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Tese de Doutoramento em Ciências da Saúde

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Declaro ter atuado com integridade na elaboração da presente tese. Confirmo que em todo o trabalho conducente à sua elaboração não recorri à prática de plágio ou a qualquer forma de falsificação de resultados. Mais declaro que tomei conhecimento integral do Código de Conduta Ética da Universidade do Minho.

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“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.”
Marie Curie

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FCT Fundação para a Ciência e a Tecnologia
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ABSTRACT

Lung development is a complex process that involves a coordinated orchestration of several signalling pathways and mechanisms in order to growth normally. Pulmonary hypoplasia emerges as a result of foetal abnormal lung development and consequently it leads to high rates of morbidity and mortality. The understanding of normal and abnormal foetal lung growth has became clinical relevant because it can offer new perspectives in the treatment of lung diseases. Regardless the recent advances in the knowledge of lung development, there are still a lot of mechanisms that need to be elucidated and investigated.

The main aim of this PhD dissertation was to investigate new physiological regulators of foetal lung growth in order to find a prenatal therapy to revert foetal lung hypoplasia in Congenital Diaphragmatic Hernia (CDH) context. Moreover, two already well-known and studied signalling pathways (retinoic acid and neuroendocrine factors) were further investigated and an important link between them was discovered/established. Using molecular/histological techniques and gain-loss of function studies with nitrofen rat model and human samples we tried to achieve our goals.

In this PhD thesis, it was demonstrated that ephrins B1, -B2 and eph B4 receptor were expressed during all foetal lung developmental stages. Moreover ephrin B1 and eph B4 receptor mainly presented mesenchymal expressions whereas ephrin B2 presented an epithelial expression. Ephrin B1 and –B2 administration contributed to increase foetal lung branching, but the observed increase has not significant biological meaning. Another interesting finding was the importance of microRNAs in human CDH. MicroRNA 10a and microRNA 200b are up-regulated in CDH human lungs when compared to normal lungs and they are also changed in the tracheal fluid of CDH babies that respond or did not respond to FETO. In FETO responders, after removing the plug, tracheal fluid presented higher expression of miR-10a and miR-200 family when compared to non-responders. Moreover, in responders the expression of those microRNAs increased significantly after removing the plug when compared to the expression observed at the moment of plug insertion. In line with the second

aim mentioned above, we decided to look into two already known and apparently unrelated signalling pathways and discover a link between them because both of them are altered in CDH (retinoic acid was down-regulated and neuroendocrine factors were up-regulated). We found that neuroendocrine factors act as regulators of lung growth, sensitizing the lungs to the action of retinoic acid through RAR α and RAR γ up-regulation.

To conclude, in this thesis, we showed that ephrins seem to be strong candidates to act as morphogens or being involved in vascular regulation instead of being a promisor mechanism to regulate lung growth. Additionally, we discovered that microRNAs are an important tool as a prognostic biomarker for CDH new-borns outcome. Moreover, we established a novel physiological link showing that neuroendocrine factors and retinoic acid signalling pathways interact with each other during foetal lung growth regulation. With this work, new insights into normal and abnormal foetal lung development were brought, discovering novel mechanisms to further explore as potential therapeutic targets into CDH field.

RESUMO

O desenvolvimento pulmonar é um processo complexo que envolve a combinação e funcionamento coordenados de várias vias e mecanismos de sinalização, a fim do crescimento pulmonar normal. A hipoplasia pulmonar surge como resultado do desenvolvimento anormal do pulmão fetal estando associada a elevadas taxas de morbilidade e mortalidade. A compreensão do crescimento do pulmão fetal normal e anormal, tornou-se clinicamente relevante pelo facto de poder oferecer novas perspetivas no tratamento de doenças pulmonares. Independentemente dos recentes avanços no conhecimento do desenvolvimento pulmonar, ainda há muito que é preciso fazer, ser elucidado e investigado.

O objetivo principal desta dissertação de doutoramento foi investigar novos reguladores fisiológicos de crescimento do pulmão fetal, a fim de encontrar uma terapia pré-natal para reverter a hipoplasia pulmonar fetal no contexto da Hérnia Diafragmática Congénita (HDC). Por outro lado, foi descoberto e estabelecido um elo de ligação entre duas vias de sinalização (via do ácido retinóico e fatores neuroendócrinos) já bem conhecidas e estudadas como sendo importantes ao longo do desenvolvimento pulmonar fetal. Foram utilizadas técnicas moleculares/histológicas e estudos de ganho-perda de função com o modelo do nitrofeno em rato e com amostras humanas.

Nesta tese de doutoramento, foi demonstrado que as efrinas B1,-B2 e o recetor eph B4 são expressos durante todas as fases de desenvolvimento pulmonar fetal. Além disso, a efrina B1 e o recetor eph B4 expressaram-se essencialmente no mesenquima enquanto que a efrina B2 apresentou ter uma expressão epitelial. A administração de efrina B1 e –B2 em culturas de explantes pulmonares, contribuiu para aumentar a ramificação pulmonar fetal, embora o aumento observado não tenha significado biológico relevante. Outro achado interessante ao longo deste trabalho de Doutoramento, foi a importância dos microRNAs na fisiopatologia da HDC em humanos. O microRNA 10a e o microRNA 200b estão aumentados em pulmões humanos com HDC quando comparados com pulmões normais e a sua expressão está também alterada no fluido traqueal em crianças que

sobrevivem ou morrem à oclusão fetal da traqueia (FETO). Nos pacientes que sobrevivem depois do FETO, após se remover o balão da traqueia, o fluido traqueal apresentou uma maior expressão de miR-10a e da família do miR-200 quando comparados com os não sobreviventes. Por outro lado, nos sobreviventes, a expressão desses microRNAs aumentou significativamente após a remoção do balão quando comparada com a expressão observada no momento da inserção do balão na traqueia. Em linha com o segundo objetivo acima mencionado, decidimos revesitar duas vias de sinalização já conhecidas e aparentemente não relacionadas, e descobrir uma ligação entre elas, pelo facto de ambas estarem alteradas na HDC (défice de ácido retinóico e aumento dos fatores neuroendócrinos nomeadamente da bombesina e da grelina). Descobrimos que os fatores neuroendócrinos atuam como reguladores do crescimento pulmonar, sensibilizando os pulmões para a ação do ácido retinóico através do aumento da expressão dos seus recetores (RAR α e RAR γ).

Para concluir, nesta tese, mostramos que as efrinas parecem ser fortes candidatos para atuar como morfogéneos ou como fatores importantes na regulação vascular ao invés de serem um mecanismo importante de regulação do crescimento pulmonar. Por outro lado, descobrimos que os microRNAs são uma ferramenta importante que poderá vir a ser utilizada como um biomarcador de prognóstico em crianças com HDC. Adicionalmente, estabelecemos uma nova ligação fisiológica mostrando que os fatores neuroendócrinos e a via de sinalização do ácido retinóico interagem entre elas durante a regulação do crescimento fetal pulmonar. Com este trabalho, hipotetizámos novas abordagens sobre o desenvolvimento/regulação do pulmão fetal normal e anormal, descobrindo-se novos mecanismos para continuar a explorar como potenciais alvos terapêuticos para o tratamento da HDC.

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ABBREVIATIONS LIST

AGO1: argonaute 1

AGO2: argonaute 2

ALDH1A2: aldehyde dehydrogenase 2

AngII: angiotensin II

AT1: type 1 angiotensin receptor

AT2: type 2 angiotensin receptor

BEAS: bronchial epithelial cells

BMP4: bone morphogenetic protein 4

BPD: bronchopulmonary dysplasia

CDH: congenital diaphragmatic hernia (Posterolateral/Bochdalek)

CRBP: cellular retinol-binding proteins

Dpc: days post conception

ECM: extracellular matrix

EGF: epidermal growth factor

EGF-R: epidermal growth factor receptor

EMT: epithelial mesenchymal transitions

EphB4: receptor ephrin B4

ERK-1/2: extracellular signal-regulated kinases-1 and -2

FETO: fetoscopic endoluminal tracheal occlusion

FGFs: fibroblast growth factors

FGFR: fibroblast growth factor receptor

FGFRL1: fibroblast growth factor receptor-like 1

FOXA: forkhead box proteins

GATA: A/TGATA/G -binding transcription factor

HGF: hepatocyte growth factor

HIF1: hypoxia-inducible transcription factor 1

HIF2: hypoxia-inducible transcription factor 2

HIP: hedgehog interacting protein

HPH: hypoxia induced pulmonary hypertension

IGF: insulin-like growth factor

JNK: c-Jun NH_2-terminal kinases

LHR: lung to head ratio

LNA: locked nucleic acid

MAPKs: mitogen-activated protein kinases

MASH: mammalian achaete-scute homologue

MKKKs: MAPK kinases kinases

MKKs: MAPK kinases

MRI: magnetic resonance imaging

NEB: neuroepithelial bodies

PDGF: platelet-derived growth factors

PDGFR: platelet-derived growth factor receptor

PI3K / Akt: protein kinase B

PNECs: pulmonary neuroendocrine cells

PPROM: preterm premature rupture of membranes

PTC: patched receptor

RA: retinoic acid

RALDH 2: retinal dehydrogenase 2

RBP: retinol-binding protein

RISC: miRNA-associated RNA-induced silencing complex

RTK: receptor tyrosine kinase

SHH: sonic hedgehog

SOX : sex determining region Y-box

SPRY: sprouty

STAT3: Signal transducer and activator of transcription 3

TGF-β: transforming growth factor β

TTF1: tyroid transcription factor (Nkx2.1)

VEGF: vascular endothelial growth factor

WNT: wingless

ZEB: zinc finger E-box-binding homeobox

CHAPTER I.

GENERAL INTRODUCTION

1. NORMAL PULMONARY DEVELOPMENT

Lung is a vital organ in all species including human. Before birth, placenta is responsible for gas exchange so respiratory problems, caused by abnormal lung development, become an issue in extra uterine life. Normal lung development is a complex process, mediated by mesenchymal-epithelial interactions see review (Bartis D*, et al.*, 2014). Moreover, lung consists of two highly branched systems, the airways and the vasculature. Mammalian lung development is a long process, starting during foetal life at 3-4 weeks of gestation in humans, and in rats at 11.5 days post-conception (dpc), continuing in the early postnatal years. It starts with a small diverticulum, the laryngotracheal groove, where the proximal portion gives rise to the trachea and the larynx. The distal portion is involved in the formation of bronchial buds, which culminate with left and right lobar branches from the main bronchial tree see review (Herriges M & Morrisey EE, 2014).

Bronchioles are originated from lung epithelial cells that differentiate from the endoderm to give rise to the epithelial tube surrounded by branching mesenchymal cells. Anatomically, human lungs present three lobs in the right lung and two in the left whereas mice and rat lungs present four lobes in the right lung and a single lobe in the left lung. Lung development has been divided into six stages in all mammalian species: embryonic, pseudoglandular, canalicular, saccular, alveolar and vascular maturation see review (Correia-Pinto J*, et al.*, 2010)(figure 1).

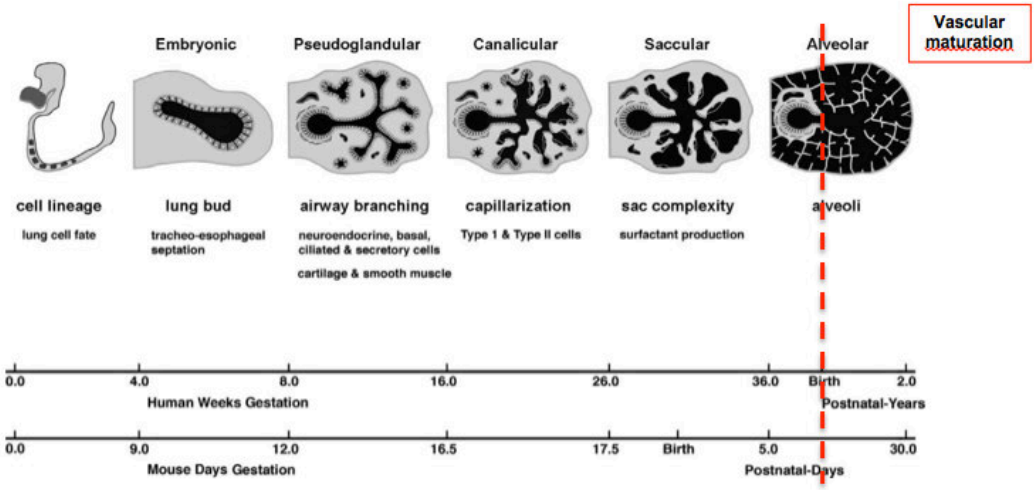

Figure 1. Overview of lung development: different phases of the developing lung in human and mouse, before and after birth. Adapted from (Kimura J & Deutsch GH, 2007).

The **embryonic** stage occurs between weeks 4 and 8 of human's gestation (9.5 to 12.5 in rat) and is mainly characterized by specification, budding of the lungs and tracheoesophageal septation. This stage characterizes the lung by the emergence of a ventral diverticulum of the foregut endoderm, separating from the oesophagus and elongating caudally. Sex determining region Y-box 2 (SOX 2) expression marks the proximal endoderm progenitor lineage. The expression of the tyroid transcription factor (TTF1, Nkx2.1) occurs mediated by Wnt signalling pathway and constitutes the first evidence of the respiratory system specification in the anterior foregut endoderm. A correct orchestration between SOX2, forkhead box 2 (FOXA2), binding transcription factor (GATA 4), GATA 6 in the endoderm and in the mesenchyme is required to establish normal lung growth. The left and right lobar branches of the bronchial tree are established as well as left and right asymmetry where FGFs and sonic hedgehog (Shh) have a crucial role. For instances, inhibition of Shh is associated with abnormal lung development. Moreover they are also important in later phases of lung differentiation.

The **pseudoglandular** stage occurs between weeks 8 and 16 of gestation in humans (12.5 to 16.5 dpc in rat). It is mainly characterized by airway branching. The mature conducting airway appears as a result of the progressive dichotomous branching as each branch bifurcates repeatedly.

The epithelial cells differentiate in a centrifugal manner and before 10 weeks after conception, the presence of cartilage can already be found around the main bronchi. Branching morphogenesis is regulated by several growth factors, receptors and signalling molecules that act as inductive signals mediating epithelial-mesenchymal interactions. Those interactions are crucial for normal lung development in early and later stages of lung growth, although their role during later stages of lung development is still unclear. For instances, FGF-10 and bone morphogenetic protein 4 (BMP4) are fundamental in specifying branching initiation sites and outgrowth (Weaver M*, et al.*, 2000). Sprouty (SPRY) and Noggin antagonize FGF and BMP signalling leading to limitation of branching specificity locations. Epidermal growth factor (EGF), Shh and Wnt are also responsible for branching regulation. FGF10 is high and Shh is low (locally suppressed by patched receptor – PTC) where a branch is supposed to occur; so Shh, FGF10 and mSPRY2 may determine FGF signal and are responsible for bud outgrowth rate at a given point and hence interbranch length (Roth-Kleiner M & Post M, 2003; Warburton D*, et al.*, 2005). In contrast, branching stops when FGF10 induces the dynamic expression of mSPRY2. mSPRY2 is synthetized at the most distal tip until it reaches maximum expression, inhibiting FGF10 expression.

The **canalicular** stage, occurs between 16 and 26 weeks of gestation in humans (16.5 to 17.5 dpc in rat) overlapping with the end of pseudoglandular stage. This stage is defined by the proximal-distal cell type specification and capillarization contributing to the beginning of blood-air interface. The pulmonary epithelium starts to differentiate into surfactant producers (type II cells) and type I cells (cells of thinned air-blood barrier). The most important transcription factors involved in this stage of lung development are: mammalian achaete-scute homologue 1 (Mash-1) and SOX 2 that are crucial for the pulmonary neuroendocrine cells and SOX 2, TTF 1, FOXA 2 and GATA 6 for the epithelial cells. In this stage the terminal bronchioles start dividing into respiratory bronchioles and alveolar ducts starting the gas-exchange region.

The **saccular** stage starts on week 26 and finishes on week 36 of gestation in humans (17.5 dpc to postnatal day 5 in rats). In this stage, lung

parenchyma increases by dichotomous branching of the terminal generations of the airway tree. Moreover the lung becomes prepared for the transition to air breathing with pulmonary surfactant production (4 different types of surfactants) by alveolar type 2 cells. The interstitium becomes thinner as a result of apoptosis and mesenchymal cells continue to differentiate. The first primitive alveoli start emerging and the capillaries grow into the mesenchyme surrounding the primary alveoli, forming two complex branching systems: airways and vasculature. In this stage the lack of amniotic fluid is associated with abnormal lung development and lung hypoplasia.

The **alveolar** stage starts at gestation week 36 and continue through early childhood. Alveoli in humans can be formed until at least 2 years of post-natal life. In rats this stage starts in the first week after birth, and culminate in the first month of life. The alveoli develop from the terminal endings of the alveolar sac and with time, their diameter increases, as well as the number of terminal saccules, alveolar ducts and alveoli (Roth-Kleiner M & Post M, 2003). Alveologenesis consist of gas-exchange capacity maturation and airway wall secondary crest septation (originate alveoli) and elongation. Furthermore, alveologenesis increase gas-exchange surface area. In this stage, platelet-derived growth factor-A (PDGF-A) and its receptor (PDGFR-α) are one of the leading factors responsible for controlling alveolarization. Regarding human lung parenchyma, it changes between a new-born and an adult lung because this stage continues to occur after birth. In contrast, the number of airways generations and branching pattern are already established at birth.

Vascular maturation is a postnatal stage occurring essentially during the first 2 to 3 years of life in humans. In this stage, capillary remodelling occurs and the thick capillary bilayer gives rise to a thin capillary monolayer (ephrins action), improving gas-exchange capacity and increasing gas-exchange surface area.

2. Regulation of Foetal Growth of Hypoplastic Lungs

Normal lung development is regulated by genetic factors; mechanical stimuli namely intra-thoracic compression, foetal breathing movements and chemical factors such as calcium concentration and relative hypoxia see review (Herriges M & Morrisey EE, 2014; Varner VD & Nelson CM, 2014).

Herein the most important factors/peptides/signalling pathways studied in hypoplastic lungs are summarized. All the basal levels are presented and compared between normal and CDH lungs.

Fibroblast Growth Factors

FGFs are involved in several processes such as cell proliferation, differentiation and migration during organogenesis (Itoh N & Ornitz DM, 2011). Concerning the embryonic lung, FGFs are crucial for the regulatory networks between epithelium and mesenchyme (Warburton D*, et al.*, 2000). There are some *in vitro* and *in vivo* studies with FGFs. FGF18, FGF10, FGF7 and fibroblast growth factor receptor-like 1(FGFRL1) show decreased expression in hypoplastic lungs late on gestation when compared to normal rat lungs (Teramoto H*, et al.*, 2003; Dingemann J*, et al.*, 2011; Takahashi H*, et al.*, 2013). In contrast, fibroblast growth factor receptor 2 (FGFR2) and FGFR3 are increased in hypoplastic lungs, and when FGF10 is administered to lung explants, it promotes lung growth and branching (Acosta JM*, et al.*, 2001; Friedmacher F*, et al.*, 2012). Moreover deficient mice for FGF10 present lung agenesis and in normal fetal rats, *in vivo* localized induced overexpression of FGF10 induces cystic lung malformations (Ohuchi H*, et al.*, 2000; Henriques-Coelho T*, et al.*, 2007; Gonzaga S*, et al.*, 2008).

Epidermal Growth Factor and Transforming Growth Factors

EGF and TGFα are members of EGF family binding and activating the same EGF receptor (EGF-R). Their mRNA expression and immunoreactivity are higher during early foetal life in the bronchiolar epithelium and EGF proteins and EGF-R are expressed in the lung epithelium (Guarino N*, et al.*, 2004).

Regarding EGF and TGF roles, it is described that EGF and TGFβ1 promote normal branching pattern and differentiation in the murine model after tracheal transection, and are involved in the regulation of normal lung development (Chinoy MR*, et al.*, 1998). In CDH human lungs and in bronchopulmonary dysplasia (BPD), EGF and TGFα expression is increased in the bronchiolar epithelium and in the proximal airways (Guarino N*, et al.*, 2004; Le Cras TD*, et al.*, 2004). Regarding TGFβ1 it is overexpressed on CDH rat lungs (Xu C*, et al.*, 2009). Moreover, *in vivo* maternal treatment with EGF promotes lung branching and maturation, increasing lung to body weight ratio in nitrofen CDH rat lungs (Li J*, et al.*, 2004). Additionally, EGFR null mutation leads to 50% branching reduction and to a lethal phenotype (Ma L*, et al.*, 2009).

Bone Morphogenetic Proteins

BMPs are implicated in regulating lung specification, branching and patterning. The BMP family comprise a branch of the TGFβ superfamily playing a key role in development and is expressed during embryonic lung development. BMP4 is expressed in pulmonary epithelium, with higher expression in the tips and adjacent mesenchyme. Moreover BMP4 expression is decreased in CDH rat lungs late in gestation when compared to normal lungs (Makanga M*, et al.*, 2013). Misexpression of BMP4 in transgenic mice resulted in smaller lungs and clearly dilated terminal sacs. On the other hand, overexpression of BMP4 induced recover of lung hypoplasia promoting lung branching (Warburton D*, et al.*, 2000). BMP4 knockouts triggered embryonic lethality.

Wingless

Wnt is expressed during lung development, playing a key role in lung morphogenesis. Its expression remains unclear especially considering early phases of lung growth, however Wnt gene products are involved in epithelial to mesenchymal interactions during lung development (Takayasu H*, et al.*, 2010). There are some published data regarding Wnt ligands and CDH. Wnt2 is decreased in CDH rat lungs in the pseudoglandular stage when

compared to normal lungs. In opposite, Wnt5a is increased on CDH rat lungs late in gestation (Doi T & Puri P, 2009; Takayasu H*, et al.*, 2010). Wnt7b inhibition decreased airway branching causing pulmonary hypoplasia and reducing lung smooth muscle (Rajagopal J*, et al.*, 2008). Indeed, Wnt5a knockouts present delayed lung maturation and respiratory failure, and Wnt7b null mice presents hypoplastic lungs and prenatal lethality, which suggests their strong association with lung development.

Sonic hedgehog

Shh is a secreted glycoprotein with several patterning roles in the developing embryo. Looking to Shh expression in rat lungs, its expression is detected in the airway epithelial cells and decreased after 18.5 dpc. Shh is crucial for branching morphogenesis regulation because inhibits FGF 10 expression to initiate and maintain lung branching. It is well known that Shh null mutants present lung hypoplasia. Shh expression is locally supressed at branch tips by the induction of its PTC receptor and hedgehog interacting protein (HIP). Consequently, the activation of PTC induces glioblastoma (GLI) transcription factors (Gli 1, Gli 2, and Gli 3). Moreover, CDH rat lungs present a decreased expression of Shh late in gestation when compared to normal foetal lungs (Unger S*, et al.*, 2003).

Angiotensin

Little was known about lung development and Angiotensin. Angiotensinogen is expressed in epithelial, endothelial and vascular smooth muscle cells. Recent data showed a rat study where Angiotensin II (AngII) promotes *in vitro* lung branching mediated by type 1 angiotensin receptor (AT1). In foetal lung explant cultures, administration of AngII and type 2 angiotensin receptor (AT2) agonist promotes lung branching whereas AT1 agonist inhibits lung growth. Moreover, when AT2 agonist is administrated *in vivo* to pregnant dams, CDH new-borns present improved lung function and the survival rate increases (Nogueira-Silva C*, et al.*, 2012b).

Table 1 presents a summary of the molecular alterations observed in CDH foetal lungs. Moreover, *in vitro* and *in vivo* studies are also summarized.

Table 1. Molecular alterations in CDH foetal lungs – *in vitro* and *in vivo* studies.

Key molecular players									
	FGF	**EGF & TGFs**	**BMP**	**Wnt**	**Shh**	**AngII**	**Ghrelin**	**Bomb**	**RA**
Basal levels	FGFR2↑ FGFR3↑ FGF18↓ FGF10↓ FGF7↓ FGFRL1↓	EGF↑ TGFα↑ TGFβ1↑	BMP4↓ BMPR2↓ BMP7↓	Wnt2 ↓ Wnt5a ↑	↓	NA	↑	↑	↓
In vitro (administration)	FGF10 ↑ LB CTR	EGF ↑ LB CTR TGF β1 ↓ LB CTR TGF β2 ↓ LB CTR	NA	Wnt7b inhibition= PH CTR	NA	↑ LB CTR	↑ LB CTR	NA	↑ LB CTR ↑ LB
In vivo (administration) & Knockouts (KO)	FGF10 = cystic lung anomalies CTR	EGF ↑ Lung maturation ↑ LB CTR TGF β3 KO =Embryonic Lethality	BMP4 KO =Embryonic Lethality	Wnt5a KO =Respiratory Failure Wnt7b KO =Hypoplastic lungs	Shh KO =Hypoplastic lungs	AT2 ↓	↓ PH	↓ PH	↓ RARα/β KO =PH

KO – Knockout; PH – Pulmonary hypoplasia; NB – Normal branching; NA – Not available; CTR – Control; ↑ - Increased; ↓ - Decreased; = - Equal

3. EPHRINS

There is a strong correlation between ephrins and its receptors with cardiovascular development, angiogenesis, cell migration, tissue border maintenance and vascular remodelling. Ephrins expression can distinguish between arteries, when ephrin B2 is expressed, and veins, when ephrin B4 receptor (eph B4) is expressed, and for this reason are considered as arterial and venous markers, respectively (Coultas L*, et al.*, 2005). Interestingly, ephrins and its receptors expression, appear before the circulatory system is defined, and are not restricted to arterial or vein endothelial cells. Moreover in lung, ephrins expression also appears in mural cells. Published data have shown that mice lacking either ephrin B2 or eph B4 die at 10.5 dpc. In fact, defects in vascular patterning in the yolk sac and within the embryo are displayed. Moreover, when ephrin B2 is downregulated, ephrin B2 treatment preserves alveolar and lung vascular growth. Ephrin B1, B2 and eph B4 knockout mice die early in gestation and display angiogenesis impairment (Gerety SS*, et al.*, 1999; Adams RH*, et al.*, 2001; Davy A*, et al.*, 2004).

Eph receptors and ephrins form the largest group of the receptor tyrosine kinase (RTK) family and mediate numerous developmental processes in multiple organisms. Ephrins are transmembrane proteins that communicate between external stimulus and the interior of the cell controlling several cell processes (Bai J*, et al.*, 2014). Indeed, ephrins are divided into two sub-groups: eph A and eph B, based on their ligand binding affinity and structure of the extracellular domain.

There are nine eph A receptors in human genome, which connect five ephrins-A and six eph B receptors, and that bind three ephrins-B ligands see review (Park I & Lee HS, 2015). Besides, upon ephrin ligand and eph receptor (cell surface proteins) binding, bidirectional signalling occurs.

Eph receptors and ephrins signalling is involved in several developmental processes namely cell adhesion, migration, chemo-repulsion, tissue morphogenesis, cytoskeleton dynamics, cell positioning, axon guidance, patterning, segmentation and vascular remodelling. All of these processes contribute to the formation of different tissues and organs. In this sense, they exert a central role in embryonic development, controlling cell

proliferation and death, cell adhesion, attraction and repulsion and cellular shape. On the other hand, in adulthood, they regulate remodelling of synapses, bone remodelling, epithelial differentiation and integrity, immune function, stem cell self-renewal and insulin secretion see review (Genander M & Frisen J, 2010). In injury conditions, eph receptors and ephrins are altered. For instance considering injuries in central nervous systems, ephrins and eph receptors are up-regulated and regarding skeletal malformations, they are down-regulated. Moreover eph receptors and ephrins can be expressed both in the same cells or they can have their own pattern depending on the tissues.

Ephrins are present in different branching organs such as pancreas, mammary gland, kidney, and also the lung. In the lung, ephrin B1 only binds to some eph Bs in contrast to ephrins B2, which binds to all eph Bs receptors and eph A4 see review (Fagotto F*, et al.*, 2014). Ephrin B2 was already tested *in vivo* and *in vitro,* in the adult rat model, and it promotes: lung repair, vascular growth, microvascular endothelial cell viability and consequently attenuates pulmonary hypertension (Vadivel A*, et al.*, 2012). Recent data showed that ephrin B2 is expressed in alveolar type II cells and ephrins B2, -B1 and eph B4 receptor are expressed in all gestational ages of lung development in rats (Bennett KM*, et al.*, 2013). Ephrins and eph are also involved in lung cancer, being up or downregulated. For instance, ephrin A1 overexpression is strongly associated with metastasis, tumour progression and bad prognosis and ephrin B3 is involved in non-small cell lung cancers (Stahl S*, et al.*, 2013; Ieguchi K*, et al.*, 2014).

3.1 INTRACELLULAR SIGNALLING PATHWAYS

Mitogen-activated protein kinases (MAPKs) constitute key downstream targets of FGF signalling pathway in numerous cell types. MAP kinases are divided into three major families: extracellular signal-regulated kinases-1 and -2 (ERK-1/2), c-Jun NH_2-terminal kinases (JNK) and p38 kinases. MAPKs are known to regulate cellular proliferation and migration. The mammalian genome contains two ERK/MAP kinase genes, Mek1 and Mek2 that activate

ERK1 and ERK2. Inactivation of Mek genes in mesenchyme results in diverse phenotypes including lung hypoplasia. Interestingly, Mek gene mutation in respiratory epithelium leads to lung agenesis. ERK1 and 2 are typically activated by FGFs and are important for normal lung development (Liu Y*, et al.*, 2004). Moreover, their role in lung development is based on the effects of RTK activation including hepatocyte growth factor (HGF), PDGF, EGF, FGF, insulin-like growth factor (IGF) and G protein activation see review (Ciccarelli A & Giustetto M, 2014). Thus, the ERK/MAPK pathway is crucial for the establishment of mesenchymal and epithelial signals required for respiratory tract development. On the other hand, JNK and p38 are involved in stress responses and apoptosis. p38/MAPK is activated through a cascade of kinases defined as MAPK kinases kinases (MKKKs) and MAPK kinases (MKKs) and is involved in controlling branching morphogenesis (Liu Y*, et al.*, 2008).

Another important signalling cascade concerning lung growth and development, cell proliferation and migration, is protein kinase B (PI3K / Akt). PI3K / AKT is an intracellular signalling pathway regulating cell cycle that communicates extracellular mitogenic signals to the nuclear transcriptional machinery (Vivanco I & Sawyers CL, 2002). AKT kinase family has three isoforms (AKT1, AKT2 and AKT3) and each one of them have different roles and functions with overlapping expression, sharing high homology see review (Hers I*, et al.*, 2011). AKT1 and AKT2 are involved in different cancer types and have different roles. There is published data showing that AKT isoforms are important in pulmonary vasculature. Indeed, AKT1 is responsible for pulmonary vascular remodelling. AKT1 knockouts mice have attenuated microvascular permeability and oedema, and are protected against hypoxia induced pulmonary hypertension (HPH).

Another important signalling pathway involved in lung growth regulation is signal transducer and activator of transcription 3 (STAT3). STAT family members are phosphorylated by the receptor-associated kinases and then they form homo or heterodimers that translocate to cell nucleus, acting as transcription activators mediating intracellular signalling pathways. There is strong evidence that STAT3 is changed in cancer.

Regarding the signalling pathways described above, all of them can be

involved in foetal lung development. There are several articles showing that these signalling pathways can be altered in CDH conditions, or after *in vitro* administration of growth factors, proteins, or molecules that interfere with branching morphogenesis (Piairo P*, et al.*, 2011; Nogueira-Silva C*, et al.*, 2012a; Nogueira-Silva C*, et al.*, 2013).

4. NEUROENDOCRINE FACTORS

Pulmonary neuroendocrine cells (PNECs) are present in the airway epithelium of human and animal lungs, playing a role in the regulation of lung growth and maturation. Moreover, PNECs are involved in pulmonary development and also in the regulation of pulmonary vascular tone. During the last decade, several studies showed that PNECs, in the early stages of lung development, are modulators of foetal lung growth and differentiation. Interestingly, at the time of birth, they act as airway O_2 sensors involved mainly in neonatal adaptation. Postnatally, PNECs / neuroepithelial bodies (NEB) are important in airway epithelial regeneration and lung carcinogenesis providing a lung stem cell niche. PNECs constitute the first cell type to differentiate in lung airway epithelium see review (Cutz E*, et al.*, 2007).

Bombesin and ghrelin are neuroendocrine peptides and have key roles in lung development and maturation during intrauterine life until postnatal period. Bombesin is produced by PNECs and ghrelin is mainly synthesized in the endocrine A-like cells of oxyntic mucosa of the stomach. Regarding bombesin, its expression is maximal during the pseudoglandular and canalicular stages. Moreover, *in vitro* or *in vivo* blocking of bombesin retards lung epithelial development. Thus, bombesin seem to play a physiological role in the induction of lung morphogenesis. In CDH rat lungs, bombesin is increased when compared to normal lungs and promotes lung branching (Warburton D*, et al.*, 2000; Cutz E*, et al.*, 2007).

A recent study showed that *in vivo* administration of bombesin to nitrofen pregnant rats, promotes lung development and rescues lung hypoplasia in CDH embryos (Sakai K*, et al.*, 2014).

Concerning ghrelin expression, ghrelin presents higher expression in the airway epithelium during the pseudoglandular stage, suggesting its role in branching morphogenesis (Volante M*, et al.*, 2002; Santos M*, et al.*, 2006; Cutz E*, et al.*, 2007). Moreover ghrelin expression has been reported in other branching organs such as pancreas, kidney, mammary gland and placenta.

Published data has shown that maternal ghrelin administration attenuates pulmonary hypoplasia in rat foetuses with CDH, and that ghrelin is

overexpressed in human and rat hypoplastic lungs. Additionally *in vitro* ghrelin administration to foetal lung explants increases lung branching in normal lungs (Santos M*, et al.*, 2006; Nunes S*, et al.*, 2008).

5. RETINOIC ACID

Retinoids (retinoic acid and related signalling molecules) play an essential role in several aspects including pregnancy maintenance, foetal and perinatal growth, embryogenesis and organogenesis, namely in lung development (its important in all stages of lung development). In adulthood, they regulate immunity, reproduction, vision and are crucial for proper functioning of lung, liver and neural system. Moreover, retinoic acid (RA) deficit, the biologically active form of vitamin A, is strongly associated with diaphragmatic defects at birth, hypoplastic lungs, pulmonary agenesis and tracheoesophageal septum development.

Indeed, Vitamin A administration in rats' diet during pregnancy and *in vivo* administration of RA to pregnant rat dams has been shown to revert lung hypoplasia in embryos and decreases the incidence of diaphragmatic defects (Thebaud B*, et al.*, 1999). Furthermore, exogenous administration of RA into nitrofen lung explant cultures, rescues abnormal lung branching (Baptista MJ*, et al.*, 2005; Montedonico S*, et al.*, 2006; Pederiva F*, et al.*, 2012). However, high concentrations of RA are associated with adverse effects, namely teratogenicity and consequently its extremely important to have a RA balanced diet (Hunt JR, 1996). Recent studies suggest that RA signalling pathway is disturbed in the nitrofen model of CDH justified by the fact that nitrofen inhibits RALDH 2 (enzyme responsible for catalysing the final step in RA production). Indeed a genetically engineered modified mice was created to test this hypothesis see review (Montedonico S*, et al.*, 2008). In normal lung development, RALDH 2 expression, is downregulated in budding regions and its high in proximal regions of the lobes. There is a proximal-distal gradient pattern of RA expression. In this sense, the morphogenesis and shape of an organ can be determined by a concentration gradient of a molecule that gives cells polarity. For instances in the limb, there are two well described gradients of signalling (antero-poterior and proximal-distal) and RA is involved in proximal-distal pattern formation (Towers M*, et al.*, 2012). Vertebrate limbs develop in a temporal proximal-distal sequence, where proximal regions are specified and generated earlier than distal ones. After limb bud formation, RA synthesis and signalling is

restricted to the proximal limb by FGF activity. Considering lung it is described that RA blocks formation of the distal lung and induces a proximal-like phenotype in lung epithelial tubules. Pattern formation is a crucial element of lung development since the epithelial phenotypes differ between proximal and distal structures (Cardoso WV, 1995). *In vitro* experiments were done and FGF10 and Shh are downregulated within the distal buds following exogenous RA administration (inhibitory effect of RA signalling upon branching morphogenesis).

In the liver, retinyl esters are metabolized into retinol. Retinol binds to retinol-binding protein (RBP), and RBP binds to cell surface receptors and is transported in the cytoplasm bound to cellular retinal-binding proteins (CRBP), where it is converted to retinal by retinol dehydrogenase (RALDH) (Figure 2). After RA synthesis in the cell, it enters in the nucleus. There are two classes of retinoic receptors: retinoic acid receptors (RAR) and retinoic X receptors (RXR) and each one of them have three isoforms α, β, γ (Hind M & Maden M, 2004). RARs can form homodimers with RAR members or heterodimers with members of the RXR subfamily.

It is described that overexpression of ALDH1A2 (aldehyde dehydrogenase) is not a primary event, but it is a consequence of CDH-induced lung injury (Coste K*, et al.*, 2015). Moreover, double knockouts of RAR α and RAR β display lung hypoplasia and failure in the oesophagus trachea separation and *in vivo* administration of RAR antagonist (BMS493) leads to mice embryos without evidence of lung buds see review (Mollard R*, et al.*, 2000). When RA is absent, FGF10 decreases and TGF β increases, reducing lung branching. RA is a major regulator of Wnt signalling and TGF β pathway, controlling FGF10 expression since early in gestation (Chen F*, et al.*, 2010). RAR α is responsible for the correct number of alveoli to develop after perinatal period in mice, whereas RAR β restricts the number of alveoli being formed and RAR γ also regulates this process (Gallot D*, et al.*, 2005; Gudas LJ, 2012). Concerning branching, RAR α downregulation is required for sacculation in the developing lung. Among RA receptors, only signalling from RAR β and RAR γ is involved in branching morphogenesis. RAR β transcripts are expressed within the proximal airways of the pseudoglandular

lung. These are downregulated distally where there is increased branching activity. In this sense, endogenous RA signalling acts via RAR β to stabilize tubule formation by limiting the number of buds that emerge during branching morphogenesis. RAR γ displays a restricted pattern of expression in the developing lung, being expressed within the distal bud epithelium. RAR γ is not involved in transducing the RA signal, which inhibits distal lung bud formation in contrast with RAR β (Mollard R*, et al.*, 2000).

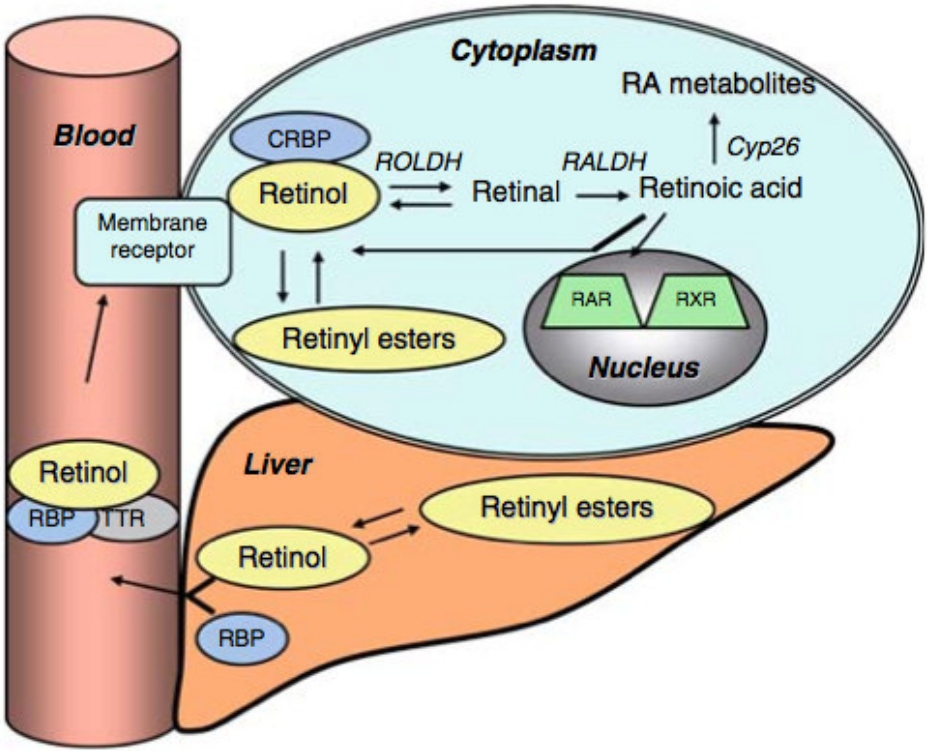

Figure 2. Schematic overview of retinoic acid signalling pathway. TTR: transthyretin; ROLDH: retinol-dehydrogenase; CRBP: cellular retinol-binding proteins; RALDH: retinal dehydrogenase; RAR: retinoic acid receptors; RXR: retinoid x receptors; RBP: retinol-binding protein (Montedonico S*, et al.*, 2008).

6. MicroRNAs

MicroRNAs are small non-coding RNAs that regulate gene expression, by targeting transcription or inhibiting translation. MicroRNAs have approximately 22 nucleotides in length and are highly conserved among animals, plants and other organisms, suggesting that microRNAs represent a highly conserved and important regulatory mechanism see review (Aghanoori MR*, et al.*, 2014). In the nucleus, primary (pri)-microRNA is transcribed by RNA polymerase II. RNase III enzyme Drosha processes pri-microRNA into precursor microRNA (pre-microRNA). Pre-miRNA is transported into the cytoplasm through exportin-5, and is digested by RNase III endonuclease, Dicer, to double-stranded microRNA molecules. Dicer cleaves the pre-microRNA and generates a mature microRNA. The mature microRNA is then separated into two single-stranded molecules: the antisense strand participates in the RNA-induced silencing complex (RISC) and is recruited by Argonaute (Ago) proteins, while the sense strand is targeted for degradation. Inside RISC, microRNA can bind to the 3'-untranslated region (3'UTR), or in some cases to the 5'UTR of target mRNA, to repress translation or induce cleavage (Figure 3). Ago 1 and Ago 2 constitute the two most highly expressed Ago proteins in mammals see review (Comer BS*, et al.*, 2015).

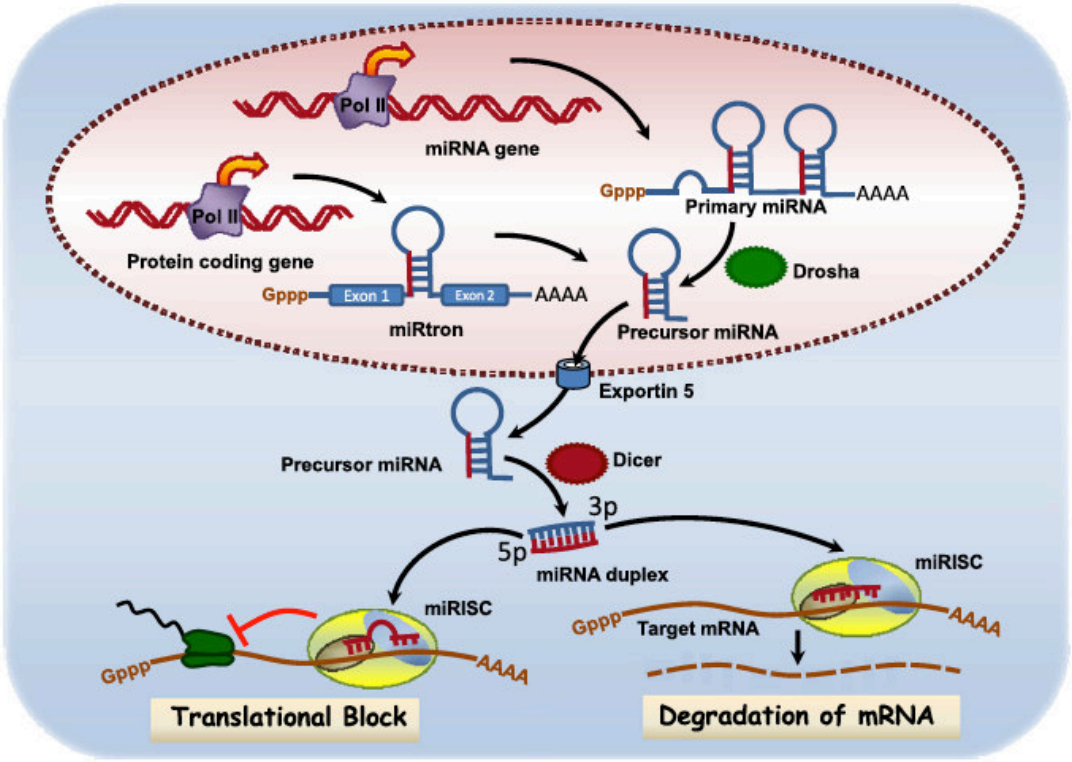

Figure 3. MicroRNA biogenesis and mechanisms of gene silencing (Comer BS*, et al.*, 2015).

The first microRNA reported was Lin-4 in 1993 and Let-7 was the second discovered in 2000. 33% of the human genome is regulated by microRNAs (Lee RC*, et al.*, 1993; Sessa R & Hata A, 2013).

Down-regulation or up-regulation of microRNAs can be associated with the origin of several diseases, such as cancer, respiratory and cardiac problems. For instance, recently published data has shown that miR-205 deregulation and miR-200 family downregulation are associated with breast cancer (Gregory PA*, et al.*, 2008). MicroRNAs are linked to cell physiology, development, differentiation, proliferation, metastasis and apoptosis. Currently, microRNAs have emerged as a future strategy to develop more effective therapies, improving drug efficiency, or to be used as potential tools in the diagnostic and prognostic of diseases. MicroRNAs are stable and easy to manipulate because they are small and can even cross the placenta. Considering normal developed tissues, deregulation of microRNAs is well tolerated, although in pathological conditions, that does not happen. Regarding oligonucleotides, locked nucleic acid (LNA), they present a modification in the ribose ring, improving nuclease resistant and conferring a

high affinity for complementary sequence, being more stable and promisor when used in *in vivo* treatments see review (Sessa R & Hata A, 2013).

6. 1 MICRORNAS IN LUNG

Regarding lung development and epigenetics, DNA methylation and histone modifications are the best-studied epigenetic mechanisms, however microRNAs have also recently emerged as an important class of members of the epigenetic machinery see review (Vasilatou D*, et al.*, 2013). MicroRNAs are present as multifunctional molecules and several studies using cells and animal trials have shown and characterized their expression.

The first evidence correlating lung development with microRNAs was observed in Dicer knockout mice and conditional cells, in which branching was abrogated in embryonic lungs or a defective airway branching in the cells was observed respectively. Moreover, concerning the embryonic stage (E 11.5), Ago 1 and Ago 2 are vastly expressed in epithelial and mesenchymal regions, showing the importance of microRNA regulation in lung remodelling and maturation see review (Johar D*, et al.*, 2014). Concerning the pseudoglandular stage, miR-17-92 cluster is important in controlling differentiation and proliferation of lung progenitor cells. Furthermore, deletion of miR-17-92 cluster leads to hypoplastic lungs, ventricular septal defects and postnatal death of all mice (Bienertova-Vasku J*, et al.*, 2014). By contrast overexpression of miR-17-92 cluster in the lung epithelium induces hyperproliferation and inhibits differentiation of proximal and distal epithelial progenitor cells.

Concerning asthma, knockdown studies have shown that microRNAs are involved in the aetiology of the disease and can be a potential therapeutic target for the disease as well (Wang Y*, et al.*, 2007; Booton R & Lindsay MA, 2014).

6. 2 MICRORNA 200 AND MICRORNA 10 FAMILIES

MiR 200 family is composed by two clusters: miR-200a, miR-200b and miR-429 share a common transcription start site on chromosome 1; miR-200c and miR-141 are transcribed as a single unit from chromosome 12 see review (Howe EN*, et al.*, 2012).

The role of miR-200 in lung development is not clear and defined, although miR-200b plays a crucial role in cancer invasiveness and tumour metastasis. MiR-200b has been associated with epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET). Moreover, the members of miR–200 family have been highlighted for its importance in the maintenance of the epithelial phenotype, since they inhibit EMT, by directly targeting and downregulating zinc finger E-box-binding homeobox (ZEB) 1 and ZEB2, resulting in enhanced E-cadherin expression.

When miR-200b is up-regulated, carcinoma cells become more epithelial leading to MET and indeed when miR-200b is downregulated the cells become more mesenchymal leading to EMT (Wang G*, et al.*, 2013; Diaz-Lopez A*, et al.*, 2015).

On the other hand, miR-200 family is altered in idiopathic pulmonary fibrosis (IPF) and miR-155 is up-regulated. MiR-155 regulates negatively TGF-β1 signalling in macrophages, silencing SMAD2 and promoting EMT, and miR-200b regulates one of the known target genes TGF-β / SMAD signalling in a complex way (Louafi F*, et al.*, 2010).

Regarding miR-10 family, less is known about these microRNAs and lung development. Two members compose miR-10 family: miR-10a and miR-10b that differ from each other just in one base. MiR-10 family is located within the Hox clusters of developmental regulators and are highly conserved for correct animal anterior-posterior patterning. They are positioned in close proximity to Hox 4 paralogues playing a role in the determination of body plans.

In cancer, miR-10 family expression was found to be deregulated however information about the role and function of miR-10 family is still missing.

MicroRNAs have been associated with two opposite roles including promotion and inhibition in every metastatic step processes, and miR-10b is known to be involved in tumour progression and metastasis. Moreover miR-10a has an important role in cellular processes such as differentiation, apoptosis, cell survival, replication and senescence in embryonic development. Additionally miR-10 family has been associated as a key player in RA induced responses. *In vitro* studies have shown that miR-10a and miR-10b are overexpressed in RA treated cells. On the other hand, inhibition of miR-10a before RA treatment in smooth muscle cells was shown to abolish their differentiation. However miR-10a by itself was unable to conduct smooth muscle cells differentiation see review (Tehler D*, et al.*, 2011).

6.3 TGF BETA

TGFβ superfamily is divided in three subfamilies: activin, TGFβ and BMP. There are three TFGβ isoforms in mammals: TGFβ1, 2 and 3. TGFβ is strongly associated with lung development and all isoforms have been found in murine embryonic lungs see review (Warburton D*, et al.*, 2005). Moreover, TGFβ is involved in embryonic development, organogenesis, tissue homeostasis and also in cell-extracellular matrix (ECM) interactions.

Published data has shown that microRNAs interfere in TFGβ pathway at multiple levels, and that TGFβ increases the maturation of microRNAs. SMAD constitutes a downstream target of TGFβ signalling pathway see review (Sivadas VP & Kannan S, 2014).

Regarding TGFβ1, it is expressed in the mesenchyme underlying distal epithelial branching points, and is responsible for structural remodelling, epithelial changes, immunosuppression processes of inflammatory airway diseases and repair of fibrosis formation (Lee CM*, et al.*, 2014). Moreover, mice lacking TGFβ1 die at few weeks after birth with multiorgan autoimmune inflammatory diseases.

TGFβ2 is localized in distal epithelium, playing a key role in branching morphogenesis. TGFβ2 knockout mice die *in utero* with severe cardiac malformations (Warburton D*, et al.*, 2005).

TGFβ3 is expressed in proximal mesenchyme and mesothelium, and is important in regulating alveolar epithelial cell proliferation during injuries. TGFβ3 mutants die after birth, presenting lung dysplasia and cleft palate.

Indeed a well regulated expression and pattern of all TGFβ is required for normal lung development, avoiding lung inflammation and lung diseases. Recently published data has shown that overexpression of TGFβ1 leads to hypoplastic phenotypes and exogenous administration of TGFβ1 to embryonic mouse lungs resulted in blocking of lung branching. In contrast, inhibition of TGFβ type II receptor stimulated lung branching (Bragg AD*, et al.*, 2001).

Additionally, TGFβ family is very important in the regulation of alveologenesis and in controlling of EMT interactions that are crucial for normal lung development. Indeed TGF-β signalling has different effects in epithelial *vs* mesenchymal cells. Recent published data revealed that, TGFβ1 addition to lung explant cultures induces arrested lung development, decreased lung differentiation and lung vasculature (Pain M*, et al.*, 2014). Moreover, null mutations of TGF-βs are associated with abnormal lung development or post-natal lung inflammation, and TGF-β blockage inhibits EMT in chicken atrioventricular explants. In fact, TGF-β2 administration induces EMT in mouse explant cultures.

7. CONGENITAL DIAPHRAGMATIC HERNIA

Congenital diaphragmatic hernia (CDH) is a congenital anomaly that does not represent a problem for babies during gestation, since they receive oxygen from the placenta; however, foetal lung hypoplasia, with associated severe persistent pulmonary hypertension, limits their survival after birth (Colvin J*, et al.*, 2005; Rocha GM*, et al.*, 2008).

Nowadays, CDH has an incidence of 1:2.500 live births with a high rate of morbidity and mortality and a poorly understood aetiology (Keller RL*, et al.*, 2010). CDH aetiology is unknown in more than 80% of the cases and no specific genetic defects or environmental factors have been associated with CDH. A better understanding of normal lung development will help to overcome lung problems, including CDH, and improve survival.

In humans, there are four types of diaphragmatic defects: posterolateral or Bochdalek type, anterior Morgagni type, central hernia or septum transversum type and diaphragmatic eventration type. The most common type is the Bochdalek hernia that has an incidence of 80% and occurs 8 times more often on the left side than on the right (due to the fact that diaphragm closure start by the right side) (Tibboel D & Gaag AV, 1996; van Loenhout RB*, et al.*, 2009). CDH is a multifactorial disease and normally appears with associated anomalies such as cardiac, renal and skeletal malformations (Holder AM*, et al.*, 2007). Interestingly, it was found in animal studies that foetal lung hypoplasia is associated with early and late gestational determinants (dual-hit hypothesis). Early determinants involve molecular disturbances and are associated predominantly with the pseudoglandular and canalicular stages of lung development, affecting branching mechanisms. Late determinants are related to mechanical factors (visceral compression) and affect mainly the saccular and alveolar stages (Keijzer R*, et al.*, 2000). However, fetal diaphragmatic surgical repair was not able to revert the ominous prognosis of CDH. In this sequence, for severe cases of CDH with a really bad prognosis, fetoscopic endoluminal tracheal occlusion (FETO) appears promising in some specific and specialized centres. Other possibilities are prenatal hormonal therapies. In chapter 2 we summarized the most recent results of this strategic research line based in

the knowledge of regulatory pathways in lung growth for which experimental models are crucial. Interestingly, recent exotic prenatal therapies have emerged as a potential therapy for these babies which clinical impact is still unknown (Lemus-Varela Mde L*, et al.*, 2014; Sakai K*, et al.*, 2014; Makanga M*, et al.*, 2015; Rhodes J*, et al.*, 2015).

7.1 CDH ANIMAL MODELS

Animal models are very useful to study early embryonic development, allowing a better understanding of normal and pathological growth (Beurskens N*, et al.*, 2007).

There are three different animal models available to study CDH: surgical, genetic and teratogenic.

Surgical models that mimic CDH are obtained by surgery where a whole in the diaphragm, of rabbits and sheeps is performed. However it does not mimic CDH properly because it is an artificial defect created on the diaphragm late on gestation and the lung is not hypoplastic. In this sense it is not a good model to understand and find the molecular mechanisms and signalling pathways that can be altered in CDH (Beurskens N*, et al.*, 2007; van Loenhout RB*, et al.*, 2009).

Considering **genetic** models, published data with knockout mice showed that the genes that are affected correspond to regions commonly deleted or duplicated in CDH in humans. Moreover, CDH is strongly associated with RA signalling pathway and double null mutant mice lacking RARα and RARβ have offspring with CDH. Furthermore, RARα and RARβ are expressed in the developing diaphragm (Mendelsohn C*, et al.*, 1994; Lohnes D*, et al.*, 1995; Greer JJ*, et al.*, 2003). Other genetic alterations that can lead to CDH are homozygote mice for Fog2 (premature stop codon), heterozygous Gata4 knockout mice, changes and deletions of COUP-TFII and retinol-binding protein (RBP)-1 and RBP-2 (Beurskens N*, et al.*, 2007).

Regarding **teratogenic** models, CDH can be induced with bisdiamine (most potent inhibitor of retinal dehydrogenase 2-RALDH 2), biphenyl carboxylic acid, SB-210661 (a benzofuranyl urea derivate) and nitrofen.

The administration of the herbicide nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) to pregnant rats on 9.5 dpc (100mg of nitrofen dissolved in 1ml of olive oil by gavage), induces congenital anomalies in the offspring, mainly diaphragmatic defects, pulmonary hypoplasia and associated malformations such as cardiac problems. The nitrofen model is the most used and it is the one that better mimic CDH in humans. However the herbicide nitrofen does not induce CDH in humans (Correia-Pinto J*, et al.*, 2003; Beurskens N*, et al.*, 2007; Kling DE & Schnitzer JJ, 2007; van Loenhout RB*, et al.*, 2009; Clugston RD*, et al.*, 2010). There are strong evidences showing that nitrofen affects RA signalling pathway, which is key in lung development and is also disturbed in CDH in humans. On the other hand, vitamin A administration to pregnant rats can reverse CDH (Mey J*, et al.*, 2003; Baptista MJ*, et al.*, 2005; Beurskens LW*, et al.*, 2009).

7.2 FETO

It is possible to diagnose CDH from 15 weeks gestation by ultrasound or magnetic resonance imaging (MRI). Additionally, liver position / herniation and estimated lung to head ratio (LHR) are good parameters to predict CDH outcomes (Figure 4). Moreover CDH associated with other congenital anomalies and malformations is associated with a bad prognosis outcome and sometimes termination of pregnancy is suggested (Dekoninck P*, et al.*, 2011; Delacourt C*, et al.*, 2012; Deprest J*, et al.*, 2014).

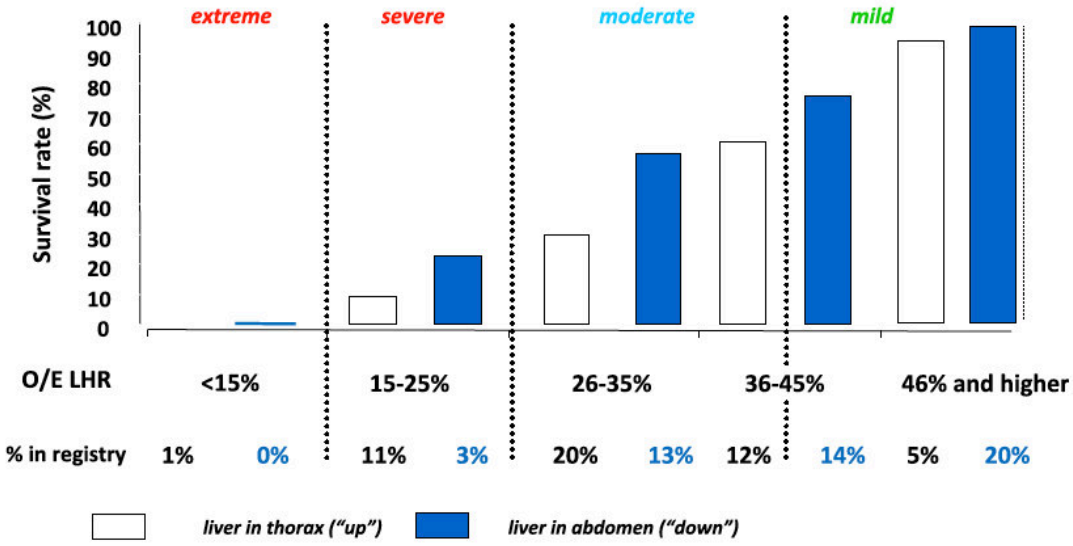

Figure 4. Survival rates of foetuses with left-sided CDH, depending on the observed/expected lung head ratio (O/E LHR) and liver position. Percentages below graphic refer to the number in each severity group (Deprest J*, et al.*, 2014).

FETO was first proposed by Jay Wilson and his team from Boston (Wilson JM*, et al.*, 1993; DiFiore JW*, et al.*, 1994). It is recommended, in severe cases of CDH, without other associated anomalies, in order to improve the outcome of these babies. Although FETO improves lung growth, it is invasive, technically demanding and limited by maternal and foetal risks. Actually FETO consists in *in utero* insertion of a balloon in the trachea around 28 weeks of gestation, and the puncture of the balloon by ultrasound guidance should be done around 34 weeks. In some cases, preterm premature rupture of membranes (PPROM) occurs and the balloon cannot be removed before birth, so it needs to be done during delivery. The tracheal occlusion prevents lung fluid to egress, thereby inducing tissue stretch

leading to lung growth and maturation. FETO increases survival in severe cases of left-sided CDH from 24.1% to 49.1% and in right-sided from 0% to 35.3% (Deprest J*, et al.*, 2011; Deprest JA*, et al.*, 2011). On the other hand, FETO still presents problems such as PPROM and the fact that it is a invasive and risky procedure. Moreover, lung maturation is still limited when compared to normal lungs, reinforcing the idea that FETO should only be done in severe cases of CDH with a really poor prognosis (Mimmi MC*, et al.*, 2015; Xie A*, et al.*, 2015).

AIMS

During the last decades, lots of efforts and advances had been done in order to treat and increase the quality of life of patients suffering from lung pathologies, namely CDH. In this sense, the main challenge of this disease is to reduce mortality and morbidity by trying to find new treatments for these patients. Improvements and sophisticated techniques such as foetal surgery, FETO, ECMO and intensive care before and after birth emerged although the morbidity rate still remains high in mild and severe cases of CDH. In order to find a potential treatment it is crucial to understand completely normal pulmonary development. The knowledge of the underlying molecular mechanisms and interactions involved in normal lung growth and maturation, will improve to the understanding of abnormal lung development. Thus the following aims were pursued in this PhD thesis:

1) To investigate the role of receptors tyrosine kinases, namely ephrins on normal foetal lung growth;

2) To predict surveillance of CDH patients analysing microRNAs present in lung and amniotic fluids before and after FETO;

3) To study the role of neuroendocrine factors and RA during normal and abnormal lung development;

4) To find new prenatal therapeutical approaches to revert foetal lung hypoplasia in CDH context.

THESIS LAYOUT

This PhD thesis is organized into seven different chapters. In **chapter I**, a general introduction on the subject of the thesis is presented, including normal lung development regulators and signalling pathways, and abnormal lung development (CDH pathophysiology). **Chapter II**, presents the first manuscript published accordingly to the aims of this thesis entitled " The role of ephrins B1 and –B2 during foetal rat lung development". In this study we investigated the expression pattern of ephrins B1, -B2 and eph B4 receptor in

rat foetal lung development and we explored the effects of ephrin B1 and –B2 supplementation in rat foetal lung explant cultures regarding the potential effects in growth regulators signalling pathways. **Chapter III**, presents a manuscript entitled “Unique tracheal fluid microRNA signature predicts response to FETO in patients with congenital diaphragmatic hernia”. This study strongly contributed to find potential biomarkers that can be useful as prognostic tools for CDH babies. **Chapter IV**, an article entitled “ Neuroendocrine factors regulate retinoic acid receptors in normal and hypoplastic lung development” is presented, where a putative link between neuroendocrine factors and RA pathway was established. The overall aim of this dissertation was to further understand lung development in order to find a potential prenatal therapy for CDH patients. On **Chapter V**, a general discussion of the most important results of this PhD work is presented. On **Chapter VI** the major conclusions are presented. The last chapter is **Chapter VII**, where all the references from the thesis are presented in alphabetical order.

CHAPTER II.

EPHRINS AND FOETAL LUNG GROWTH

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Original Paper

The Role of Ephrins-B1 and -B2 During Fetal Rat Lung Development

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Key Words
Ephrin-B1 • Ephrin-B2 • EphB4 • Fetal lung development • Branching

Abstract
Background/ Aims: The knowledge of the molecular network that governs fetal lung branching is an essential step towards the discovery of novel therapeutic targets against pulmonary pathologies. Lung consists of two highly branched systems: airways and vasculature. Ephrins and its receptors, Eph, have been implicated in cardiovascular development, angiogenesis and vascular remodeling. This study aims to clarify the role of these factors during lung morphogenesis. ***Methods:*** Ephrins-B1, -B2 and receptor EphB4 expression pattern was assessed in fetal rat lungs between 15.5 and 21.5 days post-conception, by immunohistochemistry. Fetal rat lungs were harvested at 13.5 dpc, cultured during 4 days and treated with increasing doses of ephrins-B1 and -B2 and the activity of key signaling pathways was assessed. ***Results:*** Ephrin-B1 presents mesenchymal expression, whereas ephrin-B2 and its receptor EphB4 were expressed by the epithelium. Both ephrins stimulated pulmonary branching. Moreover, while ephrin-B1 did not affect the pathways studied, ephrin-B2 supplementation decreased activity of JNK, ERK and STAT. This study characterizes the expression pattern of ephrins-B1, -B2 and EphB4 receptor throughout rat lung development. ***Conclusion:*** Our data highlight a possible role of ephrins as molecular stimulators of lung morphogenesis. Moreover, it supports the idea that classical vascular factors might play a role as airway growth promoters.

F.O. Peixoto and P. Pereira-Terra contributed equally to this manuscript.

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Introduction

Lung morphogenesis is a very complex and tightly regulated phenomenon. Transcription factors, extracellular matrix molecules, intercellular adhesion molecules and soluble growth factors have been identified as controlling factors of pulmonary development. Together, these molecular players act along the proximal-distal axis of the respiratory tract and influence local expression of specific genes that ultimately play a preponderant role on the development of the immature lung [1].

The Eph receptors and their ligands, ephrins, constitute the largest subgroup of the family of receptors tyrosine kinase (RTKs). This family of receptors is divided into two subgroups based on the similarity of their extracellular domain sequences, whereas ephrins are classified according to their structure and their affinity for the correspondent receptor subgroup [2]. Regarding ephrins-B, Eph/ephrin signaling can be bi-directional, inducing intracellular pathways downstream of both the Eph receptor and the ephrin ligand. The latter is definied as reverse signaling [3]. Albeit ephrins-A preferably bind to EphA and ephrins-B to EphB receptors, ephrins are also able to bind and activate Eph receptors from the opposite subgroup [4]. Nevertheless, some ephrins seem to have higher affinity for specific receptors as reported for ephrin-B2, which mainly binds to EphB4 receptor [5, 6].

The roles of ephrins in cell adhesion, vascular development, cell migration and tissue-border maintenance highlight a potential role in lung branching morphogenesis [3, 7]. In fact, ephrins expression has been described in other organs that, as the lung, develop through branching processes. For instance, the expression of ephrins-B1 and -B2, and EphB4 (ephrin-B2 cognate receptor) has been confirmed in adult mouse mammary gland and fetal mouse pancreas [8-10]. The expression of ephrin-B2 and its receptor has also been detected in fetal mouse kidney [11]. It is noteworthy that ephrin-B1 mutations have been found in nearly 87% of craniofrontonasal syndrome cases. Some of these cases exhibit diaphragmatic defects and consequently lung defects [12-16]. Moreover, another study described ephrin-B1 gene (*EFNB1*) duplications in fetuses that died from CDH and concomitant pulmonary hypoplasia and pulmonary hypertension [17]. On the other hand, ephrin-B2 and EphB4 receptor have been widely described as arterial and venous markers, respectively, and implicated in the development of the vascular system during embryogenesis [5, 6, 18, 19]. Ephrin-B2, ephrin-B1 and EphB4 receptor RNA was identified in sorted endothelial cells from mouse adult lung [20].

There is extensive evidence for a direct synergism between pulmonary vascular and airway development. Indeed, vascular regulatory factors play a crucial role in airway and alveolar morphogenic processes, and growth factors produced in the airway epithelium can also regulate vasculature formation [21-24].

Ephrins have been described as crucial for microvascular maturation mainly during septum maturation and capillary remodeling from double into a single capillary layer. They are also known to be involved in the process of alveologenesis, contributing for the establishment of the secondary septa and for alveolar formation [25, 26]. In fact, ephrin-B2 has been described as a contributor to normal postnatal alveolar development as its inhibition leads to arrested alveolar and abrogated lung vascular growth. Moreover, in a setting where ephrin-B2 is downregulated, ephrin-B2 treatment preserves alveolar and lung vascular growth attenuating pulmonary hypertension caused by O_2-induced arrested alveolar growth [27, 28]. Ephrin-B1 –B2 and EphB4 knockout mice die during embryogenesis and displayed several defects including angiogenesis impairment. In published data with Ephrin-B2 or EphB4 knockout mice, stained vessels were completely absent in knockouts [28, 29]. Thus, ephrin-B1, ephrin-B2 and its main receptor appear as potential key players in fetal lung development. Nonetheless, very little is known regarding ephrins-B1 and -B2 function during fetal lung development. Hence, in the present work we aimed to disclose novel roles of such proteins in normal lung development.

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Materials and Methods

Ethics Statement

Animal experiments were performed according to the Portuguese law for animal welfare ('Diário da República, Portaria 1005/92') 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). Animals were housed in an accredited 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). Moreover, all efforts were made to minimize animal suffering.

Experimental Design and Animal Model

Sprague-Dawley female rats (225g, Charles-River) 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.

Fetuses were removed by caesarean section at 13.5, 15.5, 17.5, 19.5 and 21.5 days post-conception (dpc). Fetuses were sacrificed by decapitation, its lungs dissected under a binocular surgical microscope (Leica MZFLIII, Leica Microsystems GmbH) and processed either for immunohistochemistry (IHC) or western blot studies. 13.5 dpc fetal lungs were also collected to perform explant culture.

Immunohistochemistry

IHC was performed on paraformaldehyde-fixed and paraffin-embedded lungs. Sections (4μm) were then placed on SuperFrost®Plus slides (Menzel-Glaser, J1800AMNZ). After 15' at 65°C, slides were dewaxed in xylene and rehydrated in ethanol. Antigen retrieval was achieved by boiling the slides in 1mM citrate buffer (Thermo Scientific, AP-9003-125) followed by cooling down at room temperature. Incubation with 3% hydrogen peroxide (Sigma, 95313-500 ml) in distilled water for 20' allowed quenching endogenous peroxidase. Incubation with primary antibodies occurred at 4°C overnight. The primary antibodies used were a polyclonal goat anti-ephrin-B1 in a 25μg/mL concentration (R&D Systems, Inc., AF473), a polyclonal rabbit anti-ephrin-B2 (Santa Cruz Biotechnology Inc., sc-15397) in a 1:25 dilution and a polyclonal rabbit anti-EphB4 in a 1:100 dilution (Santa Cruz Biotechnology, Inc., sc-5536). Negative control reactions included omission of the primary antibody. The incubation with the corresponding secondary antibodies occurred at room temperature and was carried according to manufacturer's instructions. Ephrin-B1: goat ImmunoCruz™ Staining System (Santa Cruz Biotechnology Inc., sc-2023). Ephrin-B2 and EphB4: UltraVision detection system anti-polyvalent horseradish peroxidase (Thermo Scientific, TP-125-H2). To visualize peroxidase activity, 3,3'-diaminobenzidine tetrahydrochloride (Dako, K3468) was used. Sections were counterstained with 50% hematoxylin.

Fetal lung explant cultures

After harvesting and dissection, 13.5 dpc lungs were transferred to 8 μm Isopore membranes (Millipore, TETP01300) previously soaked in DMEM (Lonza, 12-604F) for 1 hour. Cultures were then incubated in 200μL of complex medium, 50% Dulbecco's modified eagle medium (DMEM), 50% nutrient mixture F-12 (Invitrogen, Carlsbad, CA, USA) supplemented with 100 mg/mL streptomycin, 100 units/mL penicillin (Invitrogen), 0.25 mg/mL ascorbic acid (Sigma–Aldrich, St Louis, MO, USA) and 10% fetal bovune serum (Invitrogen) [30]. After 1 hour of incubation, recombinant ephrin-B1 (R&D Systems Inc., 473-EB-200) and ephrin-B2 (R&D Systems Inc., 496-EB-200) were added to lung explants in order to achieve a final concentration of 0.01, 0.1 and 1μg/mL. The recombinant ephrins were daily added. The four experimental groups (control and three doses for each protein) were all constituted by n=12 lungs. The explants were incubated in a 5% CO_2 incubator, at 37°C, during 96 hours. The medium was replaced at 48 hours. The explants were daily photographed to monitor branching morphogenesis. At day 0 (D_0: 0 hours) and day 4 (D_4: 96 hours) of culture, the total number of peripheral airway buds (branching), the epithelial perimeter, the external area and external perimeter were determined for all lung explants using the ImageJ 1.44 software (National Institutes of Health, USA). The results of branching, epithelial perimeter and area were expressed as D_4/D_0 ratio. At the end of the incubation time, explants were washed in PBS and stored at -80°C until use.

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Western blot

Three different pooled samples of fetal lungs for each gestational age (13.5 – 21.5 dpc) (obtained from different pregnant female rats), and cultured lungs (treated with ephrin-B1 at 1μg/mL, ephrin-B2 at 0.01μg/mL and non-treated) were processed for western blot analysis. Proteins were obtained according to Kling et al. [31]. Twenty-five or five micrograms (for explants) of protein were loaded in 12% acrylamide mini gel, electrophoresed and then transferred to nitrocellulose membranes (HybondTM -C Extra, GE Healthcare Life Sciences, 25800047). Blots were probed with polyclonal goat anti-ephrin-B1 (1:750; R&D Systems, Inc., AF473), polyclonal rabbit anti-ephrin-B2 (1:2000; Santa Cruz Biotechnology Inc., sc-15397), polyclonal rabbit anti-EphB4 (1:200, Abcam, ab123791), and with antibodies to non-phosphorylated and phosphorylated forms of p38 (8690, 4511), p44/42 (ERK1/2) (4695, 4370), JNK (9258, 4671), Akt (4685, 13038) and STAT3 (12640, 9131) (1:1000; Cell Signaling Technology Inc.) according to manufacturer's instructions. Activity of intracellular signaling pathways from lung explants was measured by the ratio between phosphorylated protein and total protein amount. The data was also normalized against the control group. For loading control, blots were probed with β-tubulin (1:200000; Abcam, ab6046).

Blots were incubated with a secondary anti-rabbit horseradish peroxidase conjugate (Santa Cruz Biotechnology Inc., sc-2004), developed with Super Signal West Femto Substrate (Pierce Biotechnology Inc., 34095) and the chemiluminescent signal was captured using the Chemidoc XRS (Bio-Rad). Quantitative analysis was performed with Quantity One 4.6.5 1-D Analysis Software (Bio-Rad Laboratories Ltd).

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed using the statistical software GraphPad Prism (version 5; GraphPad Software Inc., USA). Statistical comparison of experimental groups was achieved by t-test and one-way ANOVA on ranks. The Student-Newman-Keuls test was used for post-test analysis. Statistical significance was set at $p<0.05$.

Results

Pulmonary expression pattern of ephrin-B1, ephrin-B2 and EphB4 receptor

In order to determine ephrin-B1, ephrin-B2 and its receptor EphB4 expression pattern during fetal lung development, immunohistochemical analysis was performed for five different gestational ages: 13.5, 15.5, 17.5, 19.5 and 21.5 dpc. Ephrin-B1 exhibits strong mesenchymal expression observed in all gestational ages (Fig. 1A). Faint epithelial expression is detected at 15.5 and 17.5 dpc. Nonetheless, from 19.5 dpc on, the epithelial expression is absent, as well as endothelial expression.

Remarkably, ephrin-B2 expression pattern is highly distinct from ephrin-B1 (Fig. 1B). Ephrin-B2 is predominantly expressed in the epithelial compartment, and it is also observed in the cells surrounding the epithelium at 13.5 and 15.5 dpc. Additionally, ephrin-B2 is not detected in vascular smooth muscle cells neither in endothelial cells. At 21.5 dpc, ephrin-B2 expression appears to be restricted only to epithelial cells from terminal and respiratory bronchioles.

EphB4 expression pattern (Fig. 1C) is very similar to its cognate ligand: mainly detected in epithelial structures throughout all gestational ages. Interestingly, at 13.5 and 15.5 dpc, EphB4 receptor is also weakly expressed in potentially less differentiated cells surrounding the epithelium. This expression seems to diminish as the fetal lung maturates, becoming progressively specific to the epithelium. On the other hand, again no expression was detected in vascular structures, namely smooth muscle or endothelial cells from both arterial and venous structures.

Ephrins-B1 and -B2, and EphB4 receptor protein expression levels were assessed by western blot analysis (Fig. 2). For the gestational ages studied, EphB4 receptor, ephrins-B1 and -B2 expression was detected at relatively constant levels. Nevertheless, despite the higher variation in ephrin-B1 expression levels throughout all gestational ages, no statistical significant differences were found.

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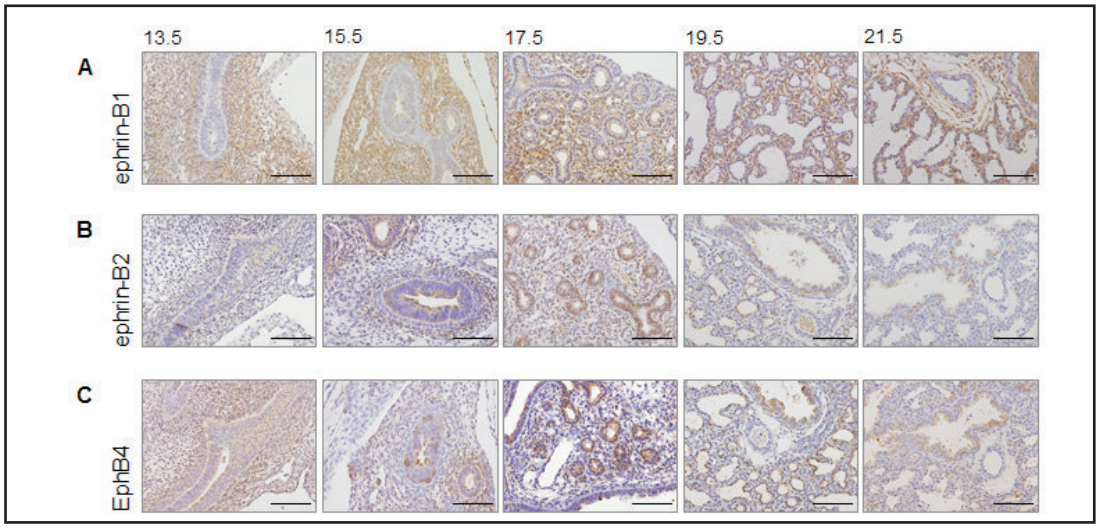

Fig. 1. Ephrin-B1 (A), ephrin-B2 (B) and EphB4 receptor (C) protein expression pattern, during fetal lung development. Five different gestational ages were analyzed: 13.5, 15.5, 17.5, 19.5 and 21.5 days post-conception (dpc). Ephrin-B2 and receptor EphB4 display a strong epithelial expression, while ephrin-B1 expression is mainly detected in mesenchymal cells. Original magnification: x200. Scale bar = 100 μm (all images at same magnification). No staining was observed in the negative controls for each protein. (data not shown).

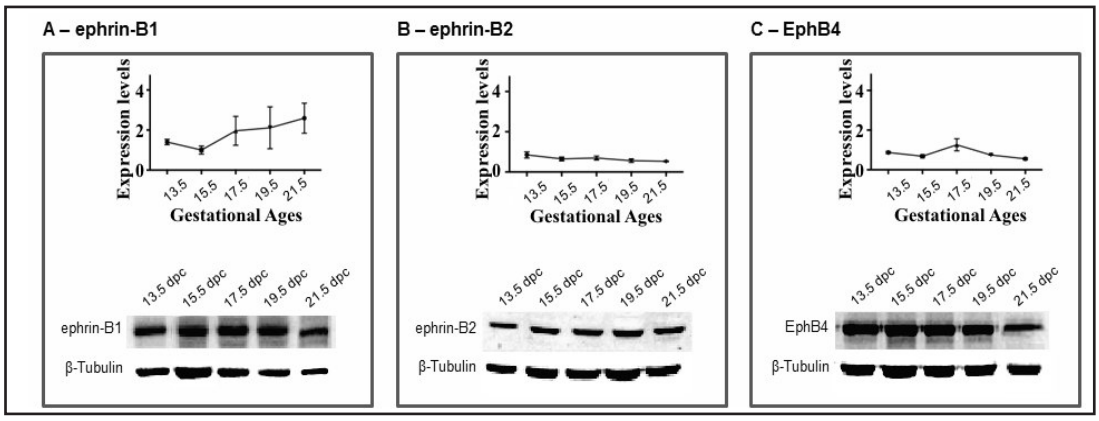

Fig. 2. Western Blot analysis of the expression levels of ephrin-B1 (A), ephrin-B2 (B) and EphB4 receptor (C) during fetal lung development. Five different gestational ages were analyzed: 13.5, 15.5, 17.5, 19.5 and 21.5 days post conception (dpc). Loading control was performed using β-tubulin. Relative protein levels were expressed in arbitrary units normalized for β-tubulin. Statistical analysis showed no significant differences between gestational stages.

Ephrin-B1 and ephrin-B2 affect fetal lung morphogenesis in rat

Aiming to unveil ephrins-B1 and -B2 potential role in fetal lung development, functional studies were performed using lung explant cultures. Explants were daily supplemented with three different doses of either ephrin-B1 or ephrin-B2 recombinant protein (selected according to literature). Morphometric analysis revealed a statistically significant effect of both ligands on lung morphogenesis. Recombinant ephrin-B1 promoted an increase in the number of peripheral airway buds regardless of the dose tested (Fig. 3B). On its turn, ephrin-B2 lowest dose (0.01μg/mL) significantly increased lung branching and the internal epithelial perimeter (Fig. 4 B and C, respectively). Moreover, ephrin-B2 treated explants present an increase in total lung area regardless of the dose studied (Fig. 4D).

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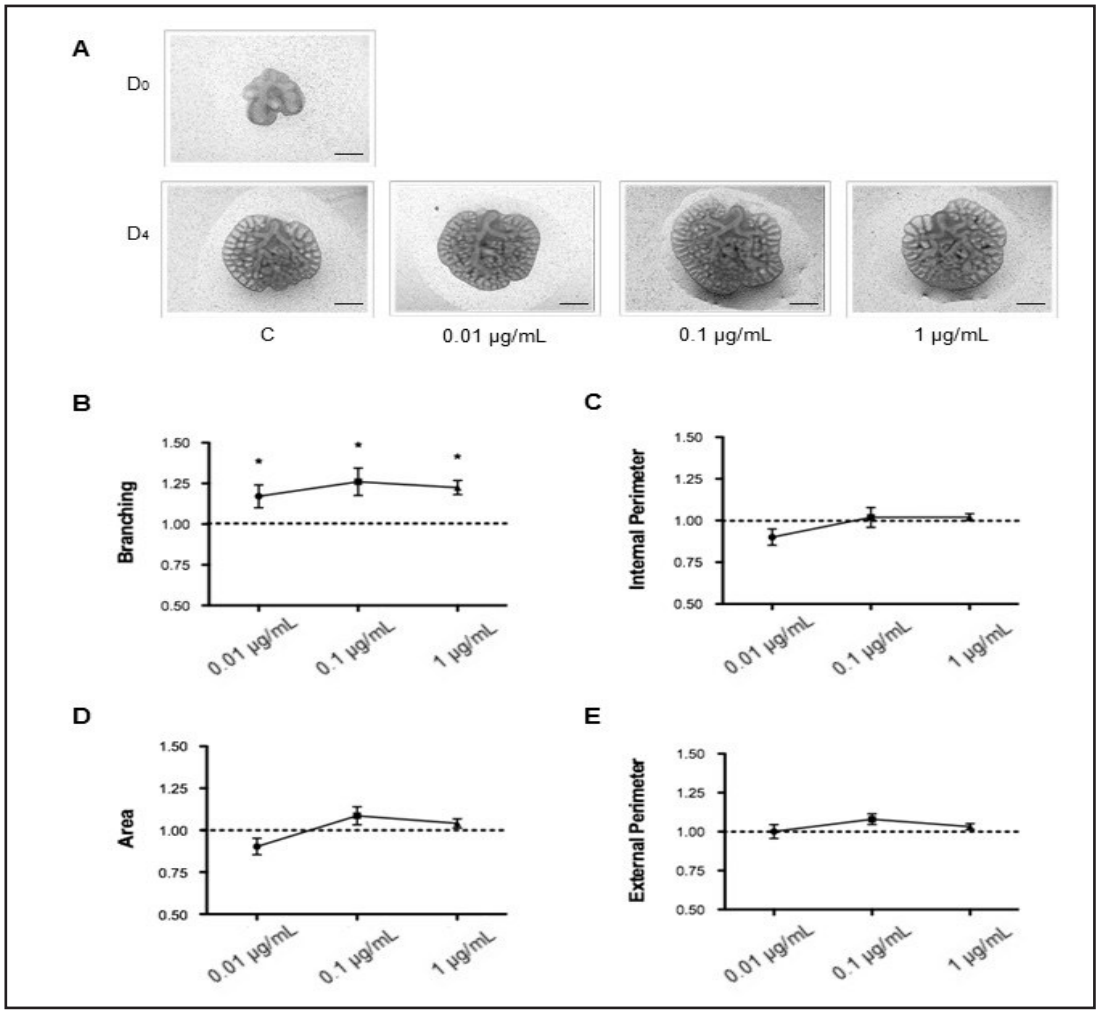

Fig. 3. Ephrin-B1 supplementation promotes branching of rat fetal lung explants. (A) Representative examples of fetal lung explants cultured with three different doses of ephrin-B1 recombinant protein (0.01, 0.1 and 1µg/mL) at day zero (D_0) and day 4 (D_4). Original magnification x25. Morphometric analysis of branching (B), internal perimeter (C), area (D), and external perimeter (E). A significant increase in the number of peripheral airway buds (branching) was observed for all tested doses. All data was normalized with the control group (C=1; dashed lines). Original magnification x25. Scale bar = 6349 µm (all images at same magnification). Results are expressed as D4/D0 ratio. $p < 0.05$: * vs. control group.

Ephrin-B2 influences the phosphorylated form of ERK, JNK and STAT3

The signaling pathways that act downstream ephrin-B1 and ephrin-B2, specifically in lung morphogenesis, are poorly understood. In order to further investigate the effect of ephrins on fetal rat lung growth, treated lung explants were assessed for intracellular signaling pathways known to regulate lung growth and development.

Pooled samples of lung explants treated with 0.01µg/mL of recombinant ephrin-B1 or 1µg/mL of recombinant ephrin-B2 (selected according to its maximal effect on lung explants growth) were evaluated for modulation of p38, p44/42 (ERK1/2), JNK, Akt and STAT3 pathways (Fig. 5). Ephrin-B1 treatment did not influence the phosphorylation levels of the analyzed pathways. Strikingly, recombinant ephrin-B2 administration promoted the inactivation of ERK, JNK and STAT signaling pathways in fetal lung explants.

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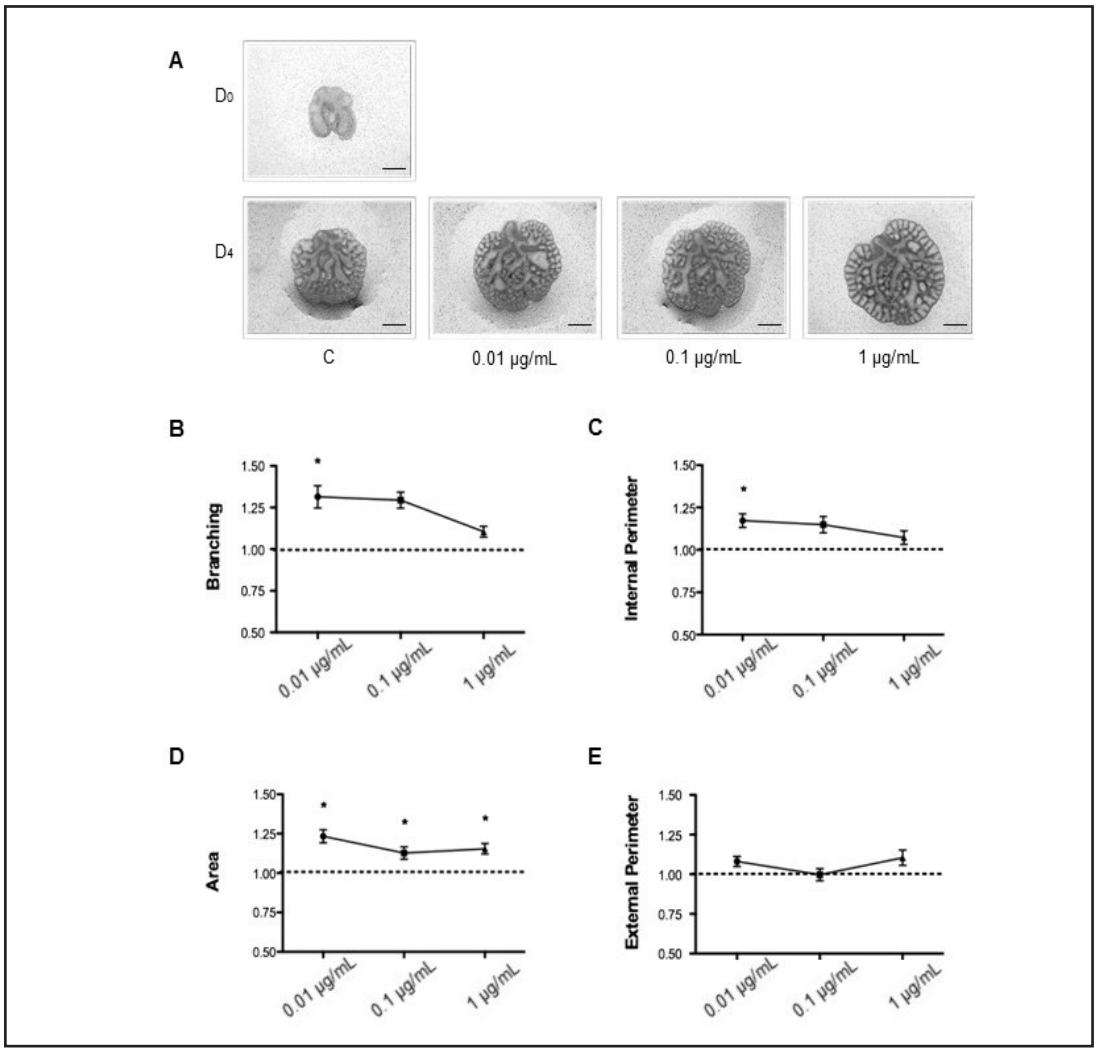

Fig. 4. Ephrin-B2 supplementation induces growth of rat fetal lung explants. (A) Representative examples of fetal lung explants cultured with three different doses of ephrin-B2 recombinant protein (0.01, 0.1 and 1µg/mL) at day zero (D_0) and day 4 (D_4). Original magnification x25. Scale bar = 6349 µm (all images at same magnification). Morphometric analysis of branching (B), internal perimeter (C), area (D), and external perimeter (E). A significant increase in the area of the explants was observed for all tested doses. The lowest dose significantly stimulated both branching and internal perimeter. All data was normalized with the control group (C=1; dashed lines). Results are expressed as D4/D0 ratio. $p< 0.05$: * vs. control group.

Discussion

There is growing evidence for the involvement of ephrins in numerous biological processes. Clearly, this family of proteins is implicated in regulation of key steps of embryonic development. This study demonstrates that two ephrin ligands - B1 and B2 - and one well-established ephrin-B2 receptor - EphB4 - are constitutively expressed during fetal lung development. Two recent studies presented strong evidence for the role of ephrin-B2 in lung morphogenesis and repair [27, 32]. Our findings not only provide further proof of ephrin-B2 function but also show how yet another ephrin ligand promotes branching of the rat fetal lung.

The constitutive and stable protein expression levels of ephrins-B1 and -B2, and EphB4 receptor particularly during early gestational stages, which are characterized by

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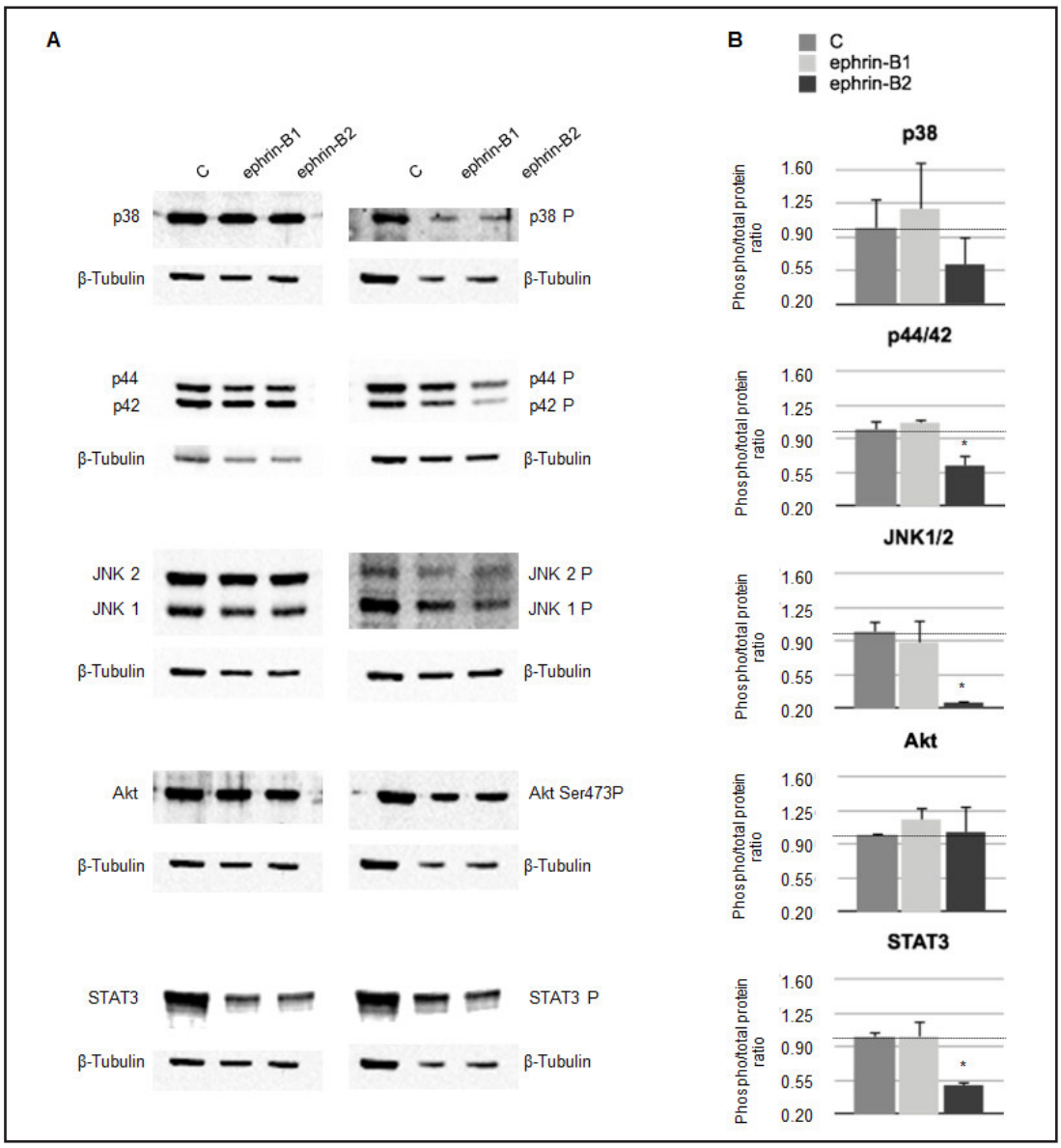

Fig. 5. Analysis of the intracellular signaling pathways that mediate Ephrin-B1 and Ephrin-B2 actions in lung morphogenesis. (A) Western blot analysis of p38, p44/42, JNK1/2, Akt and STAT3, and phosphorylated forms of p38 (p38 P), p44/42 (p44/42 P), JNK (JNK P), Akt serine 473 (Akt Ser473P) and STAT3 (STAT3 P) in control, ephrin-B1 (at 0.01μg/mL) and ephrin-B2 (at 1μg/mL) treated lung explants. Loading control was performed using β-tubulin. p38 corresponds to 38 kDa. p44 and 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 phosphorylated forms of these intracellular signaling pathways that mediate lung growth. No changes in activity levels of these pathways were observed for ephrin-B1 treated lung explants. Ephrin-B2 caused a significant decrease in p44/42, JNK1/2 and STAT3 signaling activity. Results are presented as arbitrary units normalized for β-tubulin. Activity of intracellular signaling pathways was measured by the ratio between phosphorylated protein and total protein amount. All data was also normalized against the control group (C=1; dashed lines). $p < 0.05$: * vs. control group.

intense ramification and growth phenomena, underscore them as potential players in fetal lung branching. On the other hand, the distinct expression patterns presented by the two ligands suggest distinct or, perhaps, synergistic functions. Ephrin-B1 expression was

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mainly detected in mesenchymal tissue, whereas strong epithelial expression was found for ephrin-B2 and the receptor EphB4. Interactions between epithelial structures and the surrounding mesenchyme are known to dictate and to be essential for proper branching of the lung epithelium [33-35]. Therefore, our results suggest a so far undervalued contribution from this RTK subfamily of ligands to such mesenchymal-epithelial interactions that guide lung branching morphogenesis.

Although ephrin-B2 and its receptor have been widely described as angiogenic factors [5, 6, 18, 19], epithelial expression of ephrin-B2 has been described in fetal mouse kidney and mammary gland [9, 11], also branching organs. Both ephrin-B1 and ephrin-B2 are also expressed by the pancreas epithelium [10]. In 17 dpc mice lung, ephrin-B2 expression has been observed in the epithelium. However, at 18 dpc ephrin-B2 was also expressed in some endothelial cells and, more weakly, in non-endothelial cells with epithelial morphology [36]. In human fetal lung, ephrin-B2 is expressed in the pulmonary arterial endothelium and EphB4 expressed in endothelial cells of the pulmonary vein, primary capillary plexus and pulmonary artery [37]. In rat lung, endothelial staining was absent for the three proteins studied. These results are in agreement with observations from a recent knock-in mouse model in which the intracellular domain of ephrin-B2 was replaced by bacterial β-gal sequences. The same study showed how ephrin-B2 reverse signaling blockage results in pulmonary hypoplasia and decreased lung compliance [32]. Finally, in the adult mice lung, both ephrins are enriched in the endothelial compartment [20]. The different observations in fetal lung might be due to the chosen study model and technical approach. Nevertheless, all together these observations suggest an intense endothelial-epithelial crosstalk played by these proteins since early stages of the fetal development to the adult organism. Indeed, vascular regulatory factors are crucial for airway and alveolar morphogenic processes. In fact, growth factors produced in the airway epithelium are able to regulate vasculature formation [38, 39]. Taking all this into account, we hypothesized that both ephrins-B1 and -B2 would affect fetal lung growth and branching morphogenesis.

In order to evaluate ephrins-B1 and -B2 role in lung branching morphogenesis, fetal lung explants were cultured with increasing doses of ephrin-B1 or ephrin-B2. Ephrin-B1 supplementation stimulated the formation of peripheral airway buds. Additionally, ephrin-B2 supplementation increased not only lung branching but also the internal epithelial perimeter and area of the lung explants. These results strongly support a stimulatory role of ephrin-B1 and ephrin-B2 during fetal lung development. The importance of ephrins-B1 and -B2 in different tissues during embryogenesis is highlighted by the lethality of ephrin-B1 knockout mice [29] and angiogenesis impairment caused by ephrin-B2 gene disruption [5]. Also, in a recent study, ephrin-B2 was shown, by means of siRNA mediated gene silencing, to be implicated in normal postnatal alveolar development. Authors observed decreased alveolarization associated with fewer pulmonary vessels [27].

Aiming to identify the signaling pathways that mediate the effect of ephrins-B1 and -B2 in lung growth, the activity of p38, ERK, JNK, Akt and STAT3 proteins was studied. Many factors that regulate fetal lung branching morphogenesis activate signaling pathways that culminate with MAPK, PI3K/Akt and p38 cascades [30, 31, 40-42]. In the present study, ephrin-B1 was found not to modify the phosphorylated levels of these proteins. In a different study it has been already described that ephrin-B1 signaling does not activate the p38 and ERK signaling pathways. However, ephrin-B1 led to the phosphorylation of JNK on a specific residue. Therefore, the putative phosphorylation of JNK in different residues from the ones here studied cannot be rule out [43]. A very recent study has shown that CNK1 (Connector Enhancer of KSR1) is required for the ephrin-B1-dependent JNK phosphorylation [44]. It would be interesting in future studies to access the role of this protein in the context of ephrin-B1 mediated fetal lung branching. Moreover, it would also be interesting to check if Shh, Retinoic Acid, Bmp or Fgf signaling pathways, classical regulators of lung branching, are changed when ephrin-B1 is administrated [45-49].

In contrast, ephrin-B2 stimulation induced a decrease in phosphorylated levels of ERK, JNK and STAT3, indicating a decrease in these intracellular signaling. These results

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might reflect regulatory loops in which ephrin-B2, by stimulating other branching-promoter pathways, leads to lower activity of the specific pathways here studied. Regarding ERK, although classically this pathway has been implicated as a promoter of branching morphogenesis, it was already described that ERK pathway can be involved in lung growth inhibition [42, 50]. In a lung explant culture system similar to the one used in the present study, stimulation with leukemia inhibitory factor and concomitant activation of ERK pathway resulted in branching inhibition [50]. Recent published data has also shown that knockdown of ephrin-B2 results in increased phosphorylation levels of several kinases such as ERK, STAT, c-Jun. This indicates that multiple prominent cell signaling pathways are affected by changes of EphB2 activity [51]. The putative involvement of ERK, JNK and STAT3 in lung growth inhibition might still be an indirect or balancing effect of the activation of other pathways or simply context dependent. Further investigation will help to determine whether the inactivation of such pathways is essential for ephrin-B2 function in lung development or if the obtained results reflect crosstalk and modulation by other non-canonical intracellular pathways.

In summary, this study demonstrated that both ephrin-B2 and its cognate receptor EphB4 are constitutively expressed in pulmonary epithelium, whereas ephrin-B1 is mainly expressed in the mesenchyme of the rat fetal lung. It is now clear that both ephrin-B1 and ephrin-B2 significantly influence lung morphogenesis. However, further investigation will be necessary to properly describe the expression pattern and functional contribution of other members of the ephrin-B subfamily of ligands as they might act synergistically. Ultimately, *in vivo* studies will be crucial to further confirm these factors as promoters of lung branching morphogenesis.

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CHAPTER III.

MICRORNAS SIGNATURE IN FETO PATIENTS

ORIGINAL ARTICLE

Unique Tracheal Fluid MicroRNA Signature Predicts Response to FETO in Patients With Congenital Diaphragmatic Hernia

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Objective and Background: Our objective was to determine the fetal in vivo microRNA signature in hypoplastic lungs of human fetuses with severe isolated congenital diaphragmatic hernia (CDH) and changes in tracheal and amniotic fluid of fetuses undergoing fetoscopic endoluminal tracheal occlusion (FETO) to reverse severe lung hypoplasia due to CDH.

Methods: We profiled microRNA expression in prenatal human lungs by microarray analysis. We then validated this signature with real-time quantitative polymerase chain reaction in tracheal and amniotic fluid of CDH patients undergoing FETO. We further explored the role of miR-200b using semi-quantitative in situ hybridization and immunohistochemistry for TGF-β2 in postnatal lung sections. We investigated miR-200b effects on TGF-β signaling using a SMAD-luciferase reporter assay and Western blotting for phospho-SMAD2/3 and ZEB-2 in cultures of human bronchial epithelial cells.

Results: CDH lungs display an increased expression of 2 microRNAs: miR-200b and miR-10a as compared to control lungs. Fetuses undergoing FETO display increased miR-200 expression in their tracheal fluid at the time of balloon removal. Future survivors of FETO display significantly higher miR-200 expression than those with a limited response. miR-200b was expressed in bronchial epithelial cells and vascular endothelial cells. TGF-β2 expression was lower in CDH lungs. miR-200b inhibited TGF-β-induced SMAD signaling in cultures of human bronchial epithelial cells.

Conclusions: Human fetal hypoplastic CDH lungs have a specific miR-200/miR-10a signature. Survival after FETO is associated with increased miR-200 family expression. miR-200b overexpression in CDH lungs results in decreased TGF-β/SMAD signaling.

Keywords: congenital diaphragmatic hernia, miR-10a, miR-200, pulmonary hypoplasia, TGF-β signaling, tracheal occlusion

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Congenital diaphragmatic hernia (CDH) is a developmental defect of the diaphragm, allowing herniation of abdominal viscera into the chest. It occurs in 1 in 2000 to 3000 live births.[1] Although postnatal treatment has become more standardized, substantial morbidity and mortality result from the associated pulmonary hypoplasia and abnormal vascular development of the newborn. A subset of fetuses with liver herniation and a smaller lung size, represented by an observed overexpected lung-to-head ratio (O/E LHR) under 25%, has higher mortality and morbidity rates and, therefore, today are offered in utero fetal surgery. Fetoscopic endoluminal tracheal occlusion (FETO)[2] prevents normal egress of airway fluid, which in turn induces tissue stretch, acting as a signal for lung growth. To stimulate lung maturation, it was proposed to reverse the occlusion already in utero (plug-unplug sequence),[3] which also clinically seems to improve survival and morbidity.[4,5] These preliminary observations are now being evaluated in a randomized controlled trial [http://www.totaltrial.eu, NIH NCT00763737 (moderate hypoplasia) and NIH NCT01240057 (severe hypoplasia)].[6]

A lack of understanding of the molecular mechanisms underlying pulmonary hypoplasia in CDH hampers progress for potential in utero therapies and case selection. Although about 10% of CDH patients have chromosomal anomalies, a common genetic cause for CDH is unknown.[7] However, it is widely accepted that the diaphragmatic defect and pulmonary hypoplasia result from a shared developmental insult.[8,9] MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression through posttranscriptional silencing of messenger RNAs.[10] MicroRNAs are essential for normal organogenesis during embryonic development. For example, targeted deletion of miR-1 to 2 leads to congenital heart defects in mice.[11] Previous studies have identified differential miRNA expression between various stages of lung development, but these studies did not provide much functional information.[12] Whether specific miRNAs play a role in the pathogenesis of human congenital lung diseases remains unknown. Isolated CDH is characterized by abnormal lung development. The first objective of this study was to determine whether severe human hypoplastic CDH lungs display specific microRNA expression. The second objective was to determine if the obtained microRNA signature could be used as a biomarker, by evaluating its expression in tracheal and amniotic fluid samples of CDH patients before and after forced lung growth. The third objective was to determine how abnormal miR-200b expression influences target gene expression. This is

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the first study to identify abnormal miR-200/miR10a expression in hypoplastic CDH lungs and in response to forced lung growth. The expression of these microRNAs in tracheal fluid can be used to distinguish survivors of FETO from nonsurvivors. We also demonstrate that increased miR-200b expression results in decreased target gene expression via decreased TGF-β/SMAD signaling.

MATERIALS AND METHODS

MicroRNA Screen of Lung Specimens

RNA Isolation

Erasmus MC-Sophia's institutional review board approved the study protocol, and all the experiments were performed adhering to the relevant guidelines of the Research Ethics Board of the University of Manitoba. Surrogates provided consent for the use of human tissues and biofluids. We biopsied lungs from 3 fetuses with isolated and severe CDH and 3 age-matched controls without lung disease (22, 22, and 25 weeks of gestation) undergoing termination or pregnancy. Total RNA of these lung specimens was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA).

MicroRNA Profiling

The expression profile of 319 human miRNAs was investigated using a liquid-phase bead-based array according to the manufacturer's instructions. Five microgram of total RNA was biotinylated at the 3' end using the FlexmiR MicroRNA labeling kit (Luminex, Austin, TX). The labeled RNA was hybridized to locked nucleic acid (LNA) capture probes. Each probe is bound to a fluorescently dyed xMAP bead. After washes, the biotinylated miRNAs were detected by reaction with streptavidin-phycoerythrin. The samples were analyzed on a Luminex-200 instrument. The measured intensities were subtracted from a background control reaction and normalized against a set of ubiquitously expressed small nucleolar RNAs (snoRNA). Heat maps and statistical analyses were generated with the Institute for Genomic Research MultiExperiment Viewer.

Real-Time Quantitative Polymerase Chain Reaction Validation of MicroRNA Profile

We used the miRCURY LNA PCR system according to the manufacturer's instructions (Exiqon, Vedbaek, Denmark). LNA primer sets were purchased from Exiqon (Table 1). After reverse transcribing of the isolated RNAs, we used the SYBR Green assay (Bio-Rad, Hercules, CA) for real-time quantitative polymerase chain reaction (RT-qPCR) amplification. We included 3 small RNA species as endogenous controls (SNORD38B, 5S rRNA and U6 snRNA). Of these, SNORD38B had the most stable expression between different samples and was used to normalize the data.

Biomarker Screen of Biofluids (Amniotic and Tracheal Fluids)

Clinical Evaluation

Ultrasound evaluation was performed less than 48 hours before FETO plug (further referred to as baseline, typically around 26–28 weeks) and less than 48 hours before balloon removal (typically around 34 weeks). The degree of pulmonary hypoplasia was estimated using 2-dimentional ultrasound and was expressed as an LHR as described before.[13,14] The LHR is corrected for gestational age by expressing the LHR of the index case as a proportion of what is normally expected for a gestational age-matched normal fetus (observed/expected: O/E LHR). The relative increase in O/E LHR compared to the baseline value was expressed as follows: [(O/E LHR unplug − O/E baseline)/O/E baseline] × 100%. In addition, a functional status of the pulmonary circulation was evaluated using a maternal hyperoxygenation test. The relative difference between pre- and posthyperoxygenation values was expressed as deltaPI: [(baseline PI − PI_{O2}) / baseline PI] × 100%.[15,16]

RNA Isolation

Amniotic and tracheal fluid were collected at the time of balloon insertion and its removal (n = 21). Amniotic fluid was retrieved at first entry into the amniotic cavity; tracheal fluid was sampled below the vocal cords, through the fetoscopic sheath, taking care not to contaminate it by irrigation fluid. The Ethics Committee of the UZ Leuven has approved the fetal treatment and prospective follow-up program of patients with CDH, as well as the use of fetal fluid specimens to improve prenatal prediction of outcomes. We selected archived (−80°C) sample pairs from 11 consecutive "responders" (defined as having a marked increase in O/E LHR from baseline and who were eventually surviving) and 10 "nonresponders" (ie, poor or absent increase in lung size and eventually neonatal death[17]) (Table 2).

Blinded to clinical outcome, total RNA was extracted for RT-qPCR using the miRCURY RNA Isolation Kit for Biofluids (Exiqon). For normalization of sample-to-sample variation, 1 μL of synthetic *Caenorhabditis elegans* miRNA (cel-miR-39) was added to each denatured sample.[18,19] Small RNAs were then enriched and purified according to the manufacturer's protocol.

TABLE 1. LNA Primer Sets (Exiqon) Used in RT-qPCR

LNA MicroRNA Primer Set	Target Sequence	Description
SNORD38B	TCTCAGTGATGAAAACTTTGTCCAGTTCTGCTACTG ACAGTAAGTGAAGATAAAGTGTGTCTGAGGAGA	endogenous control
5S rRNA (hsa)	GTCTACGGCCATACCACCCTGAACGCGCCCGATCTCGTCTGAT CTCGGAAGCTAAGCAGGGTCGGGCCTGGTTAGTACTTG GATGGGAGACCGCCTGGGAATACCGGGTGCTGTAGGCTTT	endogenous control
U6 snRNA (hsa, mmu)	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGA ACGATACAGAGAAGATTAGCATGGCCCCTGCGCAA GGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT	endogenous control
hsa-miR-10a	UACCCUGUAGAUCCGAAUUUGUG	tested microRNA
hsa-miR-27a	UUCACAGUGGCUAAGUUCCGC	tested microRNA
hsa-miR-195	CCAAUAUUGGCUGUGCUGCUCC	tested microRNA
hsa-let-7a	CUAUACAAUCUACUGUCUUUC	tested microRNA
hsa-miR-1	UGGAAUGUAAAGAAGUAUGUAU	tested microRNA
hsa-miR-200b	UAAUACUGCCUGGUAAUGAUGA	tested microRNA

Real-Time Quantitative Polymerase Chain Reaction

To optimize RT-qPCR performance, we did a dilution curve to determine what input volume affected RT-qPCR performance. On the basis of the outcome, 4μL of small RNAs from the biofluids samples were reverse transcribed using the miRCURY LNA Universal RT microRNA PCR protocol (Exiqon) in a total reaction volume of 10μL. The cDNA was diluted 1:40 μL in nuclease free water to be used as a PCR template. PCR reactions for quantification of miR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-10a, and cell-miR-39 were performed in triplicate and 20μl was used as the reaction volume. The RT-qPCR reactions were performed using an ABI 7500 Real-Time PCR System with the following cycling conditions: 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 1 minute. The cycle threshold (Ct) values were calculated with ABI 7500 v1.4.0 software. The target microRNAs sequences of the LNA mix primers are summarized in Table 3.

Calculation of miRNA Expression

The average expression levels of amniotic and tracheal fluid miRNAs were normalized against cel-miR-39[18,20,21] using the $2^{-\Delta Ct}$ method. Differences between the groups are presented as ΔCt, indicating the difference between the Ct value of the miRNA of interest and the Ct value of the normalizer miRNA. To ensure consistent measurements and reproducibility throughout all assays, for each PCR amplification reaction, one of the RNA samples was loaded in triplicate in all the plates, as internal control to account for any plate-to-plate variation, and the results from each plate were also normalized against an internal normalization control. The expression levels of microRNAs were normalized with the *C. Elegans* cel-miR-39 Spike-in kit.

In Situ Hybridization and Immunohistochemistry

We obtained postmortem neonatal lung tissues from 3 postnatal CDH cases and 3 age-matched controls, without lung disease (35, 37, and 40 weeks of gestation). All cases died within 1 hour after birth. In situ hybridization was carried out on 5 μm sections of formalin-fixed tissue as previously described.[22] Our protocol was first validated with an LNA control probe against U6 snRNA. A scramble-miR probe was used as a negative control and did not produce a signal. In situ hybridization with a probe against miR-10a

TABLE 2. Demographic Information for CDH Fetuses Undergoing FETO

Baseline Characteristics of the Embryos			
Characteristics	**Responders (n = 11)**	**Nonresponders (n = 10)**	***P***
O/E LHR at first evaluation (%)	22.5 (23.6–17.5)	21.6 (24.0–15.9)	0.87
Liver herniated	10 (91%)	9 (100%)	1.00
Fetal gender (male/female)	7 (m)/4 (f)	4 (m)/5 (f)	0.65
FETO Pregnancy Data and Neonatal Outcome			
FETO Data/Outcome	**Responders (n = 11)**	**Nonresponders (n = 10)**	***P***
Gestational age at plug (wk)	28.1 (28.7–27.1)	27.9 (29.1–27.0)	0.84
Gestational Age at unplug (wk)	34.0 (34.1–33.4)	34 (34.5–33.9)	0.36
Occlusion days	39 (49–35)	43 (50–34)	0.64
Relative increase in O/E LHR	161.7 (252.4–140.8)	29.8 (10.4–50.2)	<0.0001
Interval removal-delivery <24 h	1 (9%)	0 (0%)	1.00
PPROM	5 (45%)	3 (33%)	0.67
Gestational Age at PPROM (wk)	35.0 (35.9–32.6)	35.4 (36.6–29.3)	1.00
Gestational Age at delivery (wk)	37.0 (38.0–35.0)	38.0 (38.4–36.4)	0.25
Birth weight (g)	2780 (3180–2160)	3195 (3278–2650)	0.30
Oxygen at day 28	6 (55%)	NA	
NICU days	45 (61–30)	NA	
Day of neonatal death	NA	1 (2–0)	

All median (IQR) or n (%).
NA indicates not applicable. NICU indicates neonatal intensive care unit; PPROM, preterm premature rupture of membranes.

TABLE 3. MicroRNA Target Sequences for RT-qPCR and LNA Probes (Exiqon) Used for In Situ Hybridization

Target MicroRNA	**Sequence**		
hsa-miR-200a-3p	UAACACUGUCUGGUAACGAUGU		
hsa-miR-200b-3p	UAAUACUGCCUGGUAAUGAUGA		
hsa-miR-200c-3p	UAAUACUGCCGGGUAAUGAUGGA		
hsa-miR-141-3p	UAACACUGUCUGGUAAAGAUGG		
hsa-miR-429	UAAUACUGUCUGGUAAAACCGU		
hsa-miR-10a-5p	UACCCUGUAGAUCCGAAUUUGUG		
cel-miR-39-3p	UCACCGGGUGUAAAUCAGCUUG		
Probe	**Sequence**	**Concentration**	**Hybridization Temperature**
hsa-miR-200b	TCATCATTACCAGGCAGTATTA	100 nM	52°C
hsa-miR-10a	CACAAATTCGGATCTACAGGGTA	100 nM	53°C
scramble-miR	GTGTAACACGTCTATACGCCCA	100 nM	57°C
U6, positive control	CACGAATTTGCGTGTCATCCTT	0.1 nM	54°C

produced a very weak signal in postnatal lung tissues. MicroRNA species were hybridized with double-digoxigenin-labeled LNA probes (Exiqon) for 1 hour (Table 3). We detected the hybridized probes with an alkaline phosphatase–conjugated antidigoxigenin antibody (1:500) (Roche, Mannheim, Germany). Sections were immunostained with 1-step NBT/BCIP solution, containing 1 mM levamisole (Thermo Scientific, Rockford, IL). The slides were counterstained with methyl green (Sigma-Aldrich, St Louis, MO). We performed immunohistochemistry on neonatal lung tissue using an anti-TGF-β2 antibody (1:100) (Abcam, Cambridge, MA) as previously described.[23]

Microscopy and Image Analysis

Digital microscopy was performed with the ScanScope CS system (Aperio, Vista, CA). Semiquantitative measurements of the in situ hybridization studies were obtained as follows. Images up to 200× magnification were obtained and analyzed using ImageScope software (http://www.aperio.com). The entire area of each lung section was digitally mapped using the colocalization algorithm. The blue and green stainings were first calibrated with the color deconvolution tool using positive (U6 probe without counterstain) and negative (scramble probe with methyl green counterstain) control slides, respectively. The average optical densities of each stain in the red, blue, and green channels were then entered into the colocalization algorithm. The program creates a digital map of the slide made up of 3 colors: blue for positive staining, green for nuclear counterstaining, and aqua for colocalized blue and green staining. This digital map was visually checked against the original image to ensure accuracy. The program outputs the area of each color as a percentage of all 3 colors. Positive staining was calculated by adding the percentages of blue and aqua (% positive staining).

Luciferase Assay

We used the Cignal SMAD Reporter kit (SABiosciences, Frederick, MD) to investigate the effects of miR-200b on the TGF-β-induced signal transduction pathway.[24] The construct encodes the firefly luciferase reporter gene under the control of a minimal (m)CMV promoter and tandem repeats of the SMAD transcriptional response element. The assay was carried out according to the manufacturer's instructions. Cultured human bronchial epithelial cells, BEAS-2B (ATCC, Manassas, VA), were cotransfected with 0.5 μg/mL of the SMAD reporter construct and 0.01 μg/mL of LNA-oligonucleotide—inhibitor, mimic, or control (Exiqon)—using the X-tremeGENE siRNA Transfection Reagent (Roche). After 48 hours, luciferase activity was measured with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Western Blots

BEAS-2B cells were transfected with 0.01 μg/mL of LNA-oligonucleotides. After 48 hours, the cells were washed and protein extracts were prepared in lysis buffer: 10 mM Tris-HCl (pH 6.8), 5 μM β-glycerophosphate, 20 μM EDTA, 5% SDS, a protease inhibitor cocktail tablet, and phosphatase inhibitors (1 mM sodium orthovanadate, 2 mM EDTA, 10 mM sodium pyrophosphate, 30 mM sodium chloride). The supernatant protein concentration was determined using RC DC Protein Assay (Bio-Rad, Hercules, CA). Fifteen micrograms of total protein was reduced with mercaptoethanol, size fractionated with SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). Specific proteins were detected with the following antibodies: anti-SMAD2/3 (1:750) (Cell Signaling, Danvers, MA), anti-phospho-SMAD2 (1:750) (Cell Signaling), anti-ZEB2 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GAPDH (1:10000) (Abcam). Primary antibodies were detected using HRP-conjugated goat–anti-rabbit antibody (1:7000) (Bio-Rad) and HRP-conjugated goat–anti-mouse antibody (1:7000) (Bio-Rad). Exposed films were scanned and band densities were obtained after background subtraction using ImageJ software in a blinded fashion. Band densities were normalized against the corresponding GAPDH values.

Statistical Analysis

All quantitative data are presented as mean ± standard error of the mean or median and interquartile range (IQR) where appropriate. Statistical analyses were performed using the statistical software SigmaStat (version 3.5; Systat Software Inc). Statistical comparisons were performed using the unpaired Student *t* test and nonparametric Mann-Whitney *U* test where appropriate. Paired analysis was performed using a Wilcoxon signed rank test. Differences were considered significant at $P < 0.05$.

RESULTS

miR-200b and miR-10a Expression Are Increased in Fetal Hypoplastic CDH Lungs

The expression profile of the 319 human miRNAs tested is shown as a heat map in Figure E1 of the online data supplement (available at http://links.lww.com/SLA/A738). Two miRNAs, miR-200b and miR-10a, were more abundant in prenatal hypoplastic CDH lungs compared to age-matched normal control lungs ($P < 0.01$) (Fig. 1A). Both miR-200b and miR-10a had an approximately threefold greater expression in CDH lungs compared to age-matched control lungs (Fig. 1B). Four other miRNAs—miR-27a, miR-195, let-7a, and miR-1—were tested on the basis of near significant differences in the microarray analysis (Fig. E1, available at http://links.lww.com/SLA/A738). None of these had a statistically significant difference in expression in CDH lungs compared to control lungs using RT-qPCR (results not shown).

Tracheal Fluid: Responders Have Higher Expression of miR-200 Than Nonresponders After FETO

On the basis of these observations, we focused on validating and further exploring the expression of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-10a in tracheal and amniotic fluid samples of CDH patients at baseline (plug) as well as following forced lung growth by FETO (unplug). Responders (ie, with measurable lung growth and eventually surviving) to FETO had increased expression of the miR-200 family in their tracheal fluid. Expression of miR-200 was higher after FETO (unplug) in responders compared to nonresponders (Figs. 2A–E). However, their baseline expression was not different. Conversely, tracheal fluid miR-10a expression was lower in responders than in nonresponders at baseline (plug). After FETO (unplug), the expression of miR-10a increased in responders but remained unchanged in nonresponders (Fig. 2F).

Expression of miR-200 Family and miR-10a in Amniotic Fluid Decreases After FETO

After FETO (unplug), the amniotic fluid levels of the investigated miRNAs did not mirror what was observed in the tracheal fluid. The amniotic fluid levels were overall lower. Because the lungs contribute largely to the amniotic content in the third trimester and were occluded during FETO, this was anticipated.[25] The expression profiles of all investigated microRNAs exhibited a similar trend: FETO responders had significantly higher expression than nonresponders (Fig. 3). More specifically, expression of miR-200a and miR-200c significantly decreased after FETO (unplug) (Figs. 3A and C). Expression of miR-200a, miR-200c, miR-141, and miR-10a was higher in amniotic fluid of survivors than in nonsurvivors after FETO (unplug) (Figs. 3A, C, D, and F).

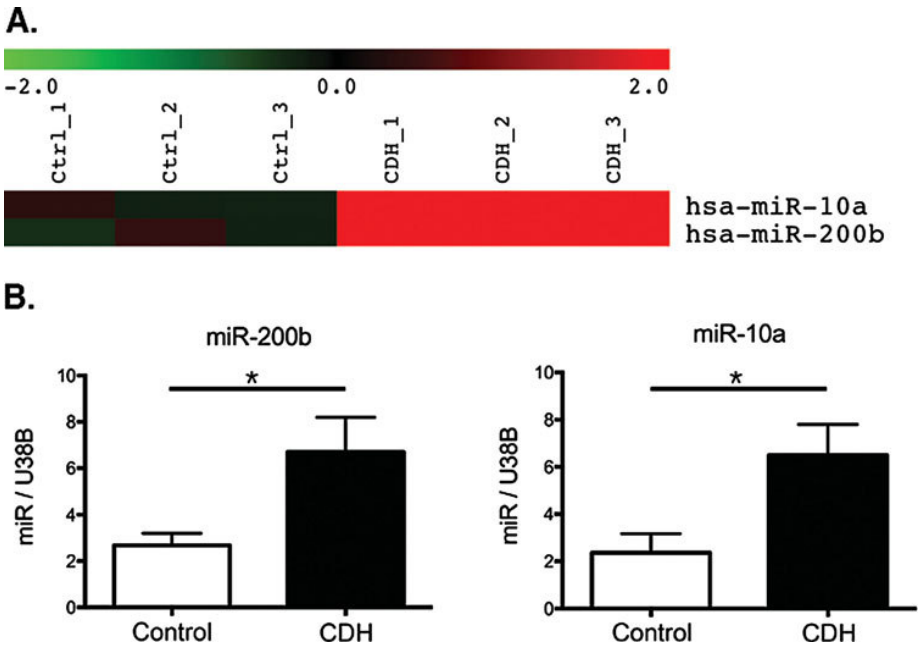

FIGURE 1. Upregulation of miR-200b and miR-10a in prenatal lungs in CDH. Total RNA was extracted from 3 CDH cases and 3 age-matched controls (Ctrl). A, Heat map representation of normalized microarray data (log 2 scale). Of the 319 miRNAs tested, miR-200b and miR-10a were overexpressed in hypoplastic lungs ($P < 0.01$). B, Real-time PCR confirmed overexpression of miR-200b and miR-10a in CDH ($^*P < 0.05$). miRNA expression is normalized relative to SNORD38B (U38B).

A Good Pulmonary Response After FETO Also Alters Pulmonary Vascular Reactivity to Oxygen

The median relative increase in O/E LHR in fetuses surviving after birth was significantly higher than in nonresponders [161.7 (IQR: 252.4–140.8) vs 29.8 (IQR: 10.4–50.2); $P < 0.0001$] (Table 2). Vascular reactivity could be assessed in 4 of 10 poor responders and 6 of 11 good responders. In fetuses with poor response after FETO, we did not observe a change in median deltaPI [−2.0% (IQR: −10.3 to 31.0) vs 13.6% (IQR: 1.3–28.2); $P = 0.63$]. In contrast in fetuses with a good response, median deltaPI increased significantly after FETO [4.6% (IQR: −1.3 to 11.9) vs 23.0% (IQR: 3.4–39.8); $P = 0.03$].

Expression of miR-200b Is Higher in the Distal Part of Postnatal Hypoplastic Lungs

We performed in situ hybridization to determine miR-200b expression in a separate set of postnatal lung sections. Hypoplastic CDH lungs were characterized by increased miR-200b expression, particularly in the terminal saccules and alveoli (Fig. 4A). We used image analysis software to generate color-coded maps and quantify the area of positive staining for each lung section. Using this method, neonatal hypoplastic CDH lungs displayed increased miR-200b expression compared to age-matched control lungs (Fig. 4B).

In situ hybridization for miR-200b produced a highly specific staining pattern in normal neonatal lungs. Bronchial epithelial cells were intensely positive for miR-200b (Fig. 4C, *thick black arrow*). In contrast, parabronchial smooth muscle cells were predominantly negative for miR-200b (Fig. 4C, *thick white arrow*). Terminal saccules contained a mixed population of positive (alveolar type II cells) and negative-staining cells. In blood vessels, endothelial cells (inner layer) were positive for miR-200b (Fig. 4C, *black arrowhead*). Perivascular smooth muscle cells, on the other hand, were negative (Fig. 4C, *white arrowhead*). Mesothelial cells of the pleura were positive for miR-200b (results not shown).

TGF-β2, a miR-200b Target Gene, Expression Is Decreased in Hypoplastic CDH Lungs

Others have identified components of the TGF-β-induced signal transduction pathway as the major targets of the miR-200 family.[26] We used immunohistochemistry to assess TGF-β2 expression in postnatal lung tissues. The cellular distribution of TGF-β2 was very similar to miR-200b, that is, expressed in bronchial epithelial cells and vascular endothelial cells, but absent from parabronchial and perivascular smooth muscle cells. CDH lungs displayed decreased TGF-β2 expression in terminal saccules compared to age-matched control lungs (Fig. 5A).

miR-200b Downregulates TGF-β Signaling in Human Bronchial Epithelial Cells

Downstream effects of TGF-β-induced signal transduction are initiated through SMAD2/3 phosphorylation and its subsequent nuclear translocation.[27] We used a luciferase bioluminescent assay to measure SMAD-induced gene expression and the impact of changing miR-200b expression in cultures of human bronchial epithelial cells. In a pilot experiment measuring miR-200b expression in different cell lines relevant to lung, we demonstrated that these cells exhibit abundant miR-200b expression (results not shown). In absence of exogenous TGF-β, bronchial epithelial cells displayed very little SMAD-dependent luciferase activity. This low baseline activity did not change when miR-200b mimics were added to the culture medium. On the contrary, inhibitors of miR-200b increased SMAD-luciferase activity by several orders of magnitude (Fig. 5B). Collectively, these data suggest that basal TGF-β-induced signaling

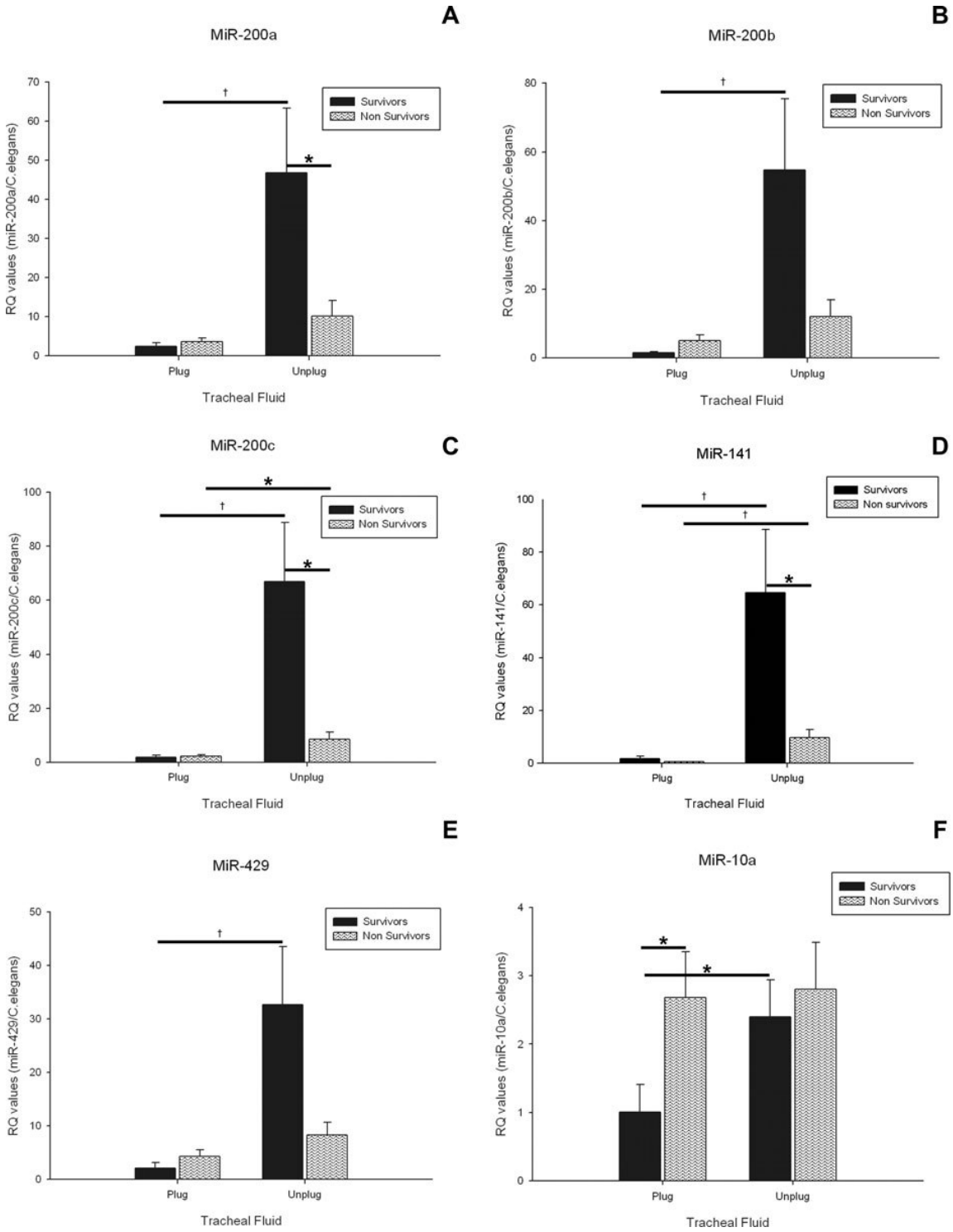

FIGURE 2. miR-200 family members and miR-10a expression analysis in tracheal fluid samples of surviving and nonsurviving patients with CDH undergoing FETO. Expression levels of miR-200a (A), miR-200b (B), miR-200c (C), miR-141 (D), miR-429 (E), and miR-10a (F) in tracheal fluid samples. Expression levels of these microRNAs were normalized to cel-miR-39. Statistical analysis was performed using the Mann-Whitney *U* test for independent comparisons between surviving and non-surviving patients and a Wilcoxon signed-rank test for paired comparisons between plug and unplug, $^{*}P < 0.05$; $\dagger P < 0.01$; $\ddagger P < 0.001$.

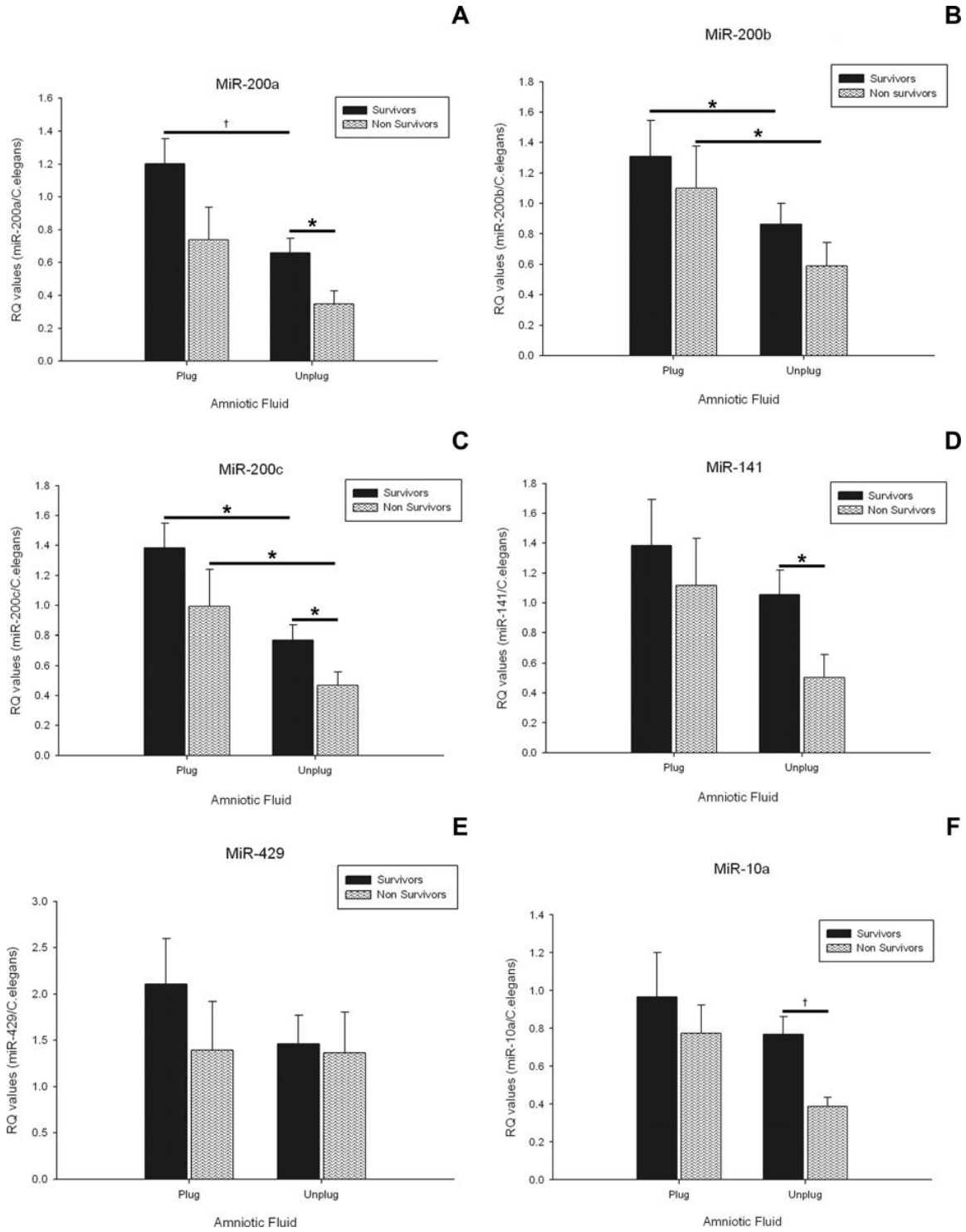

FIGURE 3. miR-200 family members and miR-10a expression analysis in amniotic fluid samples of surviving and nonsurviving patients with CDH undergoing FETO. Amniotic fluid levels of miR-200a (A), miR-200b (B), miR-200c (C), miR-141 (D), miR-429 (E), and miR-10a (F). Expression levels of these microRNAs were normalized to cel-miR-39. Statistical analysis was performed using the Mann-Whitney *U* test, for independent comparisons between surviving and non-surviving patients and a Wilcoxon signed-rank test for paired comparisons between plug and unplug, $^{*}P < 0.05$; $\dagger P < 0.01$.

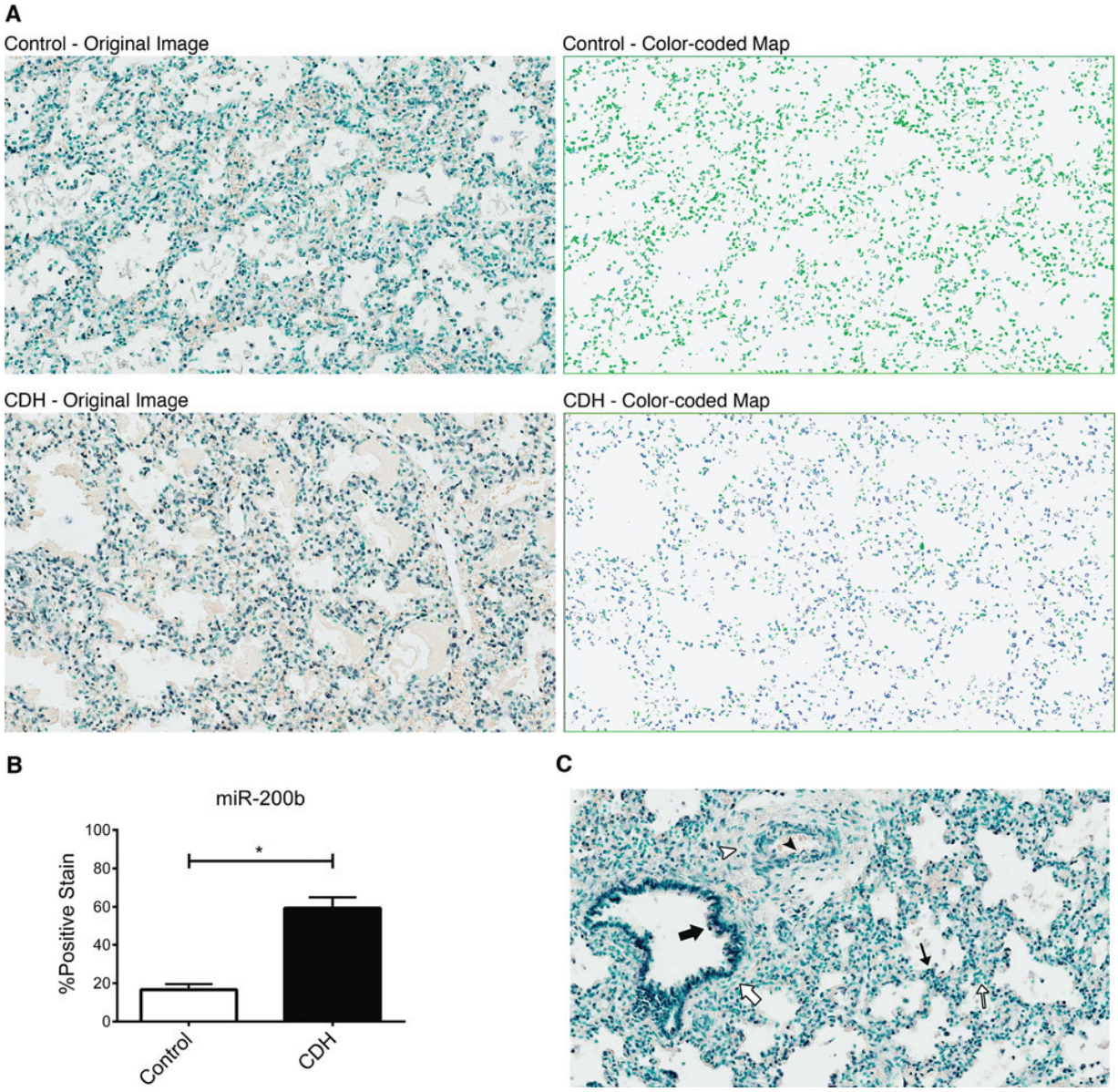

FIGURE 4. In situ hybridization for miR-200b (blue stain) in postnatal lung (methyl green counterstain, 200× magnification). A, Terminal saccules of control vs CDH lungs (color-coded maps: blue = positive staining, green = negative staining, aqua = colocalized positive and negative staining). B, Area of positive staining (% blue +% aqua) was calculated for each lung section ($^*P < 0.05$). C, miR-200b expression in normal postnatal lung. In bronchioles, miR-200b is expressed in epithelial cells (black thick arrow), but is absent from parabronchial smooth muscle cells (white thick arrow). In blood vessels, miR-200b is expressed in endothelial cells (black arrowhead) but is absent from the surrounding smooth muscle cells (white arrowhead). Terminal saccules contain a mixed population of miR-200b-positive (black thin arrow) and miR-200b-negative (white thin arrow) cells.

is tempered by steady state levels of miR-200b, and that reducing miR-200b is permissive for TGF-β-induced signaling.

To clarify mechanisms that might underpin altered basal SMAD-mediated responses, we next performed Western blotting using antibodies against total SMAD2/3 and phospho-SMAD2/3 (p-SMAD). Addition of miR-200b inhibitors or mimics to cultures of bronchial epithelial cells resulted in a nearly twofold increase or decrease, respectively, in p-SMAD levels; however, total SMAD2/3 abundance was unaffected (Fig. 5C). ZEB2 is a downstream transcriptional repressor in the TGF-β/SMAD signal transduction pathway and a putative target of miR-200b based on sequence complementarity.[27] Addition of miR-200b inhibitors or mimics to cultures of bronchial epithelial cells resulted in a nearly twofold increase or decrease, respectively, in ZEB2 levels (Fig. 5C).

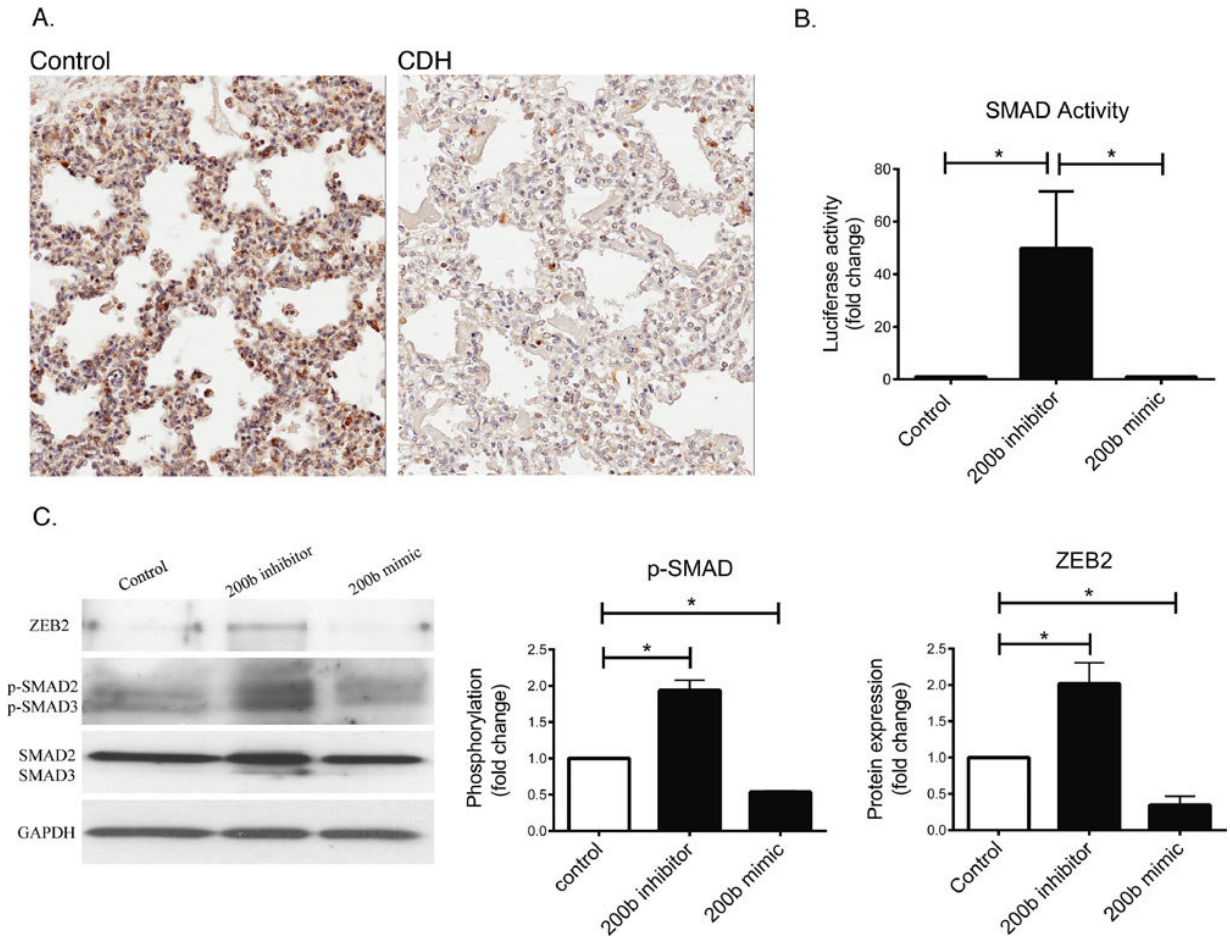

FIGURE 5. miR-200b Target Gene Expression. A, Immunohistochemistry for TGF-β2 (brown stain) in control vs CDH postnatal lungs (hematoxylin counterstain, 200× magnification). B, SMAD-luciferase reporter assay in cultures of bronchial epithelial cells. Luciferase activity was measured after a 48-hour incubation with miR-200b inhibitor, mimic, or control oligonucleotide. C, Immunoblotting for ZEB2 and phospho-SMAD (p-SMAD). Bronchial epithelial cells were incubated for 48 hours with miR-200b inhibitor, mimic, or control oligonucleotide ($^*P < 0.05$).

DISCUSSION

The lack of success in identifying a pure genetic cause for CDH prompted us to investigate the role of epigenetics and more in particular microRNAs. Our study is the first to demonstrate altered miRNA expression in human congenital lung disease. Homogenates of hypoplastic lungs from CDH fetuses display miR-200b and miR-10a overexpression. We then selected tracheal and amniotic fluid samples from fetuses with appropriate or insufficient lung growth after fetal surgery. Responders could be discriminated from nonresponders by a significantly higher miR-200 expression in their tracheal fluid, suggesting that stimulated lung development is associated with an increase in miR-200 expression. Upregulation of miR-200b persists in terminal saccules of CDH patients and is associated with decreased TGF-β2 expression. In vitro, miR-200b inhibits TGF-β/SMAD signaling in bronchial epithelial cells.

Using in situ hybridization, we determined that miR-200b is overexpressed in the terminal saccules of postnatal CDH lungs compared to age-matched controls. miR-200b is a member of the miR-200 family, including also miR-200a, miR-200c, miR-141, and miR-429.[26,28–32] These miRNAs have similar sequences and are transcribed in 2 clusters: miR-200b, miR-200a, and miR-429 share a common transcription start site on chromosome 1, whereas miR-200c and miR-141 are transcribed as a single unit from chromosome 12. There is increasing evidence that members of the miR-200 family play a role in cancer. Several studies have demonstrated that miR-200 expression is upregulated in epithelial tissues, where they contribute to the epithelial cell phenotype by inhibiting mesenchymal gene expression.[29] We observed strong miR-200b expression in bronchial and alveolar epithelial cells. In contrast, parabronchial smooth muscle cells, which are differentiated mesenchymal cells, were negative for miR-200b. Mesothelial and endothelial cells were also positive for miR-200b. We have previously demonstrated that hypoplastic CDH lungs are characterized by a disturbed epithelial/fibroblast cell ratio in favor of fibroblasts.[23]

The miR-200 family functions by inhibiting several genes involved in the TGF-β/SMAD signaling pathway.[26,33] Increased TGF-β activity enhances miR-200 expression as part of a negative feedback loop.[34] Others have previously demonstrated in the surgical

sheep model of CDH that FETO increased TGF-β2 expression in hypoplastic lungs.[35] In addition, increased TGF-β expression has been observed in hypoplastic lungs in the nitrofen-induced rat model of CDH.[36–38] Here, we observed increased miR-200 expression in prenatal and postnatal lung tissues in CDH and tracheal fluid samples of CDH patients responding to FETO. Our data suggest that the increase in miR-200 might result from an inherent increased TGF-β expression in hypoplastic lungs. In addition, FETO can increase miR-200b expression even further in lungs responding to mechanical stretch via upregulation of TGF-β expression in these lungs. The primary insult leading to CDH and pulmonary hypoplasia occurs very early in gestation when a diagnosis cannot be made yet.[39,40] Because of the unavailability of human fetal tissues during these early stages of lung development, we were unable to investigate miRNA or TGF-β expression at the time of the primary insult. In postnatal lung tissues of CDH cases, we observed decreased TGF-β2 expression, particularly in the terminal saccules. During fetal lung development, endogenous TGF-β blocks hormone-induced type II epithelial cell differentiation.[41] Decreased TGF-β activity in late stages of lung development might explain why human cases of CDH display normal surfactant maturation.[42,43] In addition, exogenous surfactant has been demonstrated to be of no benefit in the treatment of pulmonary hypoplasia associated with CDH.[44,45]

We established the inhibitory effect of miR-200b on TGF-β/SMAD signaling in human bronchial epithelial cells. Given their epithelial phenotype, these cells displayed a very low level of intrinsic SMAD2/3 activity. miR-200b inhibition significantly enhanced SMAD2/3 phosphorylation, suggesting that in bronchial epithelial cells, miR-200b functions by suppressing endogenous TGF-β activity. Both increased and decreased TGF-β signaling can lead to abnormal lung development.[46–48] Our results suggest that miR-200b plays an important role in normal lung development by closely regulating TGF-β signaling.

Extracellular microRNAs have recently emerged as potential biomarkers because they have been shown to be associated with various pathological conditions including cancer. Biomarkers have also successfully been used to stratify therapy and/or to evaluate response to therapy. Both miR-200 and miR-10a have earlier served as biomarkers in some conditions. Most of these studies have focused on their role in cancer progression. Increased serum miR-200c expression is associated with colorectal cancer progression and metastasis[49] and gastric cancer.[50] In contrast, decreased miR-200a expression is associated with poor prognosis and recurrence in ovarian cancer.[51] The role of miR-10a as a biomarker is less well established. A recent study showed overexpression of miR-10a in human pancreatic cancer cells.[52] Furthermore, a combination of miR-10a and miR-200b has recently been reported to be a valuable microRNA signature for metastatic medullary thyroid carcinoma[53] and bladder cancer.[54] We have shown that tracheal fluid expression of miR-200 and miR-10a can serve as a marker of response to FETO. Although FETO decreased amniotic miRNA expression, survivors could be distinguished by higher expression of miR-200a, miR-200c, miR-141, and miR-10a.

Considering our study limitations, we are aware that this study has a relatively small sample size, no detailed phenotypic information on the termination of pregnancy patients, a narrow window of gestational age in the FETO group and most importantly, no control amniotic or tracheal fluids. For obvious reasons, it would be unethical to try and obtain tracheal fluid samples from control patients. The first experiment was carefully planned to include only prenatal lungs for identification of potential microRNAs as etiological factors of pulmonary hypoplasia in CDH. CDH is typically diagnosed at second trimester ultrasound[55] and therefore, our 3 pairs of prenatal CDH and control lungs (22–25 weeks of gestation) form a unique homogenous set of severely hypoplastic lungs, which were not exposed to confounding pre- or postnatal factors directly interfering with lung development (steroids, ventilation, etc). These lungs were obtained from terminations of pregnancies for medical reasons and processed for research purposes within 1 hour of termination. In addition, the tracheal and amniotic fluid samples were carefully collected as part of a systematic collection of biofluids in a very well characterized homogenous group of patients undergoing FETO.

CONCLUSIONS

We are the first to report altered microRNA expression in clinical cases with abnormal lung development due to severe isolated CDH. Future studies should reveal if manipulating miR-200 and miR-10a expression improves the natural course in CDH patients and their abnormal lung development.

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P.P.-T. and J.D. contributed equally to this article.

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CHAPTER IV.

RETINOIC ACID AND NEUROENDOCINE FACTORS

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NEUROENDOCRINE FACTORS REGULATE RETINOIC ACID RECEPTORS IN NORMAL AND HYPOPLASTIC LUNG DEVELOPMENT

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Running Title:

Retinoic acid and neuroendocrine factors

Keywords: Congenital Diaphragmatic Hernia, Retinoic Acid, Bombesin, Ghrelin, Retinoic Acid Receptors, Lung development

KEY POINTS:

1) Retinoic Acid (RA) and Ghrelin levels are altered in human hypoplastic lungs when compared to healthy lungs. Although there are many data about RA, ghrelin and bombesin in CDH rat model, neuroendocrine factors have never been associated with RA signalling pathway in this animal model.
2) In this study, the interaction between neuroendocrine factors and RA was explored in the CDH rat model.
3) The authors' results indicate that normal foetal lung explants treated with RA, bombesin and ghrelin present an increase in lung growth. Hypoplastic lungs presented higher RAR α/γ expression levels. Moreover bombesin and ghrelin supplementation, *in vitro*, to normal lungs increased RAR α/γ expression whereas bombesin and ghrelin antagonists administration to normal and hypoplastic lungs decreased it.
4) This data reveals for the first time that there is a link between neuroendocrine factors and RA, and that neuroendocrine factors sensitize the lung to the RA action through RAR modulation.

ABSTRACT

Congenital diaphragmatic hernia (CDH) is characterised by a spectrum of lung hypoplasia and consequent pulmonary hypertension, leading to high morbidity and mortality rates. Moreover, CDH has been associated with an increase in the levels of pulmonary neuroendocrine factors, such as bombesin and ghrelin, and a decrease in the action of retinoic acid (RA). The present study aimed to elucidate the interaction between neuroendocrine factors and RA.

To determine the link between neuroendocrine factors and RA, *in vitro* analyses were performed on Sprague Dawley rat embryos. Normal lung explants were treated with bombesin, ghrelin, a bombesin antagonist, a ghrelin antagonist, dimethylsulfoxide (DMSO), RA dissolved in DMSO, bombesin plus RA and ghrelin plus RA. Hypoplastic lung explants (nitrofen model) were cultured with bombesin, ghrelin, bombesin antagonist or ghrelin antagonist. The lung explants were analysed morphometrically, and retinoic acid receptor (RAR) α, β and γ expression levels were assessed via Western blotting. Immunohistochemistry analysis of RAR was performed in normal and hypoplastic E17.5 lungs.

Compared with the controls, hypoplastic lungs exhibited significantly higher RARα/γ expression levels. Furthermore considering hypoplastic lungs, bombesin and ghrelin antagonists decreased RARα/γ expression. Normal lung explants (E13.5) treated with RA, bombesin plus RA, ghrelin plus RA, bombesin or ghrelin exhibited increased lung growth. Moreover, bombesin and ghrelin increased RARα/γ expression levels, whereas the bombesin and ghrelin antagonists decreased RARα/γ expression.

This study demonstrates for the first time that neuroendocrine factors function as lung growth regulators, sensitising the lung to the action of RA through up-regulation of RARα and RARγ.

INTRODUCTION:

Congenital diaphragmatic hernia (CDH) is a severe developmental anomaly with a mean incidence of 1:3000 live births whose aetiology remains poorly understood (Van den Hout et al., 2009; Keller et al., 2010; Nogueira-Silva et al., 2011). This congenital anomaly is characterised by a diaphragmatic defect that allows intra-thoracic herniation of abdominal organs and maldevelopment of the alveoli and pulmonary vessels (Van Loenhout et al., 2009). Thus, new-borns with CDH develop pulmonary hypoplasia and pulmonary hypertension (PH) and, consequently, severe respiratory failure. In fact, among the known causes of severe respiratory failure in new-borns, CDH remains the most life threatening (Goshe et al., 2005).

Despite the increased understanding of the pathophysiology of CDH and recent advances in neonatal care, CDH remains a challenging condition that is associated with high morbidity and mortality rates (Pereira-Terra et al., 2015; Pereira-Terra et al, in press). Thus, the development of a less invasive, safer and cheaper approach with minimum risk to the mother and the foetus is crucial (Leeuwen and Fitzgerald, 2014). To this end, a search for less invasive antenatal approaches that promote foetal lung growth has emerged.

Retinoic acid (RA), an active metabolite of vitamin A, binds to specific RA receptors (RARs and RXRs) and functions as an essential signal for lung growth and differentiation (Thébaud et al., 1999). Indeed, RA is involved in various phenomena related to lung morphogenesis, including the formation of the lung primordium, distal bud outgrowth, differentiation of epithelial cells and the distal mesenchyme, and promotion of alveolisation by inducing the formation of secondary septae. Additionally, the retinoid signalling pathway has been shown to be involved in CDH pathophysiology. For example, decreased plasma levels of retinol and retinol-binding protein are observed in individuals with CDH (Major et al., 1998). Furthermore, CDH has been observed in patients with deletions within chromosome 15q, which contains the gene encoding cellular retinoic acid binding protein (CRABP1) (Pober, 2007). In animal studies, double RAR knockouts (RARα and RARβ knockouts) were shown to exhibit unilateral lung agenesis and contralateral lung hypoplasia (Mendelsohn et al., 1994; Beurskens et al., 2009). Moreover,

in a nitrofen-induced CDH rat model, which is the best animal model mimicking CDH, inhibition of the RA signalling pathway was reported to occur, and rescue of nitrofen-induced PH in foetal rat lungs was observed upon maternal administration of RA (Thébaud et al., 1999; Antipatis et al., 1998; Ruttenstock et al., 2011; Sugimoto et al., 2008; Asabe et al., 1999; Doi et al., 2010; Baptista et al., 2005). The neuroendocrine factors bombesin and ghrelin are produced by pulmonary neuroendocrine cells (PNECS) and appear to play important roles in lung development and maturation from intrauterine life to the postnatal period (Asabe et al., 1999). Furthermore, CDH has been associated with an increased bombesin and ghrelin levels. Moreover early maternal administration of ghrelin during gestation was shown to attenuate pulmonary hypoplasia in a CDH animal model (Santos et al. 2006, Cutz et al., 2007). Therefore, the aim of this study was to evaluate the putative association between neuroendocrine factors (bombesin and ghrelin) and RA during normal and hypoplastic foetal lung development.

MATERIALS AND METHODS:

Ethical approval:

Animal experiments were performed according to the Portuguese law for animal welfare (Diário da República, Portaria 1005/92). Animals were housed in an accredited animal facility and treated as specified by the recommendations of the NIH guidelines and the Guide for the Care and Use of Laboratory Animals, published by the National Academy Press (Doi et al., 2010; Baptista et al., 2005).

Animal model and experimental design:

Female Sprague Dawley 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 plugs. The day of plugging was defined as gestational day 0.5 for the purpose of time dating. According to the nitrofen-induced CDH rat model,

randomly selected pregnant rats received 100 mg of nitrofen (2,4-dichlorophenyl-p-nitrophenylether) dissolved in 1 ml of olive oil via gavage on gestational day 9.5 dpc (days post-conception) (hypoplastic group - N) (Nogueira-Silva et al., 2011). Control animals only received only olive oil (control group - C). The dams were anaesthetized with pentobarbital overdose (150mg/kg intraperitonial) followed by decapitation. After that, the foetuses were removed through Caesarean section and euthanized by decapitation. For lung explant cultures, the foetuses were harvested, and their lungs were dissected at 13.5 dpc and incubated with different compounds or inhibitors (Table 1) and at 17.5 dpc for immunohistochemistry.

Table 1: Schematic representation of the study design.

EXPLANTS	NORMAL	+Bombesin
		+Ghrelin
		+Bombesin Antagonist
		+Ghrelin Antagonist
		+Retinoic Acid (RA)
		+Bombesin+RA
		+Ghrelin+RA
	NITROFEN	+Bombesin
		+Ghrelin
		+Bombesin Antagonist
		+Ghrelin Antagonist

Immunohistochemistry (IHC):

Immunostaining was performed in formalin-fixed and paraffin-embedded control and hypoplastic lungs at 17.5 dpc, as previously described (Nogueira-Silva et al., 2012). All the slides were stained during the same time. The RARα antibody (Santa Cruz Biotechnology Inc, Heidelberg, Germany) was used at a 1:200 dilution, the RARβ antibody (Santa Cruz Biotechnology Inc.) at a 1:50 dilution, and the RARγ antibody (Abcam at a 1:100 dilution. Incubation with UltraVision detection system anti-polyvalent horseradish peroxidase (Lab Vision Corporation) was carried out according to the manufacturer's instructions. Omitting the primary antibodies negative controls were performed. The slides were photographed with an Olympus BX61

microscope (Olympus, Tokyo, Japan). At least three independent experiments were performed.

Foetal lung explant culture

Control and hypoplastic lungs were removed from 13.5 dpc embryos, harvested and dissected in DPBS (Lonza) under a dissection microscope (SZX16, Olympus). The lungs were then transferred to Nucleopore membranes (Isopore^{TM} membrane filters, Millipore) and cultured for 4 days as previously described (Piairo et al., 2011).

The control and hypoplastic lung cultures were supplemented daily with 1 μM bombesin (Tocris Bioscience) (Kresch et al., 1999) or 30 nM ghrelin (Peptides international, Louisville) (Nunes et al., 2008) and with one of the following treatments: 10^{-6} M RA (Sigma) dissolved in 0.1% dimethylsulfoxide (DMSO), 0.1% DMSO alone, 1 μM bombesin plus 10^{-6} M RA, 30 nM ghrelin plus 10^{-6} M RA, 10^{-7} M bombesin antagonist (Phoenix Pharmaceuticals) (Hosli et al., 1993) or 10^{-6} M ghrelin antagonist (Bachem AG) (Nunes et al., 2008). Thus, this set of experiments included the following 14 groups: control (n=27), control plus ghrelin (n=27), control plus bombesin (n=27), control plus ghrelin antagonist (n=27), control plus bombesin antagonist (n=27), control plus DMSO (n=27), control plus RA dissolved in DMSO (n=27), control plus bombesin and RA (n=27), control plus ghrelin and RA (n=27), nitrofen (n=27), nitrofen plus ghrelin (n=27), nitrofen plus bombesin (n=27), nitrofen plus bombesin antagonist (n=27) and nitrofen plus ghrelin antagonist (n=27). The culture medium was replaced every 48 hours. Lungs were collected for Western blotting after 4 days in culture (96 h).

Morphometric analysis

Branching morphogenesis was monitored daily by photographing the explants using a stereomicroscope equipped with a camera (DP71, Olympus). At day 0 (D_0: 0 h) and day 4 (D_4: 96 h) of culture, branching, the epithelial and total perimeters, and the epithelial and total areas were measured using ImageJ image processing and analysis software (version 1.44, Maryland, USA).

For all experimental conditions, the results were expressed as the D_4/D_0 ratio.

Western blotting

Lung explants were processed for Western blot analysis using an RARα antibody at a 1:500 dilution, an RARβ antibody at a 1:200 dilution and an RARγ antibody (Santa Cruz Biotechnology Inc.) at a 1:500 dilution.

Protein lysates of pooled lung explants (n=9) from each group were obtained via homogenisation of the foetal tissue with a pellet pestle motor (Kontes, Chicago, USA) as previously described (Nogueira-Silva et al., 2013). Different pooled lung samples were used, and three independent experiments were performed. Ten μg of protein was loaded onto 8% acrylamide minigels, and the procedure was performed as previously described (Piairo et al., 2011). The blots were incubated with RARα, RARβ or RARγ antibodies overnight at 4ºC, followed by an appropriate secondary anti-rabbit (1:5000 dilution) (Santa Cruz Biotechnology Inc.) or anti-mouse (1:2000 dilution) (Cell Signalling Technology) horseradish peroxidase-conjugated antibody. The blots were subsequently developed with the Super Signal West Femto Substrate (Pierce Biotechnology), and the chemiluminescent signal was captured using a Chemidoc XRS system (Bio-Rad, Philadelphia, USA). As a loading control, the blots were probed with a β-tubulin antibody (1:150,000 dilution; Abcam). Quantitative analysis was performed using Quantity One analysis software (version 4.6.5 1D, Bio-Rad).

Statistical analysis

All quantitative data are presented as the mean ± SEM. Statistical analysis was performed via one-way ANOVA using SigmaStat 3.5 (Systat Software Inc., California, USA). The Bonferroni test was applied for post-test analysis. The threshold for statistical significance was set at $p<0.05$.

RESULTS:

RAR expression pattern in normal and hypoplastic 17.5 dpc rat lungs

Immunohistochemistry analyses revealed that RARα, β and γ were expressed in the epithelial and mesenchymal cells of both control and hypoplastic lungs (Figure 1). Moreover, RARα and RARγ expression appeared to be higher in hypoplastic lungs than in control lungs (Figure 1).

Bombesin, ghrelin, RA, bombesin plus RA and ghrelin plus RA supplementation analyses

To evaluate the effects of neuroendocrine factors (bombesin and ghrelin), RA and bombesin or ghrelin plus RA on lung growth, control and hypoplastic foetal lung explants were performed. Figure 2A shows representative examples of control foetal lung explants treated with bombesin (1 μM), ghrelin (30 nM), RA (10^{-6} M), bombesin plus RA or ghrelin plus RA. In normal lungs, bombesin, ghrelin, RA and the combination of bombesin or ghrelin with RA appear to stimulate lung growth (Figure 2A). In fact, all of these compounds increased the total number of peripheral airway buds, and bombesin, RA, bombesin plus RA and ghrelin plus RA also increased the epithelial perimeter (Figure 2B). Moreover RA increases the total area of the lungs (Figure 2B).

In contrast, bombesin and ghrelin did not interfere with hypoplastic lung growth, as shown in Figure 2C and confirmed through morphometric analysis (Figure 2D).

RAR protein expression after bombesin, ghrelin, bombesin plus RA and ghrelin plus RA supplementation

To investigate the possible mechanisms underlying the effects of bombesin, ghrelin, and neuroendocrine factors plus RA interactions, the protein levels of RARα, β and γ were assessed. RARα and RARγ expression levels were higher in untreated hypoplastic lungs than in control lungs (Figure 3A, C). Bombesin and ghrelin supplementation induced RARα and RARγ expression

in normal lungs (Figure 3A, C). Bombesin plus RA and ghrelin plus RA also induced RARα expression (Figure 3A). In contrast, ghrelin-treated hypoplastic lungs exhibited decreased RARα and RARγ expression (Figure 3A, C). The expression of RARβ was similar in all of the studied groups (Figure 3B).

Effect of RA on RAR expression in normal foetal lungs

To assess the effect of RA on RARα, β and γ, the levels of these three proteins were evaluated. In control lungs, RA led to decreased RARα and RARγ expression but increased RARβ expression (Figure 4).

Effects of bombesin and ghrelin antagonists on normal and hypoplastic lung growth and RAR expression

To confirm the roles of bombesin and ghrelin in normal and hypoplastic lung development, antagonists of these peptides were added to lung explants. The bombesin and ghrelin antagonists significantly inhibited lung growth, as indicated by decreases in the total number of peripheral airway buds and the epithelial perimeter (Figure 5 and 7A). However, the total area remained unchanged after treatment with the antagonists (data not shown). Regarding RAR protein expression, explants treated with the bombesin and ghrelin antagonists exhibited a statistically significant decrease in RARα expression (Figure 6A and 7B). In contrast, RARβ expression in treated explants was similar to that in untreated explants, and a slight decrease in RARγ expression was observed in explants treated with these antagonists (Figure 6B, C and 7B, C).

DISCUSSION:

Congenital diaphragmatic hernia (CDH) is associated with pulmonary hypoplasia, and it has been shown that RA signalling is involved in the establishment of this disease. Furthermore, CDH has been linked to increased levels of neuroendocrine factors. This report describes a putative link between neuroendocrine factors and the RA pathway in normal and hypoplastic lung development for the first time.

Retinoids such as vitamin A and its derivatives are crucial for growth, development and tissue differentiation (Blomhoff and Blomhoff, 2006). RA acts by binding to nuclear receptors that exist in 3 different isoforms: α, β and γ (Mactier and Weaver, 2005). RA receptors are expressed from the earliest stages of embryonic lung development into postnatal life. RARα is ubiquitously expressed at consistent levels over time during lung development, whereas RARβ is excluded from the distal epithelium during branching but is still present in the epithelial cells of proximal and midsized airways (Wongtrakool et al., 2003; Roth-Kleiner and Post, 2005). In this study, we show that in the canalicular stage, RARs are expressed in epithelial and mesenchymal cells in both normal and hypoplastic lungs; moreover, the levels of RARα and RARγ appear to be increased in hypoplastic lungs, in contrast to what Rajatapiti (Rajatapiti et al., 2006) have been showed previously in qualitative analysis (Figure 1).

Classically, RA is described as one of the major stimulators of lung development. In CDH, several growth factors, including RA, have been observed to promote lung branching when exogenously administered (Baptista et al., 2005, Keller et al., 2010). Furthermore, the RA signalling pathway has been shown to be involved in CDH pathogenesis and pulmonary hypoplasia. For instance, the incidence rate of CDH is higher in litters born to dams fed vitamin A-deficient diets (Andersen et al., 1941). RARα/RARβ double knockout mice display diaphragmatic hernia (Chen et al., 2003); however, when mutations affect only one isoform of one receptor, no foetal anomalies are detected, suggesting functional redundancy between various receptors and isoforms (Azais-Braesco and Pascal, 2000). Nitrofen

suppresses the RA response element (Major et al., 1998), and *in vitro* assays have shown that nitrofen inhibits RALDH2 (Mendelsohn et al., 1994). In humans, infants with CDH exhibit lower plasma retinol levels than healthy infants (Mey et al., 2003; Beurskens et al., 2007). Additionally, it has been demonstrated that antenatal administration of vitamin A reduces the incidence of CDH and restores lung maturation in the nitrofen rat model and that RA improves lung branching in CDH lungs in foetal lung explant cultures (Thébaud et al., 1999; Thebaud et al., 2001; Babiuk et al., 2004).

Focusing on neuroendocrine cells, studies conducted during the last decade have revealed a complex functional role for PNECs. In the early stages of lung development, PNECs act as modulators of foetal lung growth and differentiation, whereas at the time of birth, they act mainly as airway O_2 sensors involved in neonatal adaptation (Andersen et al., 1941). The major neuroendocrine peptides in the lung are bombesin and ghrelin. Recent data from our group showed that ghrelin is overexpressed in human hypoplastic lungs and the lungs of CDH model rats compared with normal lungs (Santos et al., 2006). Bombesin expression has been shown to be higher in CDH lungs than in normal lungs (Cutz et al., 2007; Santos et al., 2006). These increases in some growth factors observed in CDH lungs might represent a compensatory response of hypoplastic lungs attempting to recover normal levels of lung growth (Santos et al., 2006).

Given that PNECs behave as lung sensors and that RA is a classical lung growth factor and the levels of both neuroendocrine peptides and RA are affected in hypoplastic lungs, we hypothesised a putative link between these two important lung regulators.

In the present study, it was demonstrated that bombesin, ghrelin and particularly RA or bombesin or ghrelin combined with RA promote normal lung branching and increase the lung epithelial perimeter (Figure 2A and 2B). Moreover, exogenous supplementation of bombesin or ghrelin in normal lungs increased RARα and RARγ expression but did not affect RARβ expression (Figure 3). In contrast, inhibition of bombesin or ghrelin decreased RARα and RARγ expression as well as lung branching (Figure 6 and 5). Thus, these results demonstrate, for the first time, that there is a link between neuroendocrine factors and the RA signalling pathway. Moreover,

RARα and RARγ appear to be the major mediators between these two apparently different pathways. Indeed, these results suggest that the action of neuroendocrine factors during lung growth is at least mediated by an increase in RARα and RARγ expression, indicating that RA pulmonary sensitisation is mediated by neuroendocrine factors through RARα and RARγ.

Furthermore, the results showed that RA supplementation in normal lungs decreased RARα and RARγ expression and increased RARb expression (Figure 4). A possible explanation for this observation is that neuroendocrine cells sense the increase in lung growth mediated by RA and consequently decrease the production of neuroendocrine factors, which in turn leads to decreased RARα and RARγ expression. However, the possibility of a direct down-regulation effect of RA on RAR expression cannot be excluded. RARβ expression was stimulated by RA supplementation and remained unchanged by bombesin and ghrelin modulation. These results exclude the involvement of RARβ in the RA/PNEC interaction with respect to lung growth mechanisms.

Regarding pulmonary hypoplasia, according to the literature, RARα and RARγ are overexpressed in hypoplastic lungs compared with normal lungs. *In vitro* bombesin and ghrelin supplementation in hypoplastic lung explant cultures did not affect the examined lung morphometric parameters (Figure 2B and 2D), probably because these receptors are already overexpressed. The expression of RARα and RARγ slightly decreases in hypoplastic lungs after ghrelin supplementation (Figure 3). We do not have a plausible explanation for this result. However, this finding reinforces the idea that there is an association between neuroendocrine factors and RA signalling. Moreover, inhibition of bombesin or ghrelin significantly decreased RARα expression as well as lung branching (Figure 7) reinforcing our hypothesis.

It has largely been demonstrated that RA significantly increases lung branching in hypoplastic lungs after RA supplementation *in vitro*. Moreover, hypoplastic lungs overexpress bombesin and ghrelin (Cutz et al., 2007). In this study, we observed an increase in RARα and RARγ expression in hypoplastic lungs that might be explained by the compensatory

overexpression of bombesin and ghrelin in these lungs (Figure 3). This is in agreement with the effect of neuroendocrine factor supplementation in normal lungs. In Figure 8, a putative mechanism for the link between neuroendocrine cells and the RA pathway is presented.

In summary, based on these results, we can conclude that neuroendocrine factors act as regulators of lung growth, sensitising the lungs to the action of RA through RARα and RARγ up-regulation.

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

PPT performed the experiments and prepared the manuscript.

PPT and JCP conceived and designed the study. PPT, RSM, CNS and JCP analysed and interpreted the results. PPT, RSM, CNS and JCP drafted the manuscript for important intellectual content. Each author contributed to and approved of the final manuscript. All the experiments were done in Life and Health Sciences Research Institute (ICVS) in the University of Minho – Braga, Portugal.

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Figure 1

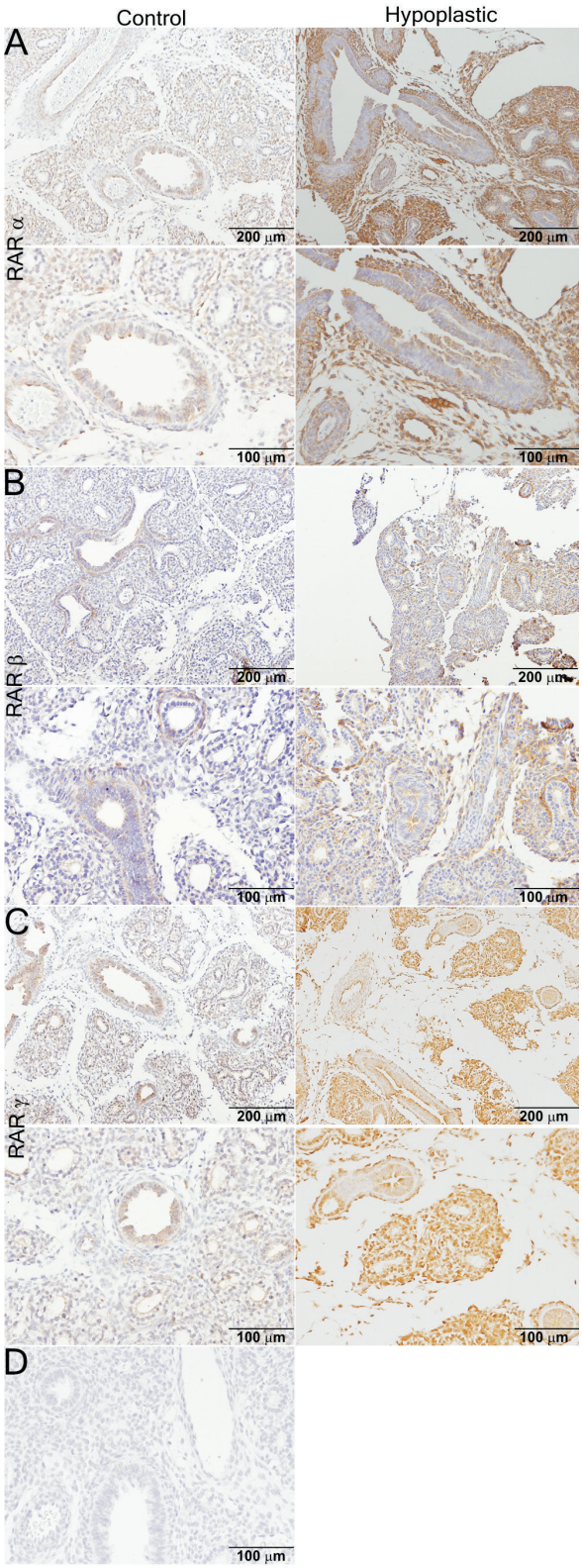

Figure 1. **Retinoic acid receptor (RAR) expression patterns in 17.5 days post conception rat lungs under normal and hypoplastic conditions. (**A) RARα. (B) RARβ. (C) RARγ. (D) Negative immunohistochemistry controls - omission of the primary antibodies.

Figure 2

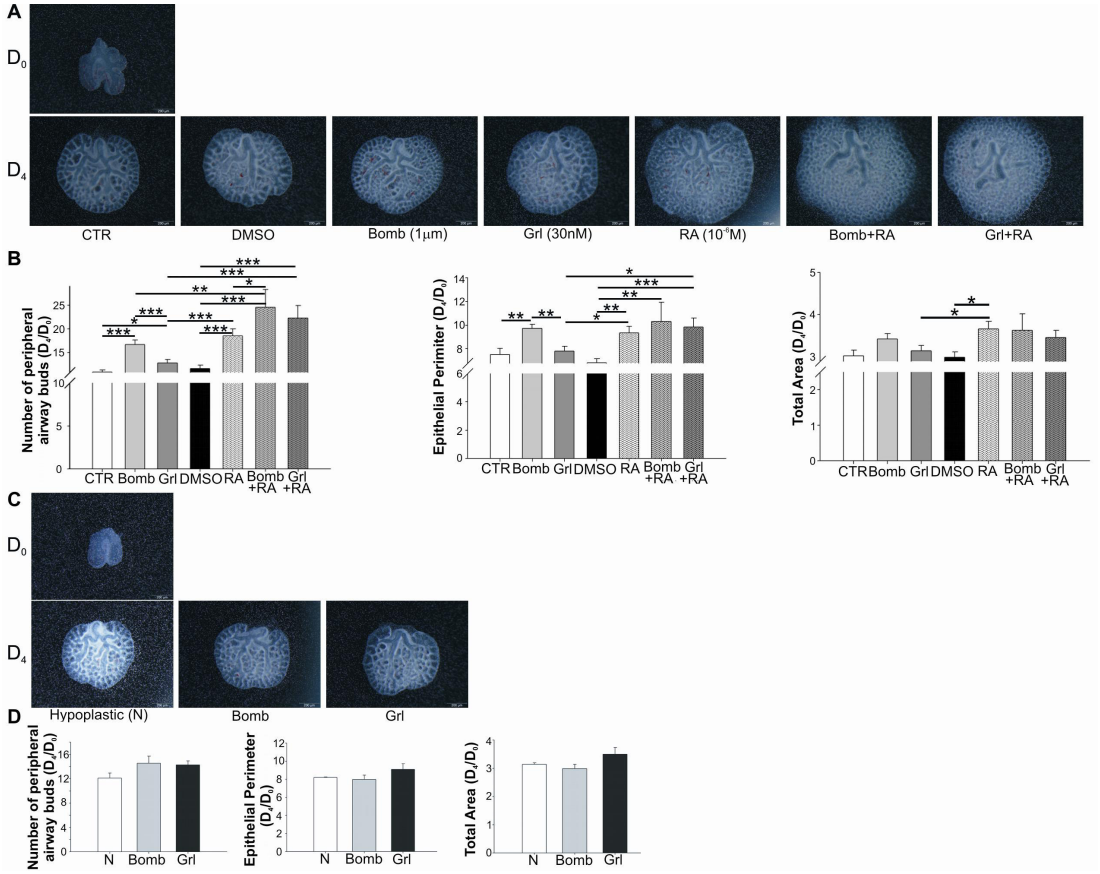

Figure 2. **Branching morphogenesis of rat lung explant cultures treated with bombesin (Bomb), ghrelin (Grl), retinoic acid (RA), bombesin plus RA (Bomb+RA) or ghrelin plus RA (Grl+RA)**. (A) The upper panel is representative of normal lung explants (CTR) at day zero (D_0); the bottom panel represents untreated lung explants (CTR) and explants treated with DMSO, bomb, grl, RA, bomb plus RA or grl plus RA on day four (D_4). (B) Morphometric analysis of the number of peripheral airway buds, epithelial perimeter and total area. (C) The upper panel is representative of the hypoplastic lung explants (N) at D_0; the bottom panel represents untreated hypoplastic lung explants (N) and hypoplastic explants treated with bomb or grl on D_4. (D) Morphometric analysis of the number of peripheral airway buds, epithelial perimeter and total area. The results are expressed as the D_4/D_0 ratio. *p<0.05; **p<0.01; ***p<0.001.

Figure 3

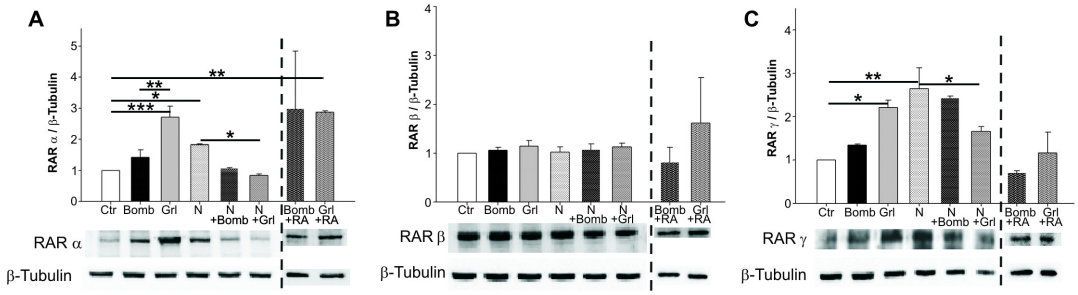

Figure 3. **Protein expression levels of retinoic acid receptors (RARs) in normal (Ctr) and hypoplastic (N) lung explants treated with bombesin (Bomb), ghrelin (Grl) as well as bombesin plus RA (Bomb+RA) or ghrelin plus RA (Grl+RA) in normal lung explants. (**A) RARα expression, (B) RARβ expression, (C) RARγ expression. Examples of representative blots are shown. Protein levels were normalised to β-tubulin, which was used as a loading control. The data are presented as the mean ± SE. *p<0.05; ** p<0.01; ***p<0.001.

Figure 4

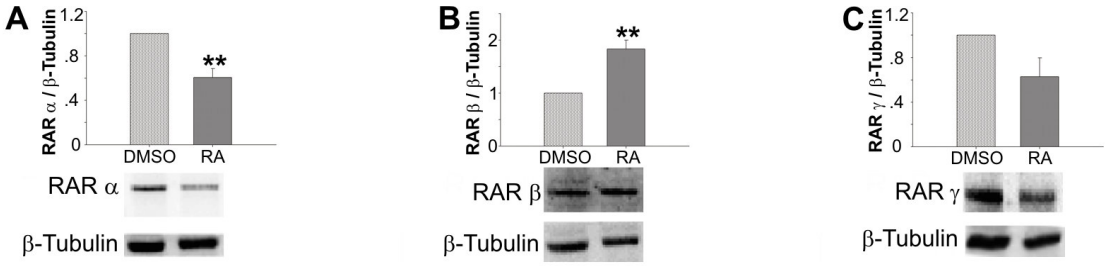

Figure 4. **Protein expression levels of retinoic acid receptors (RARs) in normal lung explants (DMSO) treated with retinoic acid (RA).** (A) RARα, (B) RARβ, (C) RARγ. Representative blots are shown. Protein levels were normalised to β-tubulin, which was used as a loading control. The data are presented as the mean ± SE. **p<0.01 vs. DMSO.

Figure 5

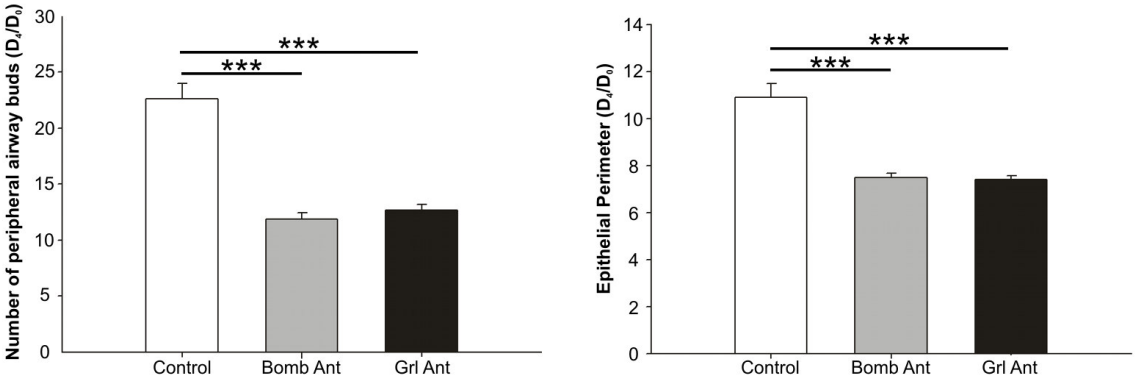

Figure 5. **Branching morphogenesis in rat lung explant cultures (Control) treated with a bombesin or ghrelin antagonist (Bomb Ant or Grl Ant).** The number of peripheral airway buds and the epithelial perimeter. The results are expressed as the D_4/ D_0 ratio. *** p<0.001.

Figure 6

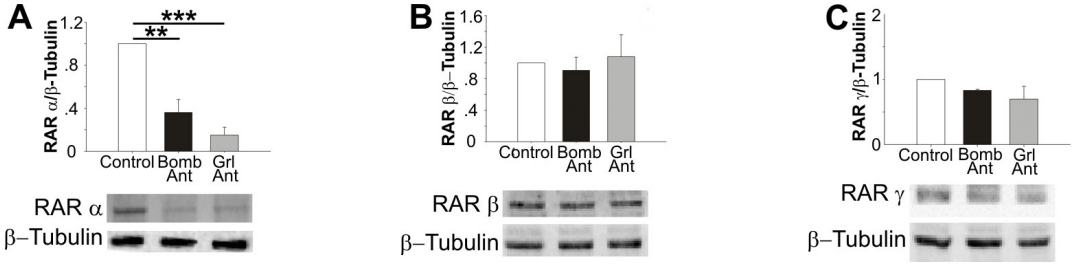

Figure 6. **Protein expression levels of retinoic acid receptors (RARs) in normal lung explants (Control) treated with a bombesin or ghrelin antagonist (Bomb Ant or Grl Ant). (**A) RARα, (B) RARβ, (C) RARγ. Representative blots are shown. Protein levels were normalised to β-tubulin, which was used as a loading control. The data are presented as the mean ± SE. **p<0.01; ***p<0.001.

Figure 7

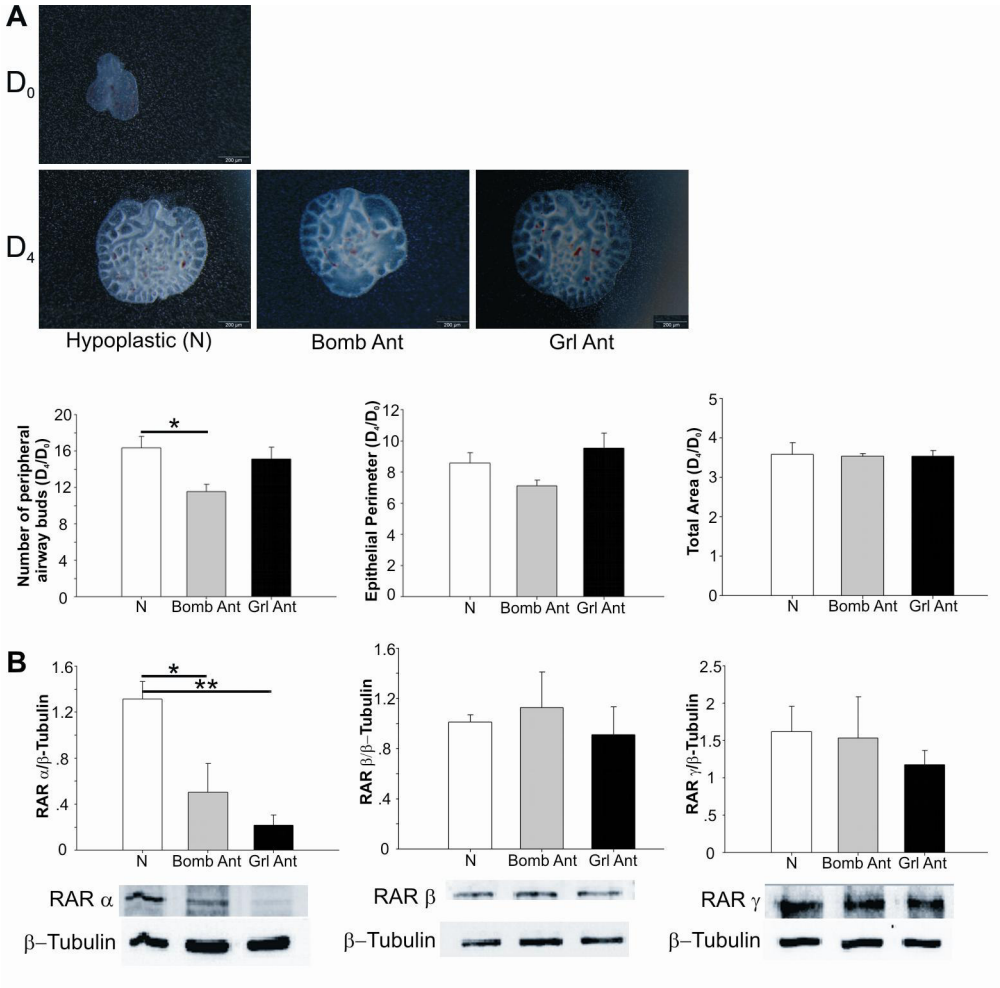

Figure 7. **Branching morphogenesis and RAR expression levels of rat hypoplastic lung explant (N) cultures treated with a bombesin or ghrelin antagonist (Bomb Ant or Grl Ant).** (A) The number of peripheral airway buds, epithelial perimeter and total area. Results are expressed as the D_4/ D_0 ratio. * $p<0.05$. (B) RARα, RARβ, and RARγ protein expression levels. Representative blots are shown. Protein levels were normalised to β-tubulin, which was used as a loading control. The data are presented as the mean ± SE. *$p<0.05$; **$p<0.01$.

Figure 8

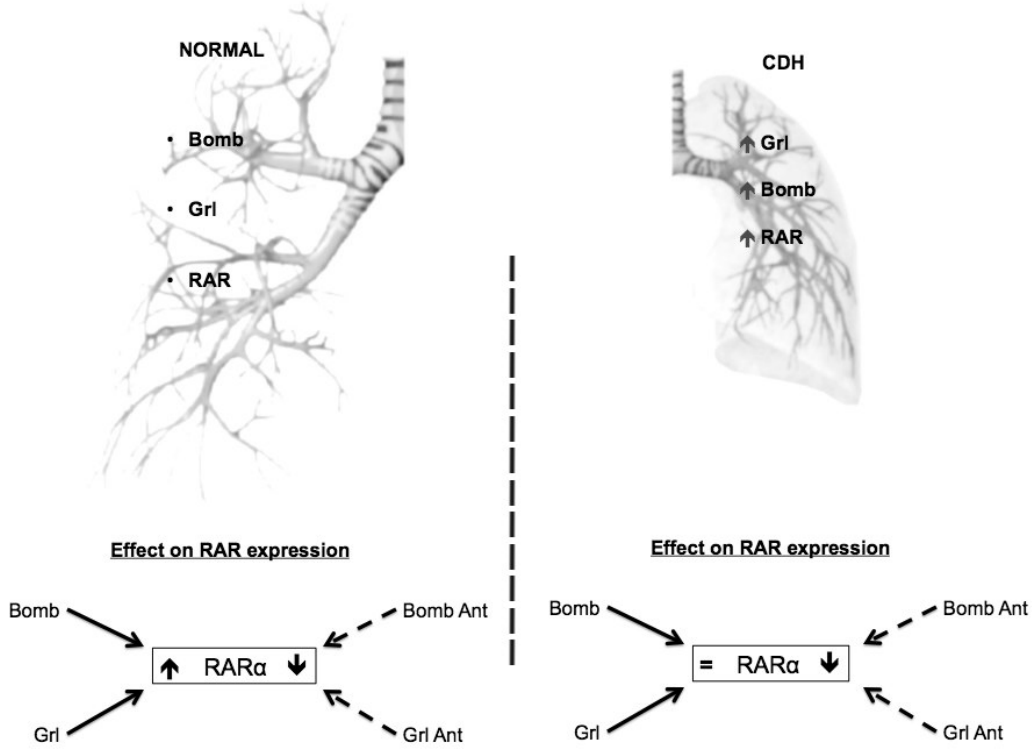

Figure 8. **Schematic representation of the putative link between neuroendocrine cells and the retinoic acid signalling pathway.** In the upper panel, on left side of the scheme we have represented the normal foetal lung expressing neuroendocrine products: Bombesin (Bomb) and Ghrelin (Grl) as well as Retinoid acid receptors (RAR). On right side of the scheme we have represented the CDH foetal lungs, which express increased (↑) levels of neuroendocrine products (Bomb and Grl) as well as RAR. On the bottom panel, we represent the RAR explants expression after modulation (agonists and antagonists) of neuroendocrine products. Focusing on isoform alfa (α), in control explants, RAR(α) expression is significantly increased (↑) and decreased (↓) with addition of agonists (full arrows, Bomb and Grl) and antagonists (dashed arrows, Bomb Ant and Grl Ant) of neuroendocrine products, respectively. Regarding CDH explants, addition of neuroendocrine products does not induce and additional increment of RAR(α) expression when compared with untreated CDH explants (=), but addition of neuroendocrine antagonists (Bomb Ant and Grl Ant) induces a significant decrease (↓) of RAR(α) expression. These findings make us to propose a mechanism of interaction between bombesin and ghrelin with the retinoic acid signalling pathway through the modulation of RAR expression that seems to be working in the hypoplastic lungs.

CHAPTER V.

GENERAL DISCUSSION

DISCUSSION

Lung development is a complex process that involves different signalling pathways (for recent review see McCulley D, *et al*., 2015). In order to understand abnormal lung development it is crucial to know normal foetal lung growth. There are several pathologies including bronchopulmonary dysplasia, lung fibrosis, pulmonary hypertension and congenital diaphragmatic hernia that result from abnormal lung development.

This PhD thesis, aimed to understand and to investigate the role of novel regulatory pathways in foetal lung such as ephrins (**Chapter II**) and microRNAs (**Chapter III**). Additionally, neuroendocrine and retinoic acid pathways were revisited as they are two out of four well-known regulators of lung growth, and a putative regulatory link between them was determined (**Chapter IV**).

Little is known about ephrins and lung development. There are just two papers associating ephrins with lung morphogenesis and repair (Vadivel A*, et al.*, 2012; Bennett KM*, et al.*, 2013). However, ephrins have been strongly associated with vascular system development, capillary remodelling, alveologenesis, microvascular maturation and angiogenesis (Wang HU*, et al.*, 1998; Adams RH*, et al.*, 1999; Gale NW*, et al.*, 2001; Shin D*, et al.*, 2001). Ephrins have already been described in other branching organs such as kidney, pancreas and mammary gland (Nikolova Z*, et al.*, 1998; Takahashi T*, et al.*, 2001). On the other hand, ephrin B1 –B2 and eph B4 knockout mice die before birth and develop several anomalies including angiogenic and lung alveolar formation impairment such as enlarged airspaces (Adams RH*, et al.*, 2001; Davy A*, et al.*, 2004; Wilkinson GA*, et al.*, 2008). Moreover, *in vivo* knockdown of ephrin B2 arrests alveolar and vascular growth, and *in vivo* supplementation attenuates pulmonary hypertension (Vadivel A*, et al.*, 2012). Considering, ephrins B1 –B2 and eph B4 importance during development and embryogenesis, its role during normal foetal lung growth as new potential lung growth regulators was assessed.

In our study, we observed by Immunohistochemistry and Western Blot analysis that ephrin B1, -B2 and eph B4 receptor are expressed during all gestational ages in lung development, with no differences between stages of foetal lung growth. This result suggests their potential role in all stages of foetal lung development. Interestingly, ephrin B1 is essentially expressed in the mesenchyme / vascular cells whereas ephrin B2 and eph B4 receptor are expressed in the epithelium, which might indicate that ephrins are involved in both processes: airway and vasculature branching, acting synergistically. In this study, when ephrin B1 and –B2 were administrated to lung explants, there was an increase in lung branching and growth reinforcing their role, namely in early stages of lung development (pseudoglandular stage). Moreover, ephrin B2 effects, lead to ERK, JNK and STAT3 signalling pathways inhibition in contrast to ephrin B1. This inhibition can be context dependent or an indirect effect of the activation of other signalling pathways. A possible explanation to the fact that there are no changes in the expression of this signalling pathways once ephrin B1 is administrated, might be the fact that ephrin B1 may be acting in other signalling pathways such as Sonic Hedgehog (Shh), RA, Bmp or FGF and does not lead to any alteration on the signalling pathways studied (Correia-Pinto J*, et al.*, 2010; Moura RS*, et al.*, 2011; Moura RS*, et al.*, 2014). It would be interesting to check weather inhibition of ERK, JNK and STAT3 are a direct consequence of ephrin B2 administration. *In vivo* studies with administration of ephrin B1 and –B2 could be done in CDH lungs in order to check if it improves foetal lung branching and reduces lung hypoplasia.

However, lung growth induced by exogenously ephrins administration in normal lungs has not biological relevance. In fact, there is no dose effect growth and the smaller dose already contributes to the differences observed between control and treated lung explants. Indeed, the higher doses do not contribute to higher increases in lung growth (no dose dependent effect).

In conclusion this study showed that ephrins and eph B4 receptor are important in foetal lung development and contribute to lung morphogenesis and branching. However, ephrins do not seem to be a key regulator of lung growth and they seem to act as a morphogen on lung development, or a vascular factor promoting lung vasculogenises. Indeed, a morphogen can be

considered a growth factor, but a growth factor is not necessarily a morphogen. In this sense, FGF, Bmps, Shh, Wnt are morphogens, because they promote *in vitro* lung development and growth, but when they are used *in vivo* (intraparenchymal microinjections) they just promote locally growth causing anomalies and cystic adenomatoid malformations (Gonzaga S, *et al.,* 2008). On the other hand, RA, neuroendocrine factors and angiotensin 2 seem to be growth factors, because *in vitro* they promote harmonious lung growth (Babiuk RP*, et al.*, 2004; Santos M*, et al.*, 2006; Nunes S*, et al.*, 2008; Nogueira-Silva C*, et al.*, 2012b).

For these reasons, a new field of research on lung development namely epigenetics and more specifically microRNAs was assessed, in normal and CDH foetal lung development (**Chapter III**). CDH is a lung disease strongly associated with high mortality and morbidity rates and pulmonary hypertension. Until now there is no specific genetic cause or molecular alteration that is responsible for CDH (Veenma DC*, et al.*, 2012).

As a PhD in Health Sciences with a master dissertation on genetics, searching and looking for a group where it is possible to deal with both fields, CDH and microRNAs, became an interest. In Canada, new highlights that can be useful using microRNAs as a new prognostic biomarker in CDH patients were found (**Chapter III**).

There are several studies reporting microRNAs as potential therapeutical targets in cancer research and also their use as biomarkers for cancer detection. Moreover microRNAs have been described to be important in numerous processes, including EMT and branching morphogenesis that are crucial during lung development and embryogenesis (Gregory PA*, et al.*, 2011; Mujahid S*, et al.*, 2013; Liu W*, et al.*, 2014; Liu W*, et al.*, 2015).

There is growing evidence that TGF-β/ZEB/miR-200 signalling network, regulates the establishment and maintenance of EMT and ZEB / miR-2OO balance, resulting in switching the cells between epithelial and mesenchymal states (Gregory PA*, et al.*, 2011). In fact, in a cell recombinant model for nitrofen-induced abnormal lung development *in vitro* it was shown a disturbed fibroblast/epithelial cell ratio in nitrofen-induced lung hypoplasia (van Loenhout RB*, et al.*, 2012).

By microarray analysis, 319 human microRNAs were scanned and a significant increase in the expression of miR-200b and miR-10a in CDH human lung tissue was observed when compared to normal lung tissue. After these interesting findings on microRNA signature and their confirmation by RT-qPCR, neonatal human postmortem lung tissue was used to do *in situ* hybridization and immunohistochemistry analysis. *In situ* hybridization showed that miR-200b expression was higher in the distal part of postnatal hypoplastic lungs when compared to normal lungs, which fits with the RT-qPCR and microarray previously done. Moreover TGF-β2 was assessed by immunohistochemistry as a target gene of miR-200b. TGF-β2 was decreased in CDH lungs when compared to normal lungs and miR-200b was increased, which corroborates with previous data reported in cancer research (Burk U*, et al.*, 2008; Gregory PA*, et al.*, 2011).

Moreover, a commercial human bronchial epithelial cells line (BEAS) was used to measure SMAD-induced gene expression, in order to establish the inhibitory effect of miR-200b on TGF-β/SMAD signalling in human BEAS.

In human BEAS, SMAD and phospho SMAD activities, increased when miR-200b decreased (administration of miR-200b inhibitor) and the opposite occurred when miR-200b was added (miR-200b mimics). Moreover ZEB2 (target gene for miR-200b) also increases when miR-200b is inhibited, and it decreases when miR-200b is administrated to normal BEAS. These results corroborate previous described data and showed a strong correlation between ZEBs and TGF-β2. Additionally, they exhibited negative correlations between miR-200 and TGF-β2, and between miR-200 and ZEBs. Moreover, it has been described that overexpression of miR-200b is associated with an epithelial phenotype (increased E-cadherin) and inhibition of miR-200b is associated with a more mesenchymal phenotype (decreased E-cadherin) (Park SM*, et al.*, 2008). In figure 5, a summary of the main results on BEAS assay is shown.

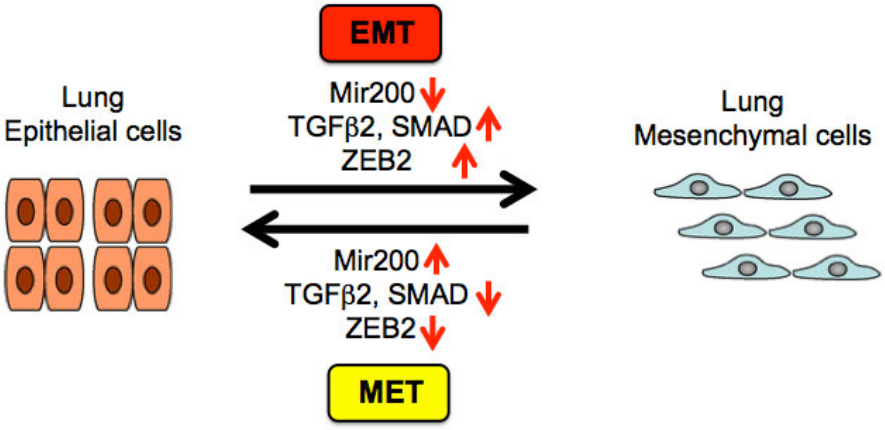

Figure 5. Schematic representation of the proposed mechanism for regulating human lung EMT / MET.

An interesting finding of this study was the fact that miR-200b expression was increased in CDH human lungs when compared to normal lungs, in opposition to what was described by Van Loenhout *et al*. 2012 in rat lungs. Moreover, TGF-β2 was increased after FETO in a CDH sheep model (Quinn TM*, et al.*, 1999; van Loenhout RB*, et al.*, 2012). A possible explanation for that might be the use of nitrofen to induce CDH in rats because in humans it may have different origins (nitrofen does not induce CDH). Moreover, the surgical model to induce CDH in sheep's, does not lead to the abnormal lung hypoplasia observed in CDH rat and human lungs, before the diaphragmatic defect. Additionally the only study published refers to *in vitro* lung epithelial and mesenchymal cells from CDH rats, and not to lung tissue and consequently the cells can change the production of different molecules or microRNAs to regulate / promote growth and development (van Loenhout RB*, et al.*, 2012).

Meanwhile, we got amniotic and tracheal fluid samples from patients with severe CDH conditions that underwent FETO, responders and non-responders patients, were analysed. Because of the significant differences found in the microarray of CDH and normal lung tissue, miR-200 family and miR-10a expression was checked in these two fluids. Regarding the tracheal fluid before FETO there were no differences in miR-200 family expression between responders and non-responders, however miR-10a was higher in non-responders and did not increase after FETO, in opposite to what happen in responders. After FETO, responders presented higher expression of miR-

200 and miR-10a than non-responders. This can be useful to predict the outcome of these patients in the future. However one of the limitations in this study is the fact that there was not any control samples of the tracheal fluid from normal patients, because it is an invasive process, and all this results need to be validated in a large sample set before being used in pre-clinical studies.

It would be interesting to further elucidate and refine this hypothesis, to investigate combinations of foetuses displaying lung growth alterations (evidenced by increase in ultrasound lung measurements) with different microRNA signatures, and on the other hand, foetuses without obvious lung growth response on ultrasound, but with a responsive microRNA signature.

Considering the amniotic fluid samples, expression of miR-200 family and miR-10a in amniotic fluid was decreased after FETO and was always lower than tracheal fluid expression. This interesting finding indicates that microRNAs in those patients form the largest component of the amniotic fluid because after FETO they decrease in the amniotic fluid and increase in the tracheal fluid. In fact, it is described that lungs contribute largely to the amniotic fluid content in the third trimester of pregnancy (Evrard VA*, et al.*, 1997). On the other hand, responders to FETO generally presented higher expression of miR-200 family and miR-10a in the amniotic fluid before plug than non-responders, so, these findings can be useful in future pre-clinical studies in order to use these microRNAs as biomarkers in the amniotic fluid to predict CDH outcome.

In the future, CDH and normal rat lung tissue will be studied during gestation in order to characterize the expression patterns of miR-200b, TGF-β2 and ZEB2.

As future perspectives, if miR-200b emerges as having a crucial role in CDH and consequently improves lung growth, maturation and development of human CDH patients, it can be used as a pre-natal therapy to help those patients. The idea is to quantify miR-200 and miR-10a expression in amniotic fluid, and to predict the prognosis for CDH patients. If the levels are lower than in the survivors, once FETO is done, miR-200 can be administrated into the lungs when the plug is inserted. Although, studies regarding doses will be crucial because overexpression or down-regulation of microRNAs are

strongly associated with cancer development and metastasis (Muller S*, et al.*, 2015; Vychytilova-Faltejskova P*, et al.*, 2015; Wong CM*, et al.*, 2015). Another interesting experiment would be analysing CDH lung tissue from responders and non-responders in order to check miR-200b and TGF-β2 expression, and correlate them with what was observed in the tracheal fluid. This study will strongly contribute to find potential biomarkers that can be useful as prognostic tools for CDH patient's outcome. In summary, these findings might be useful in the future as potential prognostic tool rather than a potential therapy.

After studying two new different signalling pathways in normal lung growth and development regulation (**Chapter II and III**), two different signalling pathways already known to be crucial and altered in foetal normal and abnormal lung development were studied (**Chapter IV**).

There are several studies reporting CDH in animal models and the best model to mimic human CDH is the nitrofen rat model (Correia-Pinto J*, et al.*, 2003; Ruttenstock E*, et al.*, 2011), justified by the fact that the lungs before being compressed by the abdominal organs are already hypoplastic. The underlying mechanisms are still unknown and a better understanding of these mechanisms will help to find a potential therapy. An important finding on CDH lungs is the capacity of catch-up growth regulation, that is the ability of the lungs to recover to normal lung growth when they are relieved from the mechanical compression and they grow *in vitro* (Nogueira-Silva C*, et al.*, 2008). Moreover, a small increase in lung parenchyma can be crucial in the adaptation of CDH foetus to extra-uterine life (Santos M*, et al.*, 2006).

Recently published data has shown that there are few molecular disturbances on CDH rat lungs at the level of vitamin A and neuroendocrine products. RA is essential for normal lung development and for diaphragm development as well (Ross SA*, et al.*, 2000). Looking into the literature, there are several articles reporting the association between nitrofen and RA disruption. RA has been described as down regulated on CDH rat lungs, and pregnant rats with a deficient diet on vitamin A result in a high rate of CDH new-borns (Clugston RD*, et al.*, 2006; Coste K*, et al.*, 2015). Moreover, prenatal treatment with RA promotes alveologenesis, increases the gas exchange surface area, lung growth and branching morphogenesis on CDH

rat lungs, attenuating lung hypoplasia (Ruttenstock E*, et al.*, 2011). In fact, the disruption on RA signalling pathway on CDH rat lungs has been largely associated with a decrease in plasma retinol and retinol-binding protein levels. Moreover the inhibition of RALDH2 induced by nitrofen is a secondary consequence of nitrofen administration. Furthermore, vitamin A has teratogenic properties and in CDH human lungs, its deficit is detected late on gestation, around 16 weeks, which limits its use as a potential therapy because it acts early on gestation (Baptista MJ*, et al.*, 2005). Regarding RAR and RXR knockouts, RAR α/ RARβ double knockout mice presented lung agenesis, hypoplasia and / or diaphragmatic defects and for this reason only RAR receptors were studied (Mendelsohn C*, et al.*, 1994) (**Chapter IV**).

Concerning neuroendocrine factors, in CDH lungs there is a pulmonary neuroendocrine cell hyperplasia, which consequently leads to bombesin and ghrelin increase, in humans and in animal models. Additionally it is known that branching organs such as kidneys, lungs and stomach are the main sources of circulating foetal ghrelin. Interestingly ghrelin knockout mice do not show lung abnormalities, although ghrelin is increased on CDH rat lungs (Sun Y*, et al.*, 2003; Santos M*, et al.*, 2006). Moreover *in vivo* administration of bombesin to nitrofen pregnant dams, decreased lung hypoplasia on CDH embryos (Sakai K*, et al.*, 2014). Published data also shows that exogenous administration of ghrelin to nitrofen pregnant rats, attenuate pulmonary hypoplasia on CDH lungs (Santos M*, et al.*, 2006; Nunes S*, et al.*, 2008).

However, the changes observed in CDH lungs concerning RA, bombesin and ghrelin expression are not well understood and the underlying mechanisms still remain unclear. In order to better clarify all this changes and mechanisms, a correlation between these two apparently different and not related signalling pathways was identified (RA *vs* neuroendocrine factors). In this study, by immunohistochemistry we observed at the end of canalicular, beginning of saccular stages, higher expression of RAR (mesenchymal and epithelial) in hypoplastic lungs when compared to normal lungs. However, Rajatapiti *et al*., have previously shown by immunohistochemistry that, there were no differences between these two groups. In fact, immunohistochemistry is a qualitative technique so we confirmed our results by Western Blot (Rajatapiti P*, et al.*, 2006). With the administration of

bombesin, ghrelin, RA, RA plus bombesin and RA plus bombesin to normal foetal lung explant cultures, an increase in the morphometric parameters analysed was observed, mainly in the number of peripheral airway buds and epithelial perimeter. RA was the major stimulator in lung growth and RA combined with neuroendocrine factors, even promoted higher growth, than when they were administrated alone. This result suggests that they are acting in a different way to promote lung growth and branching when exogenously administrated. Moreover by Western blot RAR α / γ expression increase in normal lung explants after bombesin and ghrelin administration has confirmed, the action of PNEC as potential sensors in lung growth (Santos M*, et al.*, 2006). On the other hand, when RA was administrated to the cultures, RAR α / γ expression decreased and RAR β increased. This can be explained by the fact that neuroendocrine cells sense that increase and decreased bombesin and ghrelin production, which consequently leads to a decrease in RAR α / γ expression. Another possible explanation can be a direct down-regulation effect of RA on RAR expression. This results show that there is a link between neuroendocrine factors and RA where RAR α and RAR γ develop the major roles and RAR β is apparently excluded from this interactions. Consequently these *in vitro* studies suggest that RAR α and RAR γ expression strongly sensitize for CDH pathology.

Moreover, according to this study, when RA is administrated in combination with bombesin or ghrelin, RAR α expression increases and nothing happens to the other receptors. This can be explained by the fact that RA administration decreases RAR expression, and neuroendocrine factors increases RAR expression in normal lungs, so together, the effect of each one of them cancel the effect of the other. Indeed with this experiment, the interactions between bombesin or ghrelin and RA were confirmed.

Concerning inhibitory studies, bombesin and ghrelin were inhibited in normal lung explants, and RAR expression was evaluated. Once again RAR α and RAR γ expression was decreased and branching was also reduced which reinforce the idea that neuroendocrine factors sensitize RA through RAR α and RAR γ.

Regarding hypoplastic lungs, RAR α / γ expression was increased when compared to normal lungs which was expected because hypoplastic lungs already had and increase in bombesin and ghrelin as described above. Additionally when bombesin or ghrelin were administrated to hypoplastic lungs, there were no changes in morphometric parameters probably because this PNEC stimulatory effect is already on the limit, whereas RAR α and RAR γ expression decreased. The increase on ghrelin and bombesin on CDH rat lungs can be a consequence of lung immaturity or a compensatory response of the hypoplastic lung attempting to recover to normal levels of lung growth (Santos M*, et al.*, 2006). These results suggest once again the strong link between RA and neuroendocrine factors. In the same trend bombesin and ghrelin inhibition, decreased RAR α / γ expression as well as lung branching in hypoplastic lung explants.

Interestingly, in this study RAR α and RAR γ are the major mediators involved in RA/PNEC interactions, however knockout mice that resulted in hypoplastic lungs are RAR α / RAR β double knockouts (Mendelsohn C*, et al.*, 1994; Beurskens LW*, et al.*, 2009). A possible explanation for that can be RAR redundancy because simple RAR knockout mice do not result in lung pathologies. When one isoform of RAR (α, β or γ) is knockdown, lungs look healthy, so the other two isoforms can compensate that inhibition and lungs grow and develop normally.

In future studies it would be interesting to see the expression of bombesin and ghrelin in those knockouts and to compare them with normal and nitrofen-induced lungs. On the other hand, it would also be interesting to block *in vivo* bombesin and ghrelin in pregnant rats and see what happens to RAR expression in the lungs of the foetus. Another important thing to understand would be to explore why neuroendocrine factors are up-regulated on CDH rat lungs? Is it a cause or a consequence of the disease?

In summary this study showed that neuroendocrine factors act as regulators of lung growth, sensitizing the lungs to the action of RA through RAR α and RAR γ up-regulation, contributing to understand the underlying mechanisms of these two apparently different signalling pathways.

With these work, potential prenatal therapies emerged, such as the use of RA and neuroendocrine factors in CDH babies. Regarding RA as a prenatal therapy for human CDH patients, it as a lot of complications because of RA teratogenicity. However, bombesin and ghrelin administration to CDH babies can be a potential prenatal therapy to attenuate lung hypoplasia without apparently any adverse effect.

CHAPTER VI.

CONCLUSIONS

CONCLUSIONS

Having in mind the overall aim of the present PhD thesis, to find new prenatal therapeutical approaches to revert foetal lung hypoplasia in CDH context, the main conclusions are listed bellow.

- Ephrin B1, -B2 and eph B4 receptor are constitutively expressed in foetal lung during gestation, promoting lung growth when exogenously administrated (ephrin B1 and ephrin B2). However the increase in branching growth is smaller than 20% of normal lung growth, which does not represent a big increase considering *in vivo* studies. This findings suggests that ephrins does not seem to be a promisor mechanism to regulate lung growth. Although, they can be strong candidates to act as morphogens or being involved in vascular regulation regarding foetal lung development.

- MicroRNA signature is changed in CDH human lungs when compared to healthy lungs; specifically miR-200b and miR-10a are increased. After FETO miR-200 family and miR-10a significantly increases in the tracheal fluid of responders to FETO. These results can be useful as using microRNAs as prognostic biomarkers for CDH outcome.

- Two out of four strongly studied signalling pathways (retinoic acid and neuroendocrine factors), known to promote normal foetal lung growth were shown to be correlated: ghrelin and bombesin act as regulators of lung growth, preparing the lungs to the action of RA through RARα and RARγ up-regulation.

In Figure 6 a schematic picture of the main results of this PhD work are presented. According to the aims proposed important mechanisms involved in normal and abnormal lung growth regulation, were found and some of these findings might be used in the future in pre-natal therapies in order to increase the quality of life of CDH patients. Moreover, microRNAs have the potential to be used as biomarkers to predict CDH outcome in humans.

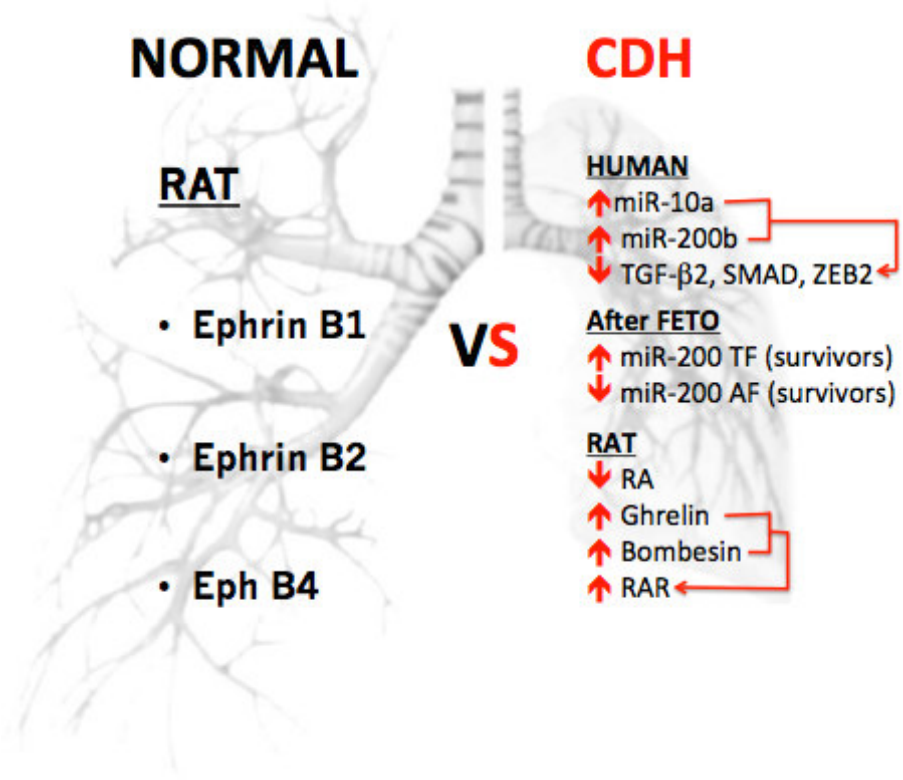

Figure 6. Schematic representation of changes found in important regulators of lung growth: normal *versus* CDH rat and human lungs

CHAPTER VII.

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