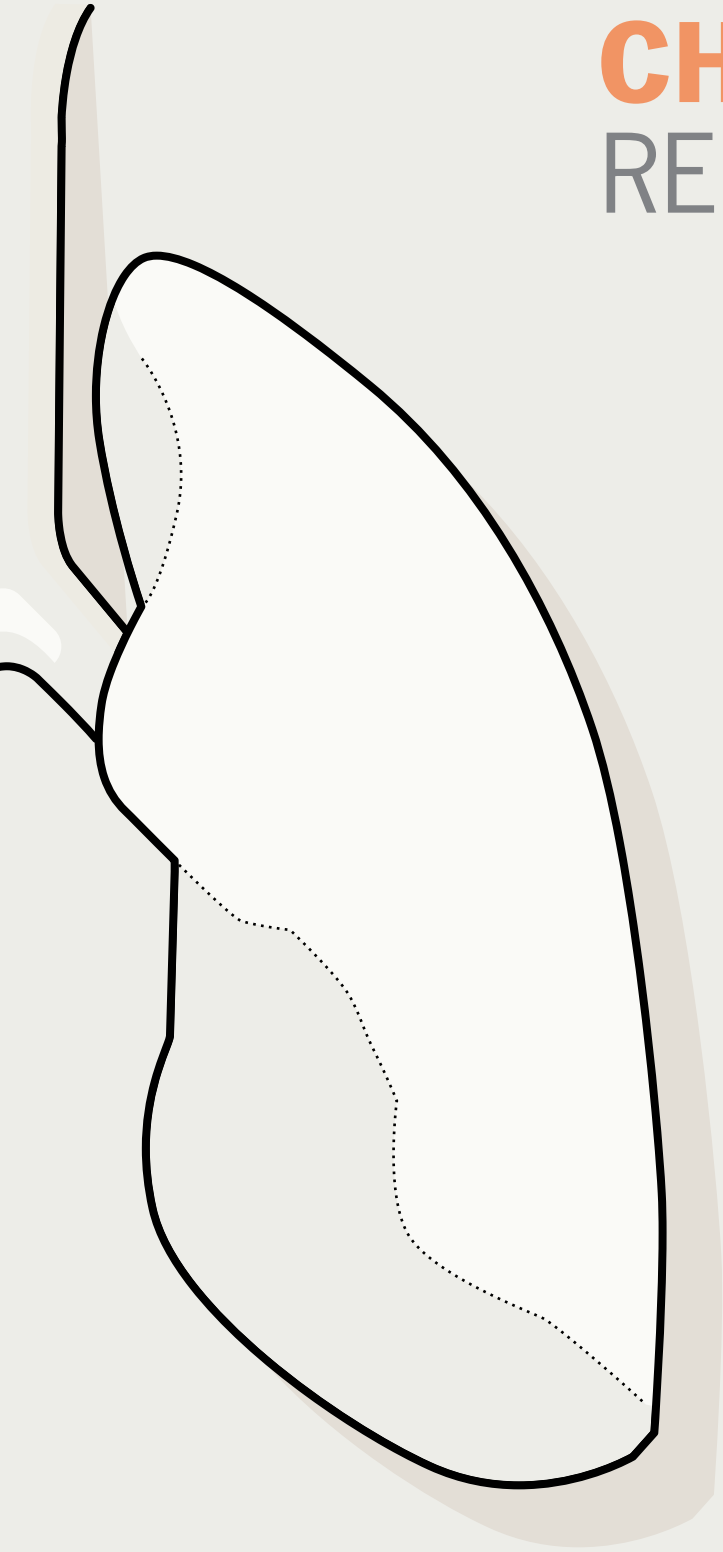
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Aqui terminam hoje estas viagens nas quais vocês me acompanharam ao longo da noite e do dia e do mar e do homem. De tudo quanto vos disse, muito mais é a vida.

Pablo Neruda

Gala Adeus SOF's, 2003

Prémio Maria Amélia de Mello, Lisboa, 2004

Braga, 2005

JOCEM, Porto, 2006

Prémio Caixa Geral Depósitos, 2007

Braga, 2007

Braga, 2007

ECS, 2009
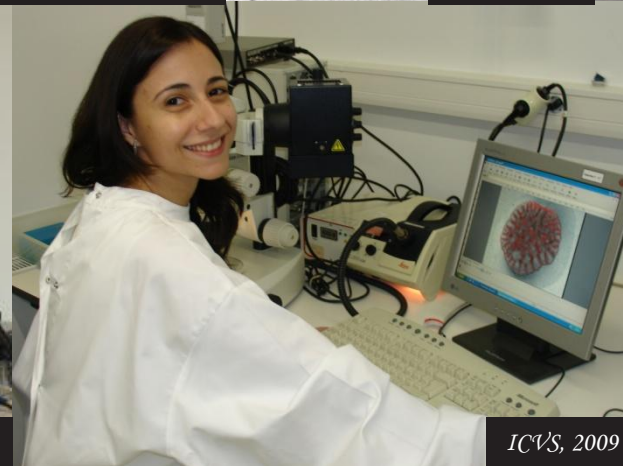
ICVS, 2009

Cristina Nogueira-Silva
INVESTIGADORA DA UNIVERSIDADE DO MINHO

A jovem investigadora viu premiado o seu trabalho no congresso das sociedades neonatais e perinatais, ao arrecadar uma medalha de ouro. Cristina desenvolveu um fármaco que pode ajudar no tratamento da malformação do pulmão fetal contribuindo, assim, para diminuir a alta taxa de mortalidade associada.

Braga, 2011

ICVS, 2012

Hospital S. Marcos, 2009

Braga, 2005

Jantar de Reis, ICVS, 2005

Juramento de Hipócrates, 2007

ECS, 2007

ECS, 2008

Istambul, 2008

ICVS, 2008

Prémio "Daniel Serrão" 2007 atribuído a jovem de Braga

Escola de Ciências da Saúde formou a melhor médica do Norte

Surgical Sciences Research Domain, Natal, 2010

Serviço de Ginecologia e Obstetrícia, Hospital de Braga, 2011

Best Moments