

Role of MicroRNA-200b in Normal Lung Development and How It Is Affected in
Abnormal Lung Development Associated with Congenital Diaphragmatic Hernia

By

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Abstract

Introduction: Congenital Diaphragmatic hernia (CDH) is a developmental defect of the diaphragm associated with abnormal lung development. A common genetic cause has not been found for CDH suggesting a role of epigenetic factors including microRNAs. We recently reported the higher level of miR-200b expression in hypoplastic lungs from preterm infants with congenital diaphragmatic hernia. MicroRNA miR-200b is best known for its role in cancer and epithelial-mesenchymal transition (EMT), but our knowledge on its role during normal lung development and how it is affected in the developmental disorders of the lung is limited. We hypothesized that miR-200b is a key determinant of normal and abnormal lung development via mechanisms associated with Epithelial-Mesenchymal Transition (EMT) and Transforming Growth Factor β (TGF- β) signaling. The aim of this thesis was to delineate the role of miR-200b in lung development and lung hypoplasia in the rat model of CDH.

Methodology: We used human lung tissues and biofluids to evaluate the expression of miR-200b and its targets using RT-qPCR, *in situ* hybridization (ISH) and immunohistochemistry (IHC). We used cultured human bronchial epithelial cells to evaluate the role of miR-200b on TGF- β /SMAD (signalling using a luciferase assay. To investigate the role of miR-200b in normal lung development, we generated a miR-200b knockout mouse (*miR-200b*^{-/-}). We compared lung functions of miR-200b knockout mice to wild type mice. We determined the lung phenotype and branching using *in vivo* micro-computed tomography scan. To study the effect of miR-200b in hypoplastic lungs associated with CDH, we used the nitrofen rat model of lung hypoplasia and CDH. We assessed miR-200b expression using RT-qPCR and *in situ* hybridization. We performed loss-of-function studies to determine the effects of miR-200b on epithelial cell phenotype and

function by immunostaining and scratch wound healing assay. Finally, miR-200b was used as a treatment to rescue lung hypoplasia *in vivo*.

Results: MiR-200b abundance in the tracheal fluid from CDH fetuses was higher in the ones with a better response to a prenatal lung growth-promoting therapy. *MiR-200b*^{-/-} mice displayed reduced distal airway branching, thickened alveolar walls, and less septation. They had a higher number of fibroblast-like cells resulting in significantly higher tissue damping and elastance and lower hysteresivity in the lung. In the rat model of lung hypoplasia and CDH, miR-200b expression was lower in early abnormal lung development and higher just before term when compared to control. Normalizing miR-200b abundance reduced CDH incidence by 33.6% and significantly reduced the severity of lung hypoplasia in *ex vivo* and *in vivo* model we employed.

Conclusions: MiR-200b is required for normal lung branching morphogenesis and lung function. Prenatal transplacental therapy with miR-200b has the potential to prevent CDH-associated lung hypoplasia.

Dedication

To my amazing Parents, Nasrin and Mohammad, for their endless love selflessness and encouragement. Thank you for making me the person I am today!

To my beloved fiancé, Ali, for his infinite love and support! Thank you for making me a happy and stronger person!

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Contributions of Authors

The presentation of this thesis is in the form of a sandwich thesis. Three manuscripts have been presented here. Naghmeh Khoshgoo is the third author of the Manuscript I and first author of the Manuscripts II and III. Manuscript I is published in *Annals of Surgery*. Manuscript II is published in *Journal of Scientific Reports* and Manuscripts III is in Press in *Annals of Surgery*.

The contributions of the authors are for each Manuscript are as follow:

Manuscript I: Unique Tracheal Fluid MicroRNA Signature Predicts Response to FETO in Patients with Congenital Diaphragmatic Hernia

Naghmeh Khoshgoo performed Luciferase assay and Western blotting on human cells and Immunohistochemistry on human tissues. she acquired and analyzed the data. *Patricia Pereira-Terra, Fuqin Zhu* and *Barbara M. Iwasiow* performed RT-qPCR on human biofluids. *Anne A. Boerema - de Munck, Jinxia Wang* and *Philip DeKoninck* performed micro array and/or PCR on human lung tissue. *Ramin Kholdebarin* performed in situ hybridization on human tissues. *Jan Deprest* and *Philip DeKoninck* acquired the tracheal and amniotic fluid samples. *Dick Tibboel* and *Robbert J. Rottier* acquired the lung samples. *Naghmeh Khoshgoo, Patricia Pereira, Ramin Kholdebarin* and *Richard Keijzer* wrote the paper and all the authors revised it. *Jan Deprest, Martin Post, Dick Tibboel* and *Richard Keijzer* delineated the hypothesis and designed the study.

Manuscript II: MicroRNA-200b regulates distal airway development by maintaining epithelial integrity

Naghmeh Khoshgoo performed LacZ staining, RT-qPCR of microRNAs, Assessing biophysical surfactant function, Scratch Assay and analysed the data for next generation sequencing and gene ontology analysis. She performed Immunostaining with help of *Landon Falk* and *Dustin Ameis* and RT-qPCR for mRNAs with help of *Chelsea A. Day* and *Fuqin Zhu*. *Robin Visser* performed lung explant culture with assistance of *Naghmeh* and *Barbara Iwasiow*. *Arzu Öztürk*, *Molly Pind*, *Agnes Fresnosa* and *Geoffrey G. Hicks* generated miR-200b knockout mice. *Naghmeh Khoshgoo* and *Barb Iwasiow* genotyped and confirmed the miR-200b knockout line. *Sujata Basu* performed lung function assessments and *Naghmeh Khoshgoo* collected and analysed the data. *Naghmeh Khoshgoo* and *Richard Keijzer* wrote the paper *Naghmeh Khoshgoo*, *Robin Visser*, *Arzu Öztürk*, *Geoffrey G. Hicks*, *Andrew J. Halayko* and *Richard Keijzer* delineated the hypothesis and designed the studies.

Manuscript III: Prenatal microRNA miR-200b therapy improves nitrofen-induced pulmonary hypoplasia associated with the congenital diaphragmatic hernia.

Naghmeh Khoshgoo performed RNA extraction and qPCR, Immunohistochemistry, luciferase assay, western blotting and *in vivo* prenatal therapy with assistance of *Barbara Iwasiow*. *Ramin Kholdebarin* performed *In-situ* hybridization of miR-200b. *Patricia terra* performed the lung explant culture and assist *Naghmeh Khoshgoo* in the *in vivo* therapy study. *Thomas H. Mahood* analyzed microarray data for miR-200 family. *Landon Falk* and *Chelsea A. Day* help *Naghmeh* with lung morphometric analysis for evaluating the outcome of miR-200b *in vivo* therapy. *Carly Fraser* assisted *Naghmeh* with preforming some of the qPCR for miR-200b. *Fuqin Zhu* and *Drew Mulhall* performed the combined ISH/IHC experiment. *Naghmeh Khoshgoo*, *Ramin Kholdebarin*, *Jorge Correia-Pinto* and *Richard Keijzer* delineated the hypothesis and designed the studies.

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List of Abbreviations

AGO	argonaute
AP	alkaline phosphatase
APC	adenomatous polyposis coli
ASM	airway smooth muscle
BMP	bone morphogenetic protein
CDH	congenital diaphragmatic hernia
CT	computed tomography
deltaPI	delta pulsatility index
DIG	digoxigenin
E	embryonic day
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
FETO	fetal endoscopic tracheal occlusion
FGF	fibroblast growth factor
FMRP1	fragile X mental retardation protein
GC	Glucocorticoid
GSK-3	glycogen synthesis kinase
Id2	Inhibitor Of DNA Binding 2
IHC	immunohistochemistry
ISH	in situ hybridization
LNA	locked nucleic acid
MET	mesenchymal-epithelial transition

miRNA	microRNA
O/E LHR	observed over expected lung-to-head ratio
PFA	paraformaldehyde
PH	pulmonary hypoplasia
PE	primordial epithelium
PPF	pleuro-peritoneal fold
PPHN	persistent pulmonary hypertension
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
RA	retinoic acid signaling
RARs	retinoid acid receptors
RXRs	retinoid X receptors
RISC	RNA-induced silencing complex
RT-qPCR	Quantitative reverse transcription PCR
SHH	sonic hedgehog
SP-A	surfactant protein A
SP-B	surfactant protein B
SP-C	surfactant protein C
SP-D	surfactant protein D
SOX2	Sex Determining Region Y-Box 2
TGF- β	transforming growth factor beta
TRBP	human immunodeficiency virus transactivating response RNA-binding protein
TTF-1	thyroid transcription factor-1

VSM	vascular smooth muscle
α -SMA	α -smooth muscle actin
UTR	untranslated region
ZEB	Zinc Finger E-Box Binding Homeobox

CHAPTER 1. General Introduction

1.1. Lung Organogenesis

Following the gastrulation stage of embryogenesis, the definitive endoderm undergoes morphogenetic changes leading to the formation of the primitive gut tube. The gut tube is comprised the foregut, the most anterior region of the gut tube, midgut and hindgut, the posterior part (Wells & Melton 1999). Lung budding starts from the ventral-lateral region of the foregut at four weeks of gestation in human, and Embryonic day (E) 9.5 in mice (Kimura & Deutsch 2007).

1.2. Stages of Lung Development

The primary goal of lung development is to establish a large surface area of a thin air-blood barrier to facilitate gas exchange that supplies organs with oxygen and remove excess CO₂. Histologically, lung development has been divided into five stages; Embryonic, Pseudoglandular, Canalicular, Saccular and Alveolar. There are no sharp boundaries between these stages; rather, the different stages represent a continuum.

1.1.1. Embryonic Stage (4-7 weeks of human pregnancy)

In the embryonic stage, lung tissue first appears as a primordial bud emerging from the ventral side of the foregut endoderm around week 4 in human (E9.5 in mice). The proximal portion of the bud develops into the larynx and trachea, and the distal portion gives rise to two ventrolateral buds. Both buds grow caudally into the surrounding mesenchyme to form the left and right main bronchi (Cardoso 2000). In humans, the lung bud on the right develops three lobar buds, and the one on the left generates two lobar buds corresponding to the mature lung lobes. In the rodent, there is one lobe on the left and four on the right. Development of the pulmonary vasculature occurs simultaneously with bronchial branching morphogenesis; the pulmonary arteries branch from the

6th pair of aortic arches and surround the lung buds where they form a vasculature plexus. By the end of the embryonic stage - around seven weeks - trachea, primary bronchi and pulmonary arteries are present, and the lung resembles a small tubulo-acinous gland which inspired the name of the next stage, the pseudoglandular stage of lung development (Joshi & Kotecha 2007).

1.1.2. Pseudoglandular Stage (5-17 weeks of human pregnancy)

During the pseudoglandular stage, the stage of branching morphogenesis, there is a constant sprouting and bifurcation of lung buds resulting in the formation of all conductive airways and terminal bronchioles by 17 weeks (Jeffery 1998). At this point, the upper lobes have 12-17 branches; the middle lobes have 18-33 branches and the lower lobes have 14-23 branches (Buchert & Reid 1961). In the pseudoglandular stage, the airways are lined with columnar epithelial cells expressing cytoplasmic glycogen. Some of these cells begin to differentiate into ciliated cells as early as eight weeks while others start to become goblet cells (Jeffery 1998). During this time, cartilage and bronchial smooth muscle cells begin to emerge (Buchert & Reid 1961). Branching morphogenesis is a highly-organized process generated by three geometrically simple modes of branching: domain branching, planar bifurcation, and orthogonal bifurcation (Fig. 1). In the domain branching mode, as lung buds grow, daughter buds appear on the lateral axis of the primary bronchial airway perpendicularly and in a proximal–distal manner, like rows of bristle on a bottle brush. This branching mode generates the main secondary branches. In planar bifurcation, two consecutive rounds of bifurcation occur in the same plane to form 4 quaternary branches. The planar bifurcation generates tertiary branches and the later generations of airway branches. Finally, orthogonal bifurcation is characterized by two rounds of planar bifurcation, but, between the two rounds, there is a 90-degree rotation of the bifurcation plane resulting in an arrangement of four second generation of branching in a rosette (Metzger et al. 2008). Each mode is controlled by

specific patterning and morphogenesis events. Domain branching is controlled by a proximal–distal periodicity generator that controls the right time and location of the new buds. A domain specifier dictates the positions and order of domains around the parent branch. Planar bifurcation requires a branch bifurcator, and orthogonal bifurcation requires both a bifurcator and a rotator.

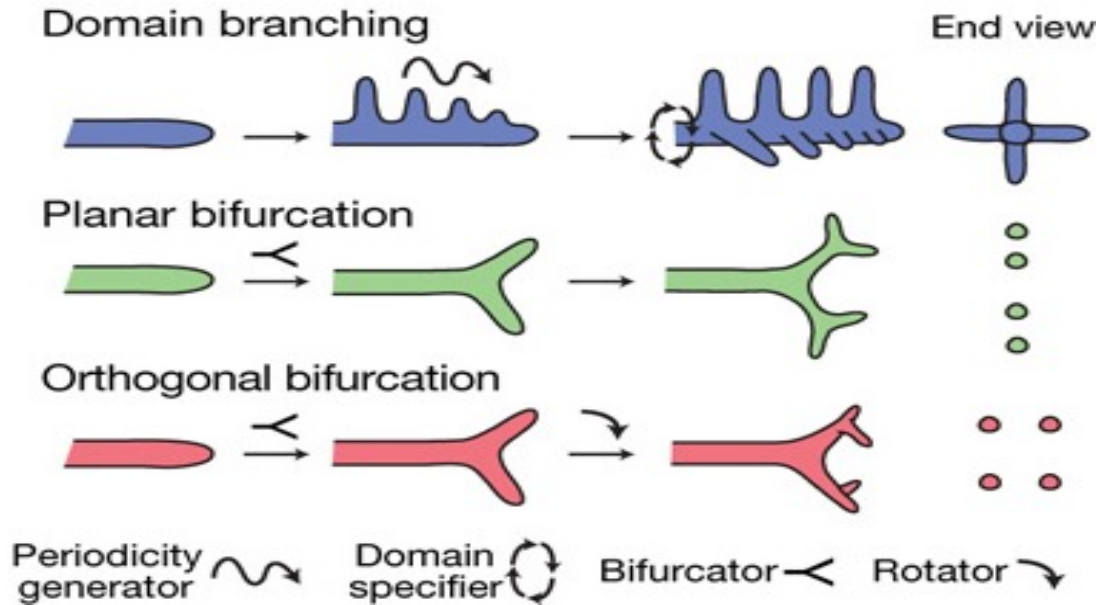


Figure 1-1: Schematic of the three types of branching: Domain branching, Planar bifurcation, and Orthogonal bifurcation (Metzger et al. 2008, used with the journal permission).

1.1.3. Canalicular Stage (16-25 weeks of human pregnancy)

During this stage, the terminal bronchioles divide into respiratory bronchioles and alveolar ducts. Vascularization and angiogenesis continue along the airways during this stage, and the numbers of capillaries increase dramatically. Around 22 weeks of gestation, smooth muscle cells are fully developed around the airways, and lymphatic vessels begin to appear. Peripheral type I and II pneumocytes can be distinguished from proximal cuboidal epithelial cells. In this stage, type II pneumocytes begin to produce surfactant (Boyden 1976; Joshi & Kotecha 2007). During fetal

lung development, the compliance and stability of the liquid-filled lung is determined mainly by the structural element of air sacs, especially the connective tissue. After birth, surfactant is required to decrease surface tension at the air-liquid interface of the alveoli to preserve elastic recoil properties of the lung. Surfactant is composed of a complex of phospholipids (mainly phosphatidylcholine) and several surfactant proteins (SP): SP-A, SP-B, SP-C, and SP-D. SP-B and SP-C are hydrophobic proteins which significantly enhance the rate of spreading of surfactant phospholipids onto an aqueous surface. However, SP-A and SP-D are important factors in pulmonary host defense and are less important in the function and processing of surfactant (Perez-Gil & Weaver 2010).

1.1.4. Saccular Stage (24-36 weeks of human pregnancy)

A dramatic increase in apoptosis and differentiation of mesenchymal cells and tremendous expansion of prospective respiratory spaces result in substantial thinning of the interstitium and formation of terminal saccules (Hashimoto et al. 2002). The walls between two saccules are still thick and comprise capillary networks forming a bilayer in the inter-saccular septa (primary septa). Squamous type I pneumocytes and secretory rounded type II pneumocytes containing surfactant vesicles (lamellar bodies) are fully differentiated. Around 26 weeks of gestation surfactant production starts, and around 30 weeks surfactant begins to be secreted into the airway lumen (Burri 1984). The complex capillary network is formed in the mesenchyme around the saccules. Toward the end of this stage, the lung can support gas exchange (Warburton et al. 2010).

1.1.5. Alveolar Stage (36 weeks in utero- 2 years)

The primary two processes taking place during the alveolar stage are secondary septation, dividing saccules into terminal alveoli, and pulmonary angiogenesis which maximizes the surface area for

gas exchange in the lung. Alveoli are small sacs comprised of 90% type I epithelial cells covering the alveolar spaces. The basement members of these cells are fused to the capillary endothelial cells to form the air-blood barrier (Burri 1984). During secondary septation, newly generated septa divide the air sac into the alveolar ducts and terminal alveoli. Epithelial cells, endothelial cells, and mesenchymal myofibroblasts are actively involved in this process. It has been shown that elastin deposition by myofibroblasts in the specific sites of primary septa correspond precisely to the location of future secondary septa buds. New septa composed of a double capillary layer and elastin localize at their tip. Later in development, microvascular maturation takes place when the double capillary network gets remodeled into a single capillary system. Septal thinning continues by the expansion of epithelial cells in the septa and apoptosis of septal myofibroblast. This phase continues for at least two years in the postnatal human lung (Warburton et al. 2010).

1.3. Molecular Regulation of Lung Development

A thorough understanding of the molecular mechanisms controlling the transformation of the small primordial buds to the mature and complex lung and the changes in these mechanism during disturbed lung development is a critical step toward discovering therapeutic approaches to improve the outcomes of neonates with abnormal lung development. Here, some of the most critical molecular pathways in lung morphogenesis will be summarized considering the major events during morphogenesis.

1.1.6. Specification of Lung Endoderm and Ventral-Dorsal Patterning

The earliest evidence of specification of the respiratory system (the trachea and lungs) is the expression of transcription factor Nkx2.1 in the ventral wall of anterior foregut around day 28 in human (E9.5 in the mouse) (Kimura et al. 1996). At the same time, a high level of Sox2 is observed

dorsally in the foregut where the future esophagus is formed (Que et al. 2007; Que et al. 2009). This ventral-dorsal patterning is dependent on signaling from the surrounding mesenchyme. The two most important signaling pathways in this stage are Wnt and BMP (Goss et al. 2009; Li et al. 2008). Wnt signaling promotes expression of Nkx2.1, and Bmp4 signaling prevents Sox2 expression in the presumptive lung endoderm (Goss et al. 2009; Domyan et al. 2011). The essential role of SHH signaling and its transducer transcription factors Gli2 and Gli3 have been shown to be important for early lung budding as well. Motoyama *et al.* have shown that *Gli2*^{-/-} / *Gli3*^{-/-} double mutant null mice die at day 10.5 without primitive lung or trachea formation (Motoyama et al. 1998).

1.1.7. Branching Morphogenesis and Epithelial-Mesenchymal Interaction

A core group of evolutionary conserved signaling pathways control elongation and branching morphogenesis of the lungs. These factors coordinate the reciprocal interaction between the epithelial cells of the conducting airways and the surrounding mesenchymal cells. Here, the most important signaling pathways during lung development will be discussed.

1.1.8. Fibroblast Growth Factor Signaling Pathway

Fibroblast Growth Factor (FGF) signaling occurs when a member of the FGF family of polypeptides bind to transmembrane tyrosine kinase receptors (FGFRs). There are 23 FGF ligands and four types of FGF receptors. Upon ligand-receptor interaction, dimerization and autophosphorylation of the receptors promote activation of different mediators that control cell proliferation, differentiation, and migration (Ornitz & Itoh 2001).

One of the most conserved roles of the FGF signaling pathway during evolution is its role in lung bud initiation and branching. Both FGF *branchless* (*Bnl*) in *Drosophila* trachea and FGF-10 in human airways act as a chemotactic factor for branching of the lung bud epithelium. FGF-10 is expressed in the mesenchyme surrounding the branch points. *Fgf10*^{-/-} mice have no distal lung despite the formation of a trachea (Min et al. 1998; Sekine et al. 1999). FGFR2 is expressed in respiratory tract epithelial cells from the embryonic stage onward and during branching morphogenesis (Peters1 ' et al. 1994). FGF-10 is a primary ligand for FGFR2 (Ohuchi et al. 2000) and FGF-10-FGFR2b signaling from mesenchymal cells surrounding the tip to the adjacent epithelial cell controls secondary and subsequent bud formation. The FGF-10 expression is regulated by other signaling factors including BMP4, sonic hedgehog (SHH) and TGF-βs showing the complexity of branch formation and growth. These interactions will be discussed below.

FGF7 is expressed later during branching morphogenesis and signals via FGFR2b as well (Post et al. 1996). The addition of FGF-7 to early lung explant culture can promote epithelial cell growth and formation of a cyst-like structure. FGF-7 is involved in epithelial cell differentiation as well (Cardoso et al. 1997; Deterding et al. 1996). However, knockout mice for FGF-7 have no significant lung defect suggesting redundancy with other FGF's (probably by FGF-10) during lung development (Guo et al. 1996).

FGF-9, is expressed in bronchial epithelium and visceral pleura at E10.5 in mice, while its receptor is expressed in the surrounding lung mesenchyme (Colvin et al. 1999). *Fgf9*^{-/-} mice demonstrate decreased mesenchyme and airway branching, however, they have normal distal airspace formation and alveolar cell differentiation (Colvin et al. 2001).

1.1.9. Sonic Hedgehog (SHH) Signaling Pathway

Sonic hedgehog signaling is one of the three hedgehog pathways that are conserved among all mammals. The SHH ligand binds to its transmembrane receptor, Patched, and releases its inhibitory effect on G protein-coupled transmembrane receptor, Smoothened. This results in activation and cell surface accumulation of the Smoothened receptor, which leads to activation of the transcription factors, Gli1, 2 or 3 (Chen & Jiang 2013). SHH signaling plays a major role in the interaction between the epithelium and mesenchyme. *SHH* is expressed at low levels in all epithelial cells, but at a higher level in the growing distal buds (Bellusci et al. 1997). *SHH* null mice produce profound lung hypoplasia and failure of trachea-esophageal separation. Also, *SHH* null lungs have abnormal FGF-10 expression. In these lungs, FGF-10 expression becomes widespread throughout the distal mesenchyme compared to its precisely restricted expression at branching points in normal lungs (Bellusci et al. 1997). This spatial restriction of FGF-10 to the mesenchyme surrounding the tips of branching airways by SHH appears to be crucial to initiate and maintain branching morphogenesis (Warburton et al. 2005).

1.1.10. Wnt Signaling Pathway

Members of the Wnt glycoprotein family control processes including cell fate specification, proliferation, polarity and migration (Logan & Nusse 2004). Wnt family members bind to one of their seven-span receptors of the Frizzled (Fz) gene family. In the canonical Wnt pathway, binding of the Wnt ligand to its receptor inhibits glycogen synthesis kinase (GSK-3)-mediated phosphorylation of the β -catenin protein in the scaffolding protein complex of adenomatous polyposis coli (APC) and axin. Hypophosphorylated β -catenin accumulates in the cytoplasm and translocates to the nucleus, where it forms an active transcription complex with T Cell Factor

(LEF1, TCF1/2/3) transcription factors to activate several target genes (Akiyama 2000; Van Noort & Clevers 2002). The involvement of the Wnt/ β -catenin signaling pathway in lung development has been demonstrated several times. β -catenin localizes in the cytoplasm and nucleus of the undifferentiated primordial epithelium (PE), differentiating alveolar epithelium and adjacent mesenchyme (Tebar et al. 2001). Conditional deletion of the β -catenin gene in mouse lung epithelial cells during development does not result in changes in the proximal lung phenotype. In contrast, the distal lung epithelium in these mice gives rise to profound perturbation of normal epithelial, mesenchymal and vascular development with decreased expression of alveolar type 2 cell marker, *Sftpc*, vascular endothelial marker, PECAM, and α -smooth muscle actin (Mucenski et al. 2003). Fibronectin (FN) accumulation at sites of epithelial bud tip splitting is essential for cleft formation in the lung airways (Sakai et al. 2003). Wnt signaling drives FN deposition between the branch tips, resulting in epithelial cleft formation (De Langhe et al. 2005).

1.1.11. Retinoic Acid Signaling Pathway

Retinoic acid (RA) is an endogenous molecule in the embryonic and adult vertebrate that is derived from Vitamin A. It can be synthesized in the cell from circulating retinol or diffused from the adjacent cell. In the absence of RA ligand, its heterodimer receptor, RA receptors (RARs)/retinoid X receptors (RXRs) can bind to RA-response elements and prevent the access to the promoters of the target genes. Upon binding of the RA ligand, a conformational change in the receptors results in recruitment of the co-activator complex and induction of gene expression (Rhinn & Dolle 2012). RA signaling is required for lung bud initiation. Vitamin A deficiency during the onset of lung development leads to lung agenesis despite the presence of a trachea. A similar phenotype is seen in *Fgf10*^{-/-} mice (Dickman et al. 1997; Sekine et al. 1999). RA signaling maintains low levels of

TGF- β signaling in the prospective lung field of the foregut to allow FGF-10 expression and lung bud initiation (Chen et al. 2007). However, during lung branching morphogenesis and cell differentiation, downregulation of RA signaling allows epithelial cell differentiation. Overexpression of RA ligand or continued activation of RA signaling in this stage results in disturbed distal lung branching and proximal-like immature airways with no type I epithelial cells (Malpel et al. 2000; Cardoso et al. 1995; Wongtrakool et al. 2003).

1.1.12. Transforming Growth Factor Beta (TGF- β) Superfamily

The TGF- β superfamily includes of several growth factors. Among them, TGF- β and bone morphogenetic protein (BMP) subfamilies have major roles during lung development. The active form of TGF- β binds to specific type II receptors, which is followed by the recruitment of type I receptors. The ligand-receptor interaction results in the activation of serine/threonine receptor kinases, resulting in the phosphorylation of a family of transcription factors which are called Smad proteins. Smad2 and Smad3 proteins are involved in the TGF- β pathway. Smad1/5/9 are involved in the BMP signaling pathway. Phosphorylation of Smads leads to recruitment of the common signaling transducer, Smad4, which in turn results in the translocation to the nucleus. The complex of Smads partner in the nucleus with other transcription factors to modulate the transcriptome in a cell-specific manner (Shi & Massagué 2003).

1.1.13. BMP Signaling Pathway

Bone Morphogenetic Proteins (BMPs) play crucial roles in regulating many processes including lung development. Among more than 20 ligands, BMP4 plays a central role in lung morphogenesis (Hogan 1996). It is expressed in distal epithelial cells around the branching point. BMP4 and SHH

signaling regulate the expression of Fgf10 and restrict it to the branching points (Figure 1-2) (Bellusci et al. 1996; Herriges & Morrisey 2014). Both overexpression or knockdown of BMP4 in embryonic epithelial cells results in abnormal lung development with dilated terminal sacs suggesting that balanced BMP4 levels are required for normal lung development (Bellusci et al. 1996; Eblaghie et al. 2006).

1.1.14. TGF- β Signalling

There are three mammalian TGF- β isoforms which are TGF- β 1, TGF- β 2 and TGF- β 3. TGF- β 1 is expressed throughout the mesenchyme during the early stages of lung development. TGF- β 2 expression is exclusively localized to the epithelial cells of the developing distal airways. TGF- β 3 is expressed in both tracheal mesenchyme and epithelium of the growing bronchioles during the pseudoglandular stage of mouse lung development (Pelton et al. 1990; Schmid et al. 1991). Exogenous overexpression of TGF- β 1 and TGF- β 2 inhibited lung branching in mouse lung explant cultures (Serra et al. 1994; Bartram & Speer 2004). Abrogation of TGF- β receptor type II promotes branching morphogenesis in mouse lung cultures (Zhao et al. 1996).

Increased TGF- β 1 expression is an early event in many lung diseases during inflammation, tissue injury, and repair phases. If these reparative phases are exaggerated and not carefully regulated, lung fibrosis occurs which is associated with over-expression of TGF- β 1 ligands and receptors. Exogenous increased expression of TGF- β 1 results in severe lung fibrosis (Sime et al. 1998; Bartram & Speer 2004). TGF- β 1 promotes lung remodeling and fibrosis by several direct and indirect mechanisms including chemotaxis of fibroblasts and macrophages, secretion of mesenchymal growth factors, ECM synthesis, fibroblast proliferation and reduction of surfactant synthesis (Bartram & Speer 2004). Several studies demonstrated that inhibition of TGF- β 1,2

ligand-receptor binding significantly reduced immunogenic or bleomycin-induced pulmonary fibrosis in rodents (Giri et al. 1993; Li et al. 1997; Denis 1994).

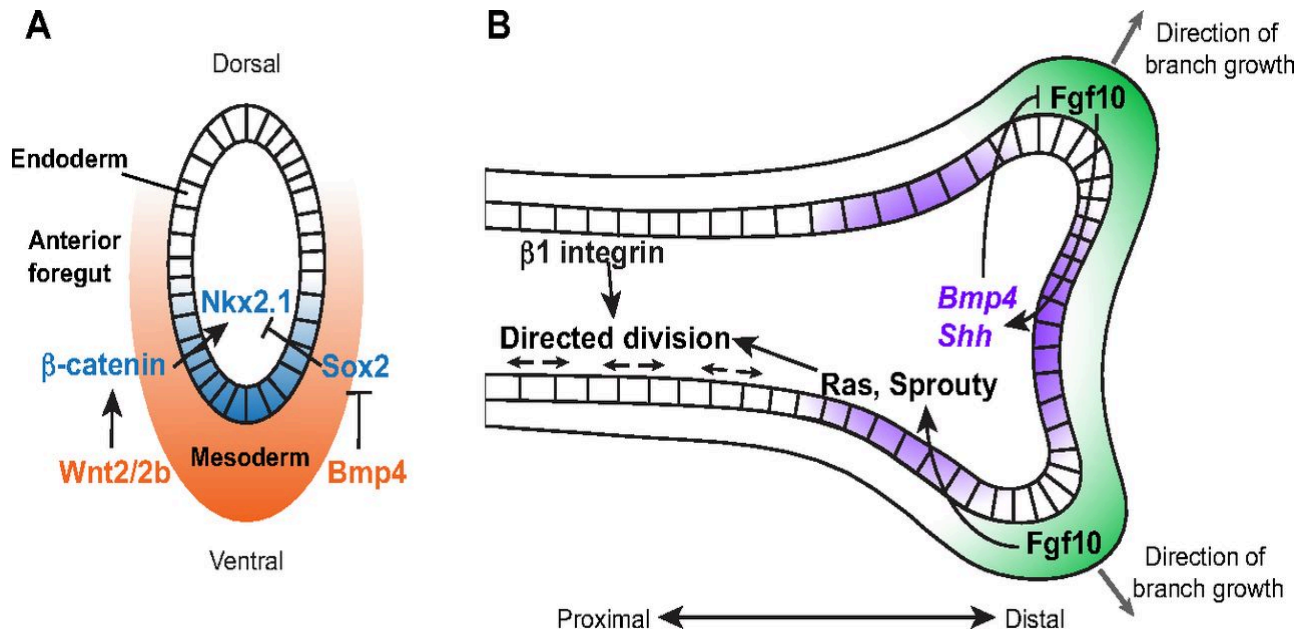


Figure 1-2: Major signaling pathways in early development of the lung. (A) Lung endoderm specification start with *Nkx2.1* expression in the ventral wall of anterior foregut (blue) at E9.5. *Wnt* and *Bmp4* signaling (orange) from the surrounding mesoderm is required for this specification. *Wnt* signaling promotes expression of *Nkx2.1*, and *Bmp4* signaling prevents *Sox2* expression in the presumptive lung endoderm. (B) *Fgf10* expression in the mesenchyme surrounding developing branch points, is essential for branching morphogenesis (green). *Shh* and *Bmp4* (purple) regulate the expression of *Fgf10*. Interaction of *Fgf10* with *Wnt*, *Bmp4* *Ras/Sprouty* signaling, establish the appropriate direction of branch growth (Herriges & Morrissey 2014, used with the journal permission).

1.1.15. Epithelial and Mesenchymal Cell Differentiation and Maturation

Our understanding of the molecular regulation of airway branching is better than our understanding of the mechanisms underlying the formation of saccules and alveoli. This is due to the complexity of these events, including the involvement of physical forces and local vascular blood flow, which

are difficult to mimic in culture. (Morrisey et al. 2013). All epithelial cell lineages are believed to be derived from common multipotent progenitors originating from the pseudoglandular stage. These progenitor cells, express transcription factors, Id2 (Inhibitor of Differentiation 2) and Sox9, throughout the lung epithelium up to E13.5. Subsequently, these cells give rise to proximal (bronchiolar) progenitor cells expressing Sox2 and distal (alveolar) progenitors (Id2⁺, Sox9⁺) (Wuenshell et al. 1996; Rawlins et al. 2009). FGF-10 signaling appears to be essential for the maintenance of the distal alveolar progenitor cell status (Volckaert et al. 2013). Also, overexpression of FGF-10 in proximal progenitor cells prevents the differentiation of a ciliated-cell lineage but facilitates their commitment to the basal-cell lineage. (Turcatel et al. 2013; Que et al. 2007). Notch signaling is another important regulator in cell fate of bronchiolar progenitors. Lack of Notch signaling in these cells promotes ciliated and neuroendocrine cell fate, whereas normal or high levels of Notch signaling favor Clara or goblet cell fates, respectively (Tsao et al. 2011). Wnt signaling is considered another important factor in maintaining epithelial proximal-distal differentiation. Loss of function of Wnt signaling leads to the inhibition of distal airway formation and enlargement of the proximal airways (Mucenski et al. 2003). Glucocorticoid (GC) signaling is highly upregulated in the late stages of lung development in the distal lung epithelium and stimulates the differentiation of distal progenitors into committed alveolar epithelial type I and II cells (Bird et al. 2014). Epithelial-mesenchymal interactions during lung development are not limited to branching morphogenesis. Adjacent mesenchymal cells are important for airway epithelial differentiation. This regulation was first discovered in tissue recombinant studies showing that distal mesenchyme can induce proximal epithelial cells to take on a more distal phenotype (Shannon et al. 1998).

The airway smooth muscle precursors are derived from FGF-10-expressing lung mesenchyme distal to the epithelial branching tips. These precursors migrate proximally and give rise to airway smooth muscle (ASM), vascular smooth muscle (VSM) and lipofibroblasts (Kumar et al. 2014; El Agha et al. 2014). These studies demonstrated that development of airway smooth muscle is dependent on WNT2. However, differentiation of adjacent proximal pulmonary vascular smooth muscle cells was not dependent on WNT2 (Goss et al. 2011).

1.4. Overview of Diaphragm Development

The diaphragm consists of a large peripheral muscular part and a central tendon. Diaphragm development begins around four weeks and is complete around 12 weeks of gestation. There are four major embryonic structures that contribute to the formation of the diaphragm which are the transverse septum, the pleuroperitoneal folds (PPF), the esophageal mesentery, and the muscular body wall. The central tendon originates from the transverse septum. Most of the diaphragm muscle is derived from the PPFs which fuses with the dorsal esophageal mesentery and the transverse septum. The medium part of the diaphragm is formed from by the dorsal esophageal mesentery, and the muscular body wall develops muscle fibers in the periphery of the diaphragm (Chavhan et al. 2010; Imaging 2012).

1.5. Congenital Diaphragmatic Hernia

Congenital Diaphragmatic Hernia (CDH) is a congenital anomaly consisting of a defect in the diaphragm, resulting in herniation of the abdominal viscera into the chest cavity, and a poorly developed lung. The condition can lead to severe respiratory failure soon after birth because of pulmonary hypoplasia (PH) and persistent pulmonary hypertension (PPH).

CDH is classified based on the anatomical position of the defect into posterolateral (Bochdalek hernia), anterior (Morgagni hernia) and central (Hiatal hernia). The posterolateral defect occurs in 70-75% of the cases. The defect is thought to arise from a failure or a delay of closure of the pleuroperitoneal folds and septum transversum to fuse properly to the intercostal muscles. 80% of posterolateral defects occur on the left side. Morgagni hernia occurs in 23-28% and the central defect in only 2-7% of the cases (Taylor et al. 2009; Veenma et al. 2012). Most of the CDH patients have a discontinuity (e.g. hole) in the diaphragm, but sometimes an under-muscularization of the diaphragm can also be seen - this is called an eventration. The degree of thinning can be variable. In severe cases, organs herniated into the chest are enclosed by a very thin structure, called a hernia sac. This sac is thought to be the focal thinning of diaphragm musculature (Pober 2007). The prevalence of CDH is 1 in 2,000 to 3,000 births (Wright et al. 2011; Stege et al. 2003). According to single institution studies, since early 1990s, advancements in pediatric surgery and neonatal intensive care have significantly increased CDH survival in recent years, now approaching 90% (Downard et al. 2003; Antonoff et al. 2011). However, population studies demonstrated no significant changes in survival with the mortality rate remaining high between 42% and 67% (Colvin et al. 2005; Mah et al. 2009). The factor that most likely influences this difference is referred to as “hidden mortality”. Hidden mortality accounts for *in utero* death or new-born CDH patients who died without being reported to referral centers. (Harrison et al. 1978; Brownlee et al. 2009; Burgos & Frenckner 2017).

1.1.16. Isolated versus Complex CDH

Isolated CDH- patients have CDH only and sometimes an associated cardiac abnormality such as left heart hypoplasia or cardiac dextroposition. This is defined as isolated CDH and accounts for 60-75% of all cases (Pober 2008). The incidence of isolated CDH over non-isolated CDH can

vary based on the reporting methods in studies. Similar to survival rate, hidden mortality is considered to contribute to this difference (Harrison et al. 1978; Brownlee et al. 2009).

Complex CDH- (Also known as non-isolated or syndromic CDH) A diagnosis of complex CDH is made when a CDH patient has additional abnormalities as part of a syndrome, a chromosomal abnormality, or non-syndromic major malformations (e.g., CDH plus cleft lip). The frequency of complex CDH is around 30-40 % of all cases. Some examples of these associated syndromes or chromosomal abnormalities are trisomy 18, tetrasomy 12p, Craniofrontonasal Dysplasia Syndrome, Fryns Syndrome and Matthew-Wood syndrome (Pober 2008; Pober 2007).

1.6. Diagnosis, Intervention, Management, and Prognosis of CDH

Around two-thirds of CDH cases are diagnosed prenatally during routine ultrasonography between 18-24 weeks (Taylor et al. 2009). The sonographic hallmarks of a left CDH include the presence of abdominal organs (e.g. a stomach bubble or left lobe of the liver) at the level of the heart, bowel loops in the thorax and a right mediastinal shift (Taylor et al. 2009). The outcome of CDH is largely based on the degree of pulmonary hypoplasia and hypertension that these infants experience. Several prenatal prognostic predictors have been proposed over the years. Liver herniation is the single most reliable predictor of mortality - CDH babies with a herniated liver have a mortality of around 65% compared to 7% when the liver position is normal (Hedrick et al. 2007). The most used predictor of mortality or severity of lung hypoplasia is fetal lung volume. It includes the observed to the expected lung-to-head ratio (O/E LHR) and MRI-based total lung volume (TLV). Fetuses in 20-26 weeks of gestation with an O/E LHR < 0.6 have a 0% chance of survival, while fetuses with a LHR > 1.35 have a 100% chance of survival (Badillo & Gingalewski 2014; Metkus

et al. 1996). Other studies have suggested that 3D MRI imaging of the lung can be used as a direct predictor of lung hypoplasia and outcome (Ruano et al. 2004).

Prenatal management of CDH is mainly limited to monitoring the pregnancy for development of prenatal complications. More recently, researchers used fetoscopic endoluminal tracheal occlusion (FETO) in severe to mild CDH using a balloon. Physiologically, plugging the trachea results in the prevention of egress of fetal lung fluid to the amniotic cavity leading to increased fetal airway pressure, promoting pulmonary proliferation and maturation of both the lung architecture and pulmonary vasculature (Kitano 2007). A recent systematic review and meta-analysis study demonstrated that FETO improves survival in isolated CDH with severe pulmonary hypoplasia compared with the standard perinatal management. However, FETO is also associated with an increased chance of premature rupture of the membranes (PROM) in these babies (Al-Maary et al. 2016).

1.7. Pulmonary Hypoplasia and Persistent Pulmonary Hypertension Associated with CDH

Pulmonary hypoplasia (PH) along with persistent pulmonary hypertension (PPH) are the most important causes of the high mortality and morbidity observed in CDH patients. Hypoplastic lungs in CDH patients are characterized by physically small lungs with fewer airway branches, a reduced number of alveoli related to each terminal airway and a thickened interstitial mesenchymal cell layer (fibrotic phenotype) (Keijzer & Puri 2010; Van Loenhout et al. 2012).

The lung vasculature develops in parallel to the airways. There are two primary mechanisms involved in pulmonary vasculature development: (a) angiogenesis, in which a new vessel sprouts from existing ones and (b) vasculogenesis, which involves the *de novo* formation of new blood vessels from *in situ* angioblasts. Angiogenesis forms the pulmonary central vasculature

and vasculogenesis establishes the distal pulmonary vasculature (Hall et al. 2002). In a patient with CDH, the pattern of the lung vasculature is abnormal, the total size of the pulmonary vascular bed is reduced, the thickness of pulmonary artery walls is increased due to excessive muscularization, and there is increased pulmonary vascular remodeling. These structural vasculature defects result in lung function abnormalities, which lead to persistent pulmonary hypertension (PPH) (Chinoy 2002). PPH is conventionally treated with inhaled nitric oxide (NO), which induces local vasodilation through activation of the endothelial nitric oxide pathway. However, inhaled NO administration is only effective in 30-40% of CDH patients compared to premature babies with pulmonary hypertension (Kool et al. 2014).

1.8. Pathophysiology of CDH

The classic teaching is that the primary defect in CDH is in the diaphragm. Abdominal organs herniate into the thoracic cavity and compress the lung resulting in pulmonary hypoplasia and pulmonary hypertension. However, studies in the nitrofen model demonstrated that hypoplasia of the lung precedes the diaphragm defect (Lritani 1984). These data led to the concept of a “dual-hit hypothesis” (Keijzer et al. 2000). This hypothesis explains pulmonary hypoplasia in the nitrofen model by two developmental insults. The first insult occurs before formation of the diaphragmatic defect and affects the development of both lungs equally. Following the formation of a diaphragmatic defect, the second insult affects the ipsilateral lung only. The herniation of the abdominal organs into the chest cause diminished fetal breathing movements, which are required for proper lung development. The dual-hit hypothesis suggests that CDH may be a combination of impaired lung development disease associated with, but not always caused by, a structural defect in the diaphragm. In the absence of nitrofen, the first insult is due to a combination of genetic, environmental or (epi-)genetic factors which are still not identified.

1.9. Signaling Pathways in the Hypoplastic Lung Associated with CDH

One of the most important signaling pathways contributing to CDH, pulmonary hypoplasia, and pulmonary hypertension is the RA signaling pathway. Experiments from over 60 years ago have shown that 25-40% of pups born to rat dams with a vitamin A deficient diet have diaphragmatic hernias (Wilson et al. 1953). These rats also have several other congenital malformations affecting the ocular, cardiac and urogenital systems. RA receptor (RAR) α/β double knockout mice have pups with CDH (Mendelsohn et al. 1994). In Human CDH, levels of retinol and retinol-binding protein are lower in first hours after birth than in age-matched controls (Beurskens et al. 2010; Major et al. 1998). Lower levels of RA can increase TGF- β signaling and decrease the level of Fgf-10 (Chen et al. 2007). Over-expression of TGF- β 1 and down-regulation of FGF-10 have been demonstrated in the nitrofen rat model of CDH at different stages of lung development (C. Xu et al. 2009; Chen et al. 2010; Teramoto et al. 2003). TGF- β 2 knockouts exhibit a lower number of distal air sacs and collapsed lungs after birth (Sanford et al. 1997). Therefore, the lower number of distal airways and sacs, as well as the higher number of fibroblasts observed in the hypoplastic lungs of CDH patients, may be caused by a dysregulation of the interaction between RA, TGF- β and FGF-10.

1.10. Animal Models for CDH

Animal models to study CDH include a teratogenic (nitrofen) model in the rat and mouse, a surgical model in the rabbit and sheep, and genetic (knockout) mouse models. There is no animal model that perfectly mimics human CDH and its associated abnormalities, but they all have shed

light on the underlying pathogenesis of the disease and have facilitated the evaluation of new interventions (Van Loenhout et al. 2009).

1.1.17. Nitrofen Rat Model

The nitrofen rat model of lung hypoplasia and CDH has been the most commonly used model for more than two decades. The use of this animal model began with toxicological studies showing that administration of nitrofen (2,4 dichlorophenyl-*p*-nitrophenyl ether), a herbicide, to pregnant rats on gestational day 9.5, results in around 70% of the fetuses developing diaphragm malformation and 100% pulmonary hypoplasia (Ambrose et al. 1971; Costlow & Manson 1981; Kluth et al. 1990). Among several animal models of CDH, the nitrofen rat model is one of the best because of its profound similarities to human CDH, such as liver herniation, a large diaphragmatic defect and asymmetrical lung hypoplasia and pulmonary hypertension. Also, unlike other models, it impacts early lung development (Van Loenhout et al. 2009). The similarities, also, hold true for the periodic occurrence of associated cardiac, palate and skeletal abnormalities (Greer et al. 2003). A big drawback of this model is that the teratogenic effects of nitrofen observed in rodents have never been demonstrated in humans. However, different studies demonstrated similar affected mechanism in nitrofen rat model and human CDH such as retinoic acid signaling pathway (Nakazawa et al. 2007; Kutasy et al. 2012). These pathological and molecular similarities make it desirable model for study CDH and the associated hypoplastic lung.

1.1.18. Surgical Models

Surgical models have been mainly used to evaluate therapeutic interventions in CDH. In this model, the defect in the diaphragm is created surgically in fetal sheep or rabbits (Lipsett et al. 1998; Wu et al. 2000). Advantages of the rabbit model over sheep are its shorter gestational period

(term in a rabbit is 31 days compared to 145-149 days in sheep), the larger litter size, easy availability and lower costs. Several therapies have been studied in the surgical model for CDH, such as the administration of corticosteroids, fetal tracheal occlusion or a combination of both in fetal lambs or rabbits (Davey et al. 2006; Roubliova et al. 2008). The diaphragmatic defect is generated relatively late in gestation in the surgical model. Therefore the surgical model does not allow studies into the etiology and early pathogenesis of the lung abnormalities in CDH (Van Loenhout et al. 2009). Moreover, other associated abnormalities (such as cardiac and skeletal abnormalities) are not observed in these surgical models either.

1.1.19. Genetic Models

Several single-gene knockout mice have been demonstrated to have an incomplete formation of the diaphragm and herniation of the abdominal organs. Deletion of Wilm's tumor 1 (*Wt1*) in the septum transversum mesenchyme results in a diaphragm defect and CDH (Carmona et al. 2016). Roundabout 1,2 (*Robo1,2*) genes encode cell-surface receptors that respond to their secreted ligands, Slit proteins, in a wide variety of cellular processes. Both *Robo1,2* and *slit* knockout mice develop a central type of hernia (Phillips et al. 2014; Domyan et al. 2013) and *Fog2* knockout mice with severe PH and a posterolateral muscularization defect (Ackerman et al. 2005). In addition, the deletion of GATA4 in the PPFs mesenchyme leads to the development of an amuscular, weak area in the diaphragm and CDH (Merrell et al. 2015). Despite the observation of a posterolateral hernia in some of these single gene knockout mice, none of these genes have been identified in human CDH except for a mutation in *Fog2* in a single patient with nonsyndromic CDH. As discussed earlier, CDH is considered a multifactorial disease with multiple developmental insults. The phenotype in CDH is so variable that it is potentially not due to a single gene mutation, but the result of the involvement of multiple genes (Van Loenhout et al. 2009).

1.11. The Etiology of CDH

Currently, the cause of ~85% of CDH cases remains unknown (Wynn et al. 2014) and a common genetic cause for CDH is unknown (Veenma et al. 2012) demonstrating our limited understanding of the etiology of CDH. It also suggests that an epigenetics and/or multifactorial etiology might play a role. This hypothesis is supported by a study showing that only one member of a monozygotic twin pair developed CDH (Pober et al. 2005b). The aim of this project is to study the role of microRNAs, as one of the epigenetic factors, in CDH.

1.12. Epigenetic Mechanisms - The Role of MicroRNAs

Epigenetic changes are mitotically heritable changes in the genome that are not encoded within the DNA sequence. The main epigenetic mechanisms include DNA methylation, histone modifications, RNA-associated silencing, and the crosstalk between these mechanisms (Piletič & Kunej 2016).

MicroRNAs (miRNAs) are short non-coding RNAs of ~22 nucleotides long. They can originate from different parts of the genome such as introns and exons of protein-coding genes or intergenic regions (Piletič & Kunej 2016). MiRNAs are involved in essential biological processes such as development, differentiation, proliferation, and apoptosis (Bartel 2004).

Biogenesis and processing of microRNAs take place in both the nucleus and cytoplasm. There are several complex protein systems involved in these processes. In the nucleus, a several-kb-long primary (pri)-miRNA is transcribed by RNA polymerase II. The pri-miRNA is then processed into a 70 nt precursor miRNA (pre-miRNA) by a microprocessor complex comprising of a ribonuclease III, known as Drosha (Lee et al. 2003) and a double-stranded RNA-binding

protein known as Pasha (DGCR8). DGCR8 acts as an anchor molecule that determines the cleavage site (Han et al. 2006). Next, Exportin 5 and its catalytic partner Ran-GTP bind the pre-miRNA and travel through the nuclear pore into the cytoplasm. In the cytosol, the pre-miRNA is processed by a ribonuclease III, DICER, into ~22-nt duplex (double-stranded) miRNA molecules. The double-stranded miRNA binds to the RNA-induced silencing complex (RISC), which consists of the Argonaute (AGO) protein, the human immunodeficiency virus transactivating response RNA-binding protein (TRBP) and the fragile X mental retardation protein (FMRP1)(Meister et al. 2004; Jin et al. 2004). Functional miRNA is produced after the complementary strand (passenger strand) is removed from the RISC complex and degraded.

Most of the target mRNAs have the complementarity binding site at their 3' UTR. But the binding site can also be at the 5' UTR, promoter or coding sequences. miRNAs bind to the target mRNA mainly by their “miRNA seed” sequences which are mostly situated at nucleotide positions 2 to 7 from the miRNA 5'-end. The downstream nucleotides (nucleotide positions 8 to 13) participate in base pairing with the target as well. The degree of complementarity between miRNA seed region and mRNA target site is considered a key determinant of the regulatory mechanism. Perfect complementarity results in the Ago-catalyzed cleavage of the mRNA strand. But imperfect complementarity leads to repression of mRNA translation. However, binding may also increase translation of protein-coding genes (Acunzo et al. 2015; Carthew & Sontheimer 2009; Piletič & Kunej 2016).

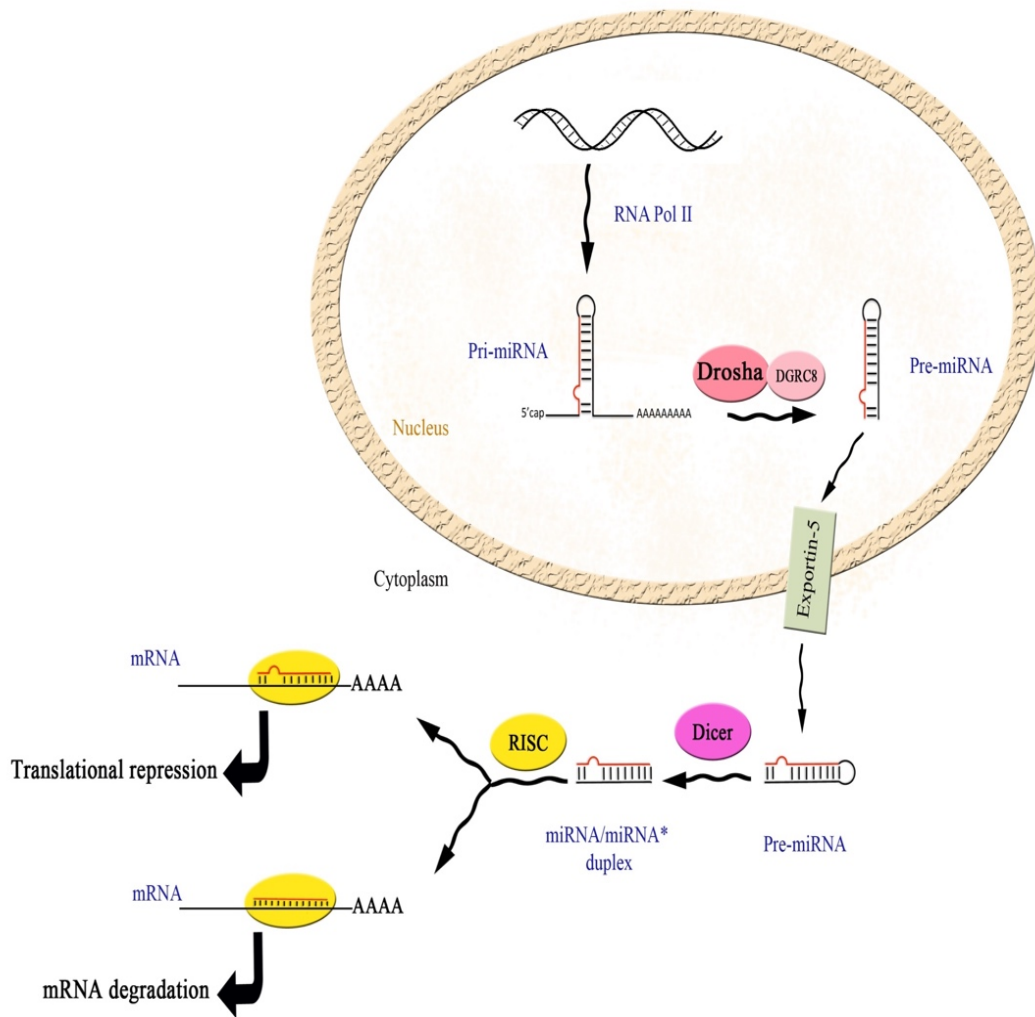


Figure 1-3: MicroRNA biogenesis and function. After transcription of a pri-miRNA by RNA pol II, this primary transcript is modified by the addition of a 5'-Cap and a 3'-poly A tail. In the next step of processing, the pri-miRNA is cleaved by the Drosha/DGCR complex into a pre-miRNA and exported to the cytoplasm by Exportin-5. The pre-miRNA in the cytoplasm undergoes further processing by Dicer into a miRNA/miRNA* duplex. After incorporation into the RNA-induced silencing complex (RISC), the miRNA guide strand (mature miRNA) leading to translational inhibition or degradation of target mRNAs. The passenger (complementary) miRNA is usually degraded (Khoshgoo et al. 2013, used with the journal permission).

1.13. MicroRNAs and Lung Development

One of the first studies demonstrating a role for miRNAs in lung development was done by Harris et al. They created a conditional knockout mouse to specifically knock out Dicer in mouse lung epithelium shortly after the initiation of lung branching. The KO lungs showed arrested branching morphogenesis with abnormal growth of the epithelial tube (Harris et al. 2006). Members of the Argonaute family are localized to branching regions during lung development. *Ago1* is expressed in the distal epithelial cells, while *Ago2* is mainly localized in the surrounding mesenchymal layer. This cell-specific localization of *Ago1* and *Ago2* suggests that they each have a distinct role in regulating genes selectively expressed in each of these cells (Lü et al. 2005).

Using a systematic profiling study of mRNA, microRNA and protein levels during different stages of lung development, *Dong et al.* showed that down-regulation of protein levels occurred independently of changes in mRNA levels in over half of the proteins analyzed indicating that the expression of these genes are regulated post-transcriptionally. These findings suggest that inhibition of protein translation by miRNAs independent of changes in mRNA levels is a prominent mechanism of regulation during lung organogenesis (Dong et al. 2010).

Our knowledge of the role of specific miRNAs during lung development is limited. One of the most studied miRNA family during development and disease is the miR-17~92 cluster which is composed of 7 miRNAs that are transcribed as a single pri-miRNA. The cluster is over-expressed in many cancer types including lung cancer (Hayashita et al. 2005). The expression of the miR-17~92 cluster is highest during the early stages of lung development but decreases during later stages of lung development. Overexpression of miR-17~92 in type II epithelial cells using a surfactant protein c gene promoter results in increased epithelial proliferation and reduced

differentiation (Lu et al. 2007). MiR-17~92 KO mice cluster die shortly after birth with lung hypoplasia and a ventricular septal defect (Ventura et al. 2008). MicroRNA profiling of embryonic lung tissue and lung cancer often produces similar results. MiRNA profiling demonstrated that some of the most highly expressed miRNAs during early embryonic lung development, including the miR-17~92 cluster, have increased expression in lung adenocarcinomas (Hayashita et al. 2005). Interestingly, miRNAs with higher expression in the adult lung than fetal lung are downregulated in tumors. One of these microRNAs is the let-7 family. In human cells, let-7 acts as a tumor suppressor by negatively regulating the Ras gene (Johnson et al. 2005). Comparing microRNA profiles between E11.5 (pseudoglandular stage) and E17.5 (late canalicular stage) in mice demonstrated that the most abundant miRNA at E11.5 was miR-17, while at E17.5, let-7 was the most abundant miRNA. Therefore, the transition from early branching morphogenesis to later differentiation is accompanied by a switch from the miR-17~92 cluster to the let-7 family (Lu et al. 2008).

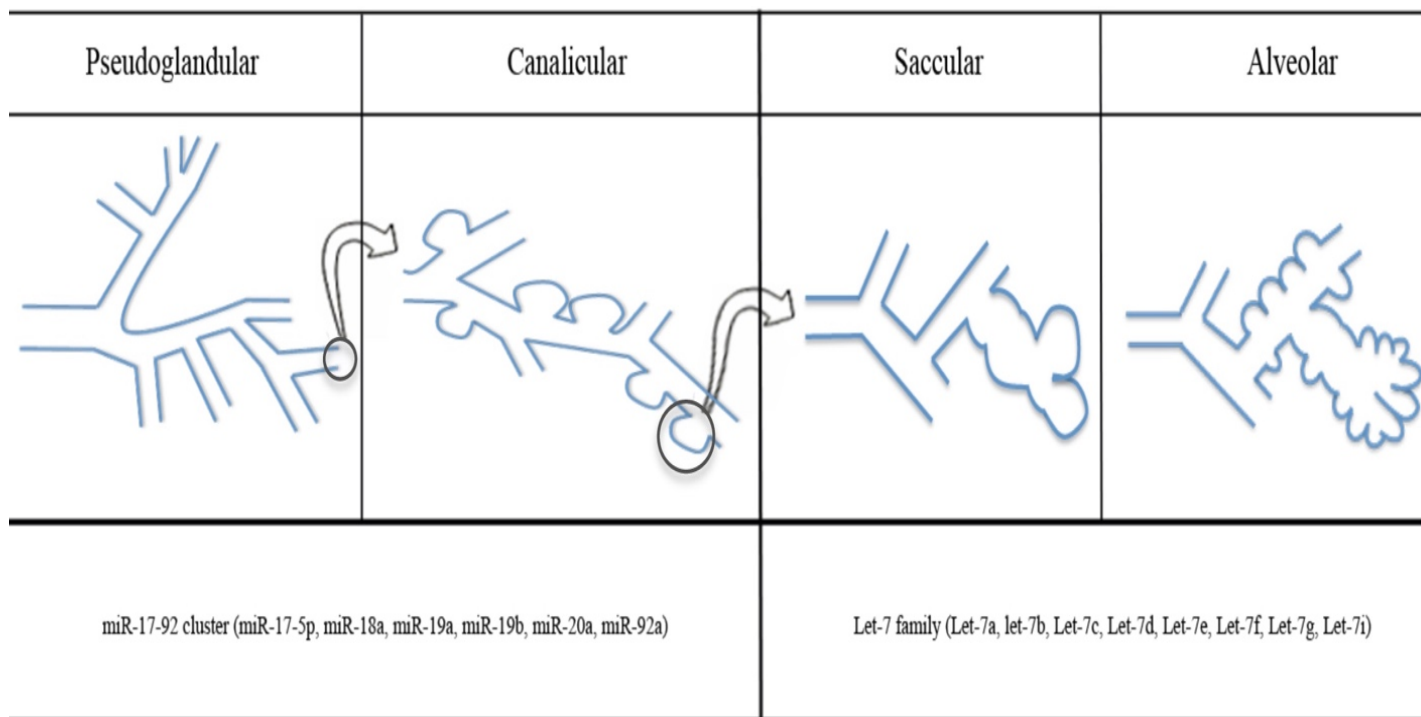


Figure 1-4: Upregulation of two important microRNA families during the different stages of lung development. The miR-17~92 cluster is up-regulated during branching morphogenesis and the canalicular stages. Let-7 family members are up-regulated during the saccular and alveolar stages. (Khoshgoo et al. 2013, used with the journal permission).

Epithelial-to-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) is a biological process that allows a polarized epithelial cell to acquire mesenchymal cell characteristics. This includes the ability to lose intercellular junctions and an enhanced migratory capacity, invasiveness, higher resistance to apoptosis, and a substantially increased, production of extracellular matrix (ECM) components (Kalluri et al. 2003). EMT is an evolutionarily conserved developmental process that contributes to the formation of the body plan and organogenesis. Epithelial cells are characterized by an apicobasal polarity. They are held together by intercellular adhesion complexes (such as E-Cadherin and integrins) and a basement membrane. Mesenchymal cells are characterized by

cellular motility, and their ability to produce ECM. Cells can transition between epithelial and mesenchymal states via EMT and the reverse process, mesenchymal-epithelial transition (MET). EMT and MET occur several times during organogenesis including the development of the neural crest (Sauka-Spengler & Bronner-Fraser 2008), kidneys (Stark et al. 1994; Carroll et al. 2005) and heart (Nakajima et al. 2000; Mercado-Pimentel & Runyan 2007).

EMT is classified into three types according to its biological (or pathological) function: Type 1– Developmental EMT; type 2– Chronic (fibrosing); and type 3– Malignancy. Type 1 EMT is distinct from 2 and 3 in that it neither causes fibrosis nor induces an invasive phenotype. Type 1 EMT also has the potential to consequently undergo MET to generate secondary epithelial cells. Type 2 EMT happens during wound healing, tissue regeneration, and organ fibrosis which are associated with inflammation. During fibrosis, ongoing inflammation result in constant type 2 EMT and ultimately organ destruction. Tissue fibrosis is a form of prolonged wound healing because of persistent inflammation. Type 3 EMT arises in the neoplastic cells with genetic and/or epigenetic changes. By losing adhesion molecules and acquiring invasive properties, EMT facilitates the delamination and metastasis of transformed epithelial cells (Kovacic et al. 2012; Kalluri et al. 2003). Depending on the tissue and signaling contexts promoting EMT, epithelial cells may show both epithelial and mesenchymal properties; this can be considered as partial EMT. Recent studies in kidney fibrosis showed that epithelial cells under inflammatory stresses could advance to various extents through an EMT. These epithelial cells keep the expression of epithelial markers along with expressing mesenchymal markers which creates the notion of partial EMT. These studies showed that, in kidney fibrosis, the profibrogenic consequences of EMT activation are not mediated by a complete transition into proliferative myofibroblasts but rather through partial EMT with impaired regenerative potential and cell-cell communication (Grande et al. 2016;

Ovadya & Krizhanovsky 2015). Also, it has been shown that COPD human bronchial epithelial cells have partially undergone EMT (Nishioka et al. 2015).

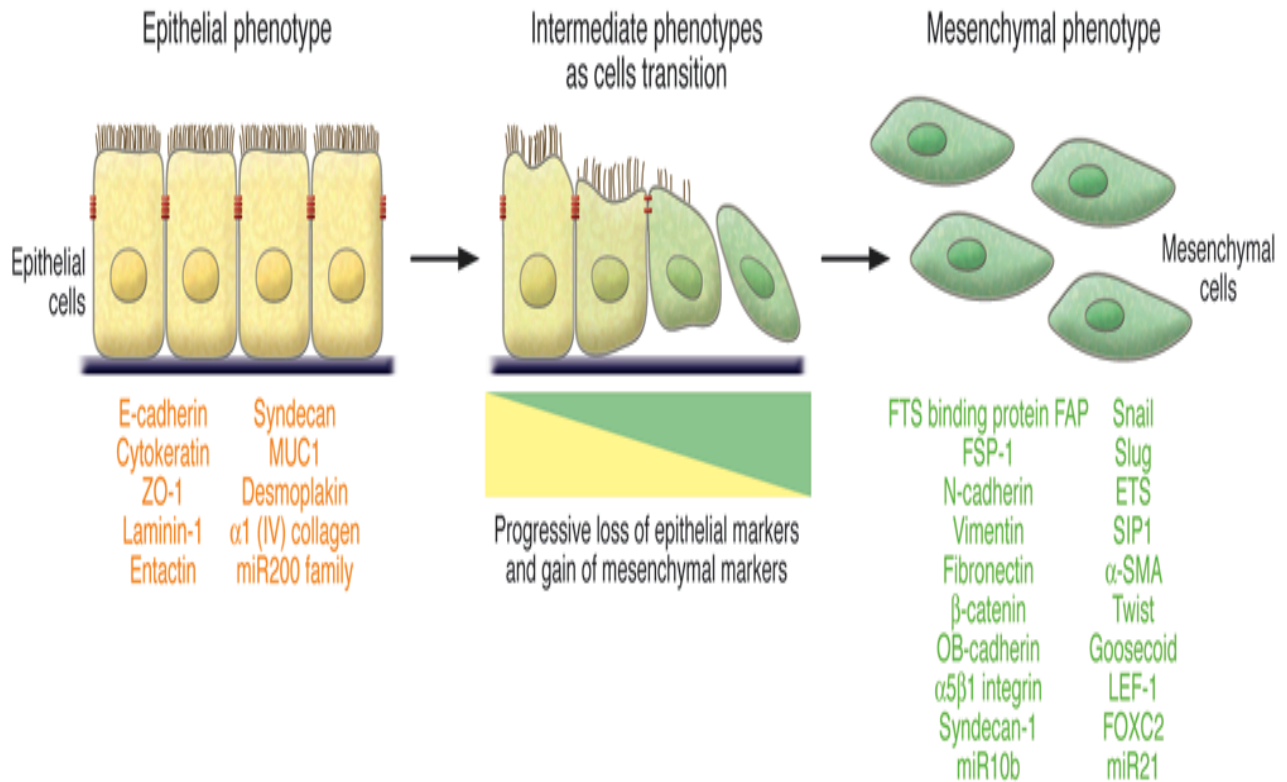


Figure 1-5: Epithelial-to-Mesenchymal Transition (EMT). EMT is a biological process that allows the functional change of polarized epithelial cells into invasive and ECM-secreting mesenchymal cells. Markers for the epithelial and mesenchymal cell are listed. Epithelial cells may show both epithelial and mesenchymal properties (co-localization of the two sets of distinct markers), which is considered as partial EMT. (Kalluri & Weinberg 2009, used with the journal permission).

1.1.20. EMT Signaling Pathway

Initiation, execution, and maintenance of EMT or MET require a complex network of gene activation and repression programs. At the cellular level, to adopt a mesenchymal phenotype, the epithelial cell must lose its epithelial markers (such as E-Cadherin, zonula occludens, cytokeratin,

and desmoplakin) and acquire expression of mesenchymal genes and proteins, such as—smooth muscle actin (SMA), collagen I and III, vimentin and fibronectin. The most widely described important signaling pathway in EMT is the TGF- β /SMAD signaling pathway. Notably, Smad2 and Smad3 seem to control activation of the EMT process (Valcourt et al. 2005). TGF- β stimulation with Smad activation leads to transcription of transcription factors, such as members of the Snail family and Zeb family, that enhance expression of mesenchymal proteins, while repressing expression of E-Cadherin and other tight junction components (von Gise & Pu 2012).

CHAPTER 2. Thesis Overview

2.1. Study Rationale

As discussed in Chapter 1, a common genetic defect has not been found for CDH suggesting a potential role for epigenetic factors including microRNAs. To explore the role of microRNAs in CDH in our lab, we performed a microarray screen of microRNAs express in human pulmonary tissues at the saccular stage of lung development. We identified two microRNAs, miR-10a and miR-200b, that had significantly higher abundance in CDH than control lungs (discussed in detail in Chapter 3, Manuscript I). In my Ph.D. research project, I evaluated the role of miR-200b in normal lung development and hypoplastic lung associated with CDH.

The miR-200 family is comprised of five members which are transcribed in two clusters. MiR-200b, 200a and 429 share a common transcription start site on chromosome 1; while miR-200c and 141 are transcribed as a single unit from chromosome 12. According to their seed sequences, they can be further divided into two subgroups; subgroup I: miR-141 and miR-200a; subgroup II: miR-200b, miR-200c and miR-429 which indicate differences in their target gene sets (Brabletz & Brabletz 2010).

MiR-200 family members are considered an epithelial marker and can regulate EMT by directly targeting E-cadherin transcription repressors, ZEB1 and ZEB2 (P A Gregory et al. 2008; Burk et al. 2008). ZEB transcription factors, induce EMT by suppressing the expression of many epithelial markers, including E-cadherin. There are eight miR-200 binding sites on the ZEB1 3' UTRs and nine miR-200 binding sites on ZEB2 3' UTRs (Korpai, Esther S. Lee, et al. 2008) (Figure 2-1). Interestingly, transcription of all miR-200 members is regulated by ZEB1 and ZEB2 (Bracken et al. 2008) as well. Several studies confirmed the ZEB/miR-200 double-negative feedback loop (Brabletz & Brabletz 2010). In addition to EMT, it has been shown that the miR-

200/ZEB2 axis can induce pluripotent stem cell generation (G. Wang et al. 2013). An essential mediator of ZEB expression is the TGF- β signaling pathway, indicating that they are essential intracellular mediators of TGF- β -induced EMT (Aigner et al. 2007). ZEB2 collaborates with the TGF- β signaling pathway by interacting with SMAD factors, and induces tumor cell invasion (Comijn et al. 2001).

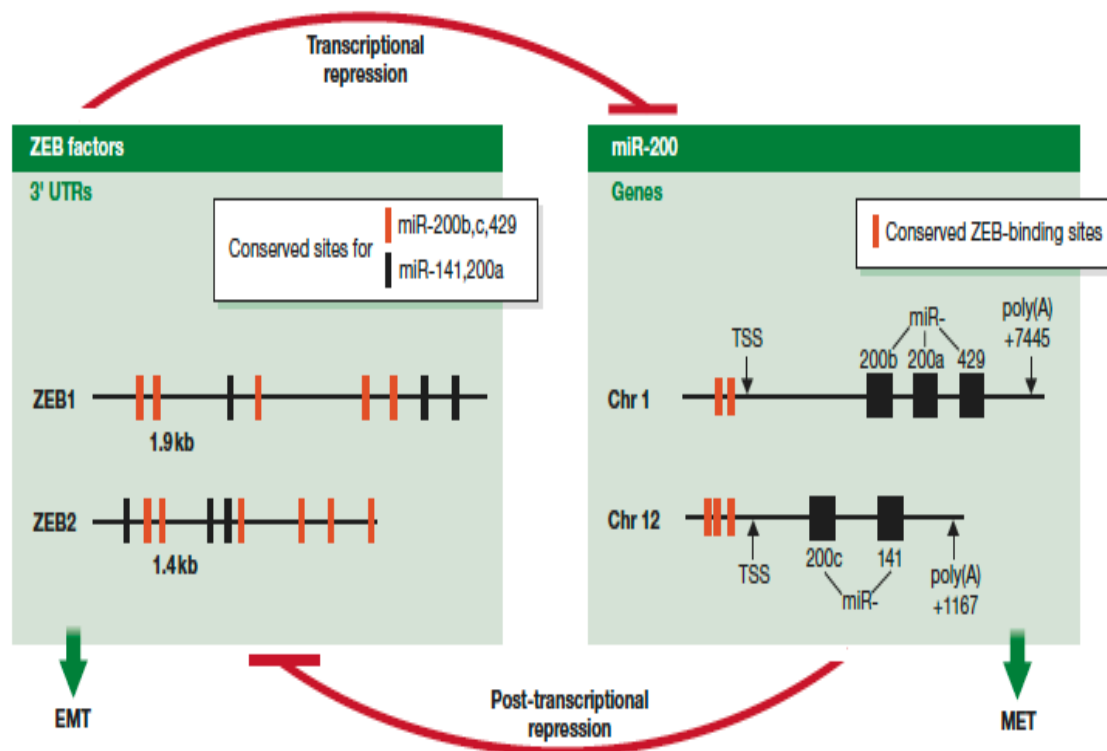


Figure 2-1: ZEB transcription factors and miR-200 family members reciprocally control the expression of each other. ZEB transcription factors repress the gene expression of all the miR-200 family members at the transcription level by binding to highly conserved recognition sequences in their promoters. miR-200 family members inhibit expression of ZEB post-transcriptionally by binding to target sites in their 3' UTRs. TSS, transcriptional start site; UTR, untranslated region (Brabletz & Brabletz 2010, used with the journal permission).

Others have shown that miR-200 is down-regulated in a mouse model of fibrotic lung disease and human patients with idiopathic pulmonary fibrosis (IPF.) They found that the onset of pulmonary fibrosis was associated with reduced miR-200b expression (Yang et al. 2012). Also, miR-200b can inhibit the migration and invasion of non-small cell lung cancer cells (Xiao et al. 2016). Using human fetal lung cultures, it has been shown that miR-200 family members regulate epithelial type II cell differentiation and function (Benlhabib et al. 2015). They found that miR-200 family inhibitors down-regulated pro-SP-C and SP-B expression.

Our own and other groups' findings summarized above led us to the derivation of a general hypothesis under which the studies in this thesis were carried out.

2.2. General Hypothesis

miR-200b is a key determinant of normal lung development that becomes altered in abnormal lung development associated with (CDH) via its regulation of EMT and TGF- β signaling.

To test the above hypothesis, the following objectives have been generated:

2.3. General Objectives:

- 1. To explore the abundance of miR-200b and its targets, their expression patterns and relation to TGF- β /SMAD signalling in lung cells, tissues and fluids from human CDH patients.**
- 2. To investigate the role of miR-200b in normal murine lung development.**
- 3. To explore prenatal miR-200b therapy for CDH in the nitrofen rat model of lung hypoplasia and CDH.**

The studies carried out to achieve the above-mentioned objective are described in Chapter 3 (Manuscript I), Chapter 4 (Manuscript II) and Chapter 5 (Manuscript III).

Manuscript I outlines most of the work we carried out on human cells, human CDH tissues and fluids. It has three main aims which are as follows:

1. *To evaluate whether severe human hypoplastic CDH lungs display specific microRNA expression.*

To achieve this aim, we used a microarray to assess the microRNA profile in human CDH lungs. As we discussed in the study rationale section, we identified two microRNAs miR-200b and miR-10a which are over-expressed in prenatal human CDH lungs. This finding provided the main rationale for the rest of this Ph.D. research project.

2. *To determine if the obtained microRNA signature could be used as a biomarker for the hypoplastic lung and/or CDH*

We performed RT-qPCR to assess the abundance of miR-200 family members and miR-10a in tracheal and amniotic fluids of fetuses with severe CDH who underwent FETO.

3. *To determine how abnormal miR-200b expression influences TGF- β / SMAD signaling and its target gene expression.*

We compared the expression of miR-200b and its known target, TGF- β 2, expression using semi-quantitative *in situ* hybridization and immunohistochemistry, respectively, 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.

Manuscript II outlines the role of miR-200b in normal murine lung development by generating miR-200b knockout mice. The aims of this manuscript are as follows:

1. *To generate miR-200b knockout mice by replacing the complete miR-200b gene with a LacZ-reporter using targeted homologous recombination.*
2. *To evaluate the expression pattern of miR-200b during lung development.*
3. *To assess the phenotype and function of lungs in miR-200^{-/-} mice.*
4. *To define target genes of miR-200b in lung tissues using miR-200b knockout mice.*

In collaboration with Dr. Geoff Hick's lab, we generated *miR-200b^{-/-}* mice. We used lacZ staining to track the promotor activity and expression pattern of the *miR-200b* gene. We used lung function studies and capillary surfactometry, *in vivo* high-resolution micro-CT scanning, Hematoxylin and eosin (H&E) staining, immunofluorescent staining and different quantitative measurements to evaluate the function and phenotype of *miR-200b^{-/-}* lungs. We performed Next Generation Sequencing on *miR-200b^{-/-}* lungs to define the changes in the transcriptome.

Manuscript III outlines the role of miR-200b in abnormal lung development in the nitrofen rat model of lung hypoplasia and CDH and evaluates prenatal miR-200b therapy for CDH in this animal model. The aims of this manuscript are as follows:

1. *To profile miR-200b expression during different stage of nitrofen-induced pulmonary hypoplasia.*

2. *To investigate the effects of nitrofen on TGF- β signaling and downstream miR-200b targets in vitro.*
3. *To evaluate miR-200b as a lung growth promoting prenatal therapy in vitro and in vivo.*

We used *in situ* hybridization, RT-qPCR and immunohistochemistry and Western blotting to evaluate the expression of miR-200b and its targets during lung development. We used a luciferase assay and Western blotting to assess the effect of nitrofen and miR-200b on TGF- β /SMAD signaling. We performed lung explants culture and *in vivo* prenatal transplacental therapy using miR-200b mimics to evaluate the potential therapeutic characteristics of miR-200b.

CHAPTER 3. Manuscript I

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

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This collaborative work was published in Annals of Surgery (2015; 262:1130–1140). Copyright held by Journal of Annals of Surgery, used with permission,

(https://journals.lww.com/annalsofsurgery/Abstract/2015/12000/Unique_Tracheal_Fluid_MicroRNA_Signature_Predicts.34.aspx)

3.1. Abstract

Objective and Summary Background Data: Our objective was to determine the *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 RT-qPCR 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- β 2 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 displayed an increased expression of two microRNAs: miR-200b and miR-10a as compared to control lungs. Fetuses undergoing FETO displayed increased miR-200 expression in their tracheal fluid at the time of balloon removal. Future survivors of FETO displayed significantly higher miR-200 expression than those with a limited response. miR-200b was expressed in bronchial epithelial cells and vascular endothelial cells. TGF- β 2 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 following FETO is associated with increased miR-200 family expression. miR-200b overexpression in CDH lungs results in decreased TGF- β /SMAD signaling.

3.2. Introduction

Congenital diaphragmatic hernia (CDH) is a developmental defect of the diaphragm, which allowing herniation of abdominal viscera into the chest. It occurs in 1 in 2,000 to 3,000 live births(Langham Jr. et al. 1996). 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 over expected 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) (Jani et al. 2009) 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),(Flageole et al. 1998) which also clinically seems to improve survival and morbidity.(Doné et al. 2013; Deprest et al. 2011) These preliminary observations are now being evaluated in a randomized control trial [<http://www.totaltrial.eu> NIH NCT00763737 (moderate hypoplasia) and NIH NCT01240057 (severe hypoplasia)] (DeKoninck et al. 2011).

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 30% of CDH patients have chromosomal anomalies, a common genetic cause for CDH is unknown (Veenma et al. 2012). However, it is widely accepted that the diaphragmatic defect and pulmonary hypoplasia result from a shared developmental insult (Keijzer et al. 2000; Allan & Greer 1997). MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression through post-transcriptional silencing of messenger RNAs.(Lachman et al. 1998). MicroRNAs are essential for normal organogenesis during embryonic development. For example, targeted deletion of miR-1-2

leads to congenital heart defects in mice (Zhao et al. 2007). Previous studies have identified differential miRNA expression between various stages of lung development, but these studies did not provide much functional information (Mujahid et al. 2013). 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 a characteristic microRNA profile. 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 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 post-FETO from non-survivors. We also demonstrate that increased miR-200b expression results in decreased target gene expression via decreased TGF- β /SMAD signaling.

3.3. Results

miR-200b and miR-10a expression are increased in fetal hypoplastic CDH lungs.

The expression profile of the 319 human miRNAs was tested using a microarray. Two miRNAs, miR-200b and miR-10a, were more abundant in prenatal hypoplastic CDH lungs as compared to age-matched normal control lungs ($P < 0.01$) (Fig. 3-1A). Both miR-200b and miR-10a had an approximately three-fold greater expression in CDH lungs as compared to age-matched control lungs (Fig. 3-1B). Four other miRNAs - miR-27a, miR-195, let-7a and miR-1 - were tested based on near significant differences in the microarray analysis. None of these were statistically significantly different in expression between CDH lungs and control lungs using RT-qPCR (results not shown).

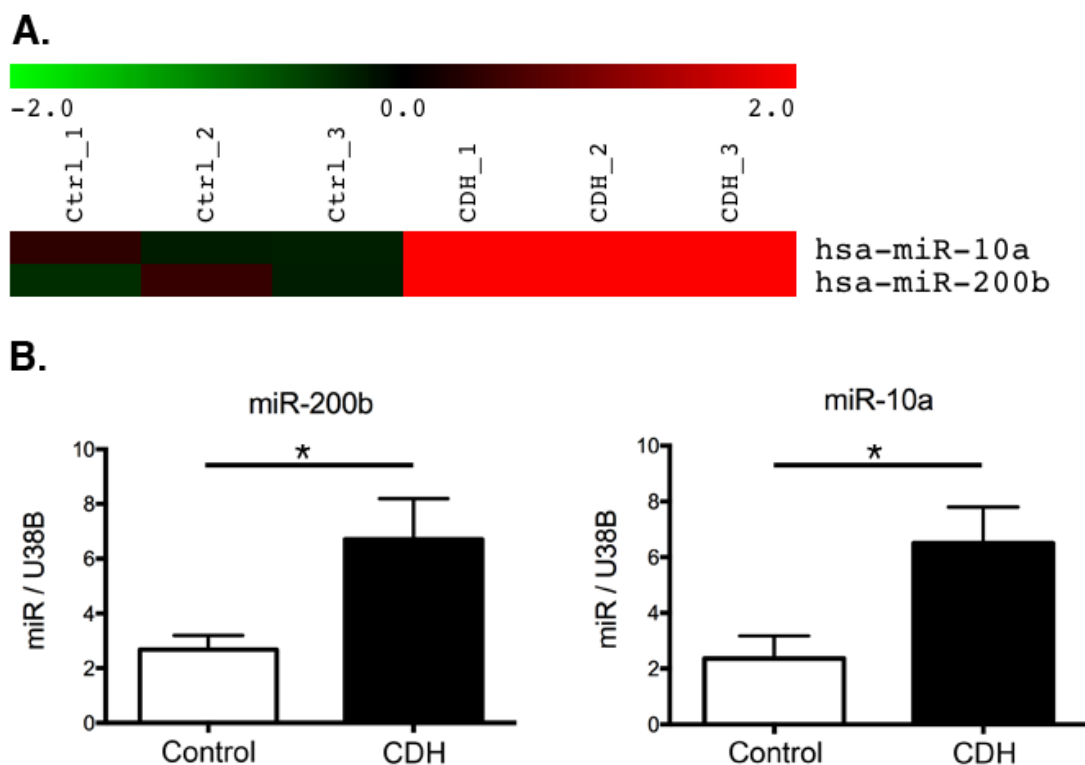


Figure 3-1: Upregulation of miR-200b and miR-10a in prenatal lungs in congenital diaphragmatic hernia (CDH). Total RNA was extracted from 3 CDH cases and 3 age-matched controls (Ctrl). (A) Heat map representation of normalized microarray data (log 2 scale). Of the

*319 miRNAs tested, miR-200b and miR-10a were overexpressed in hypoplastic lungs ($P<0.01$). (B) Real-time quantitative PCR confirmed overexpression of miR-200b and miR-10a in CDH ($*P<0.05$). miRNA expression is normalized relative to SNORD38B (U38B).*

Tracheal fluid: responders have higher expression of miR-200 than non-responders after FETO.

Based on 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 (i.e. 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 non-responders (Fig. 3-2A-E). However, their baseline expression was not different. Conversely, tracheal fluid miR-10a expression was lower in responders compared to non-responders at baseline (plug). Following FETO (unplug), the expression of miR-10a increased in responders, but remained unchanged in non-responders (Fig. 3-2F).

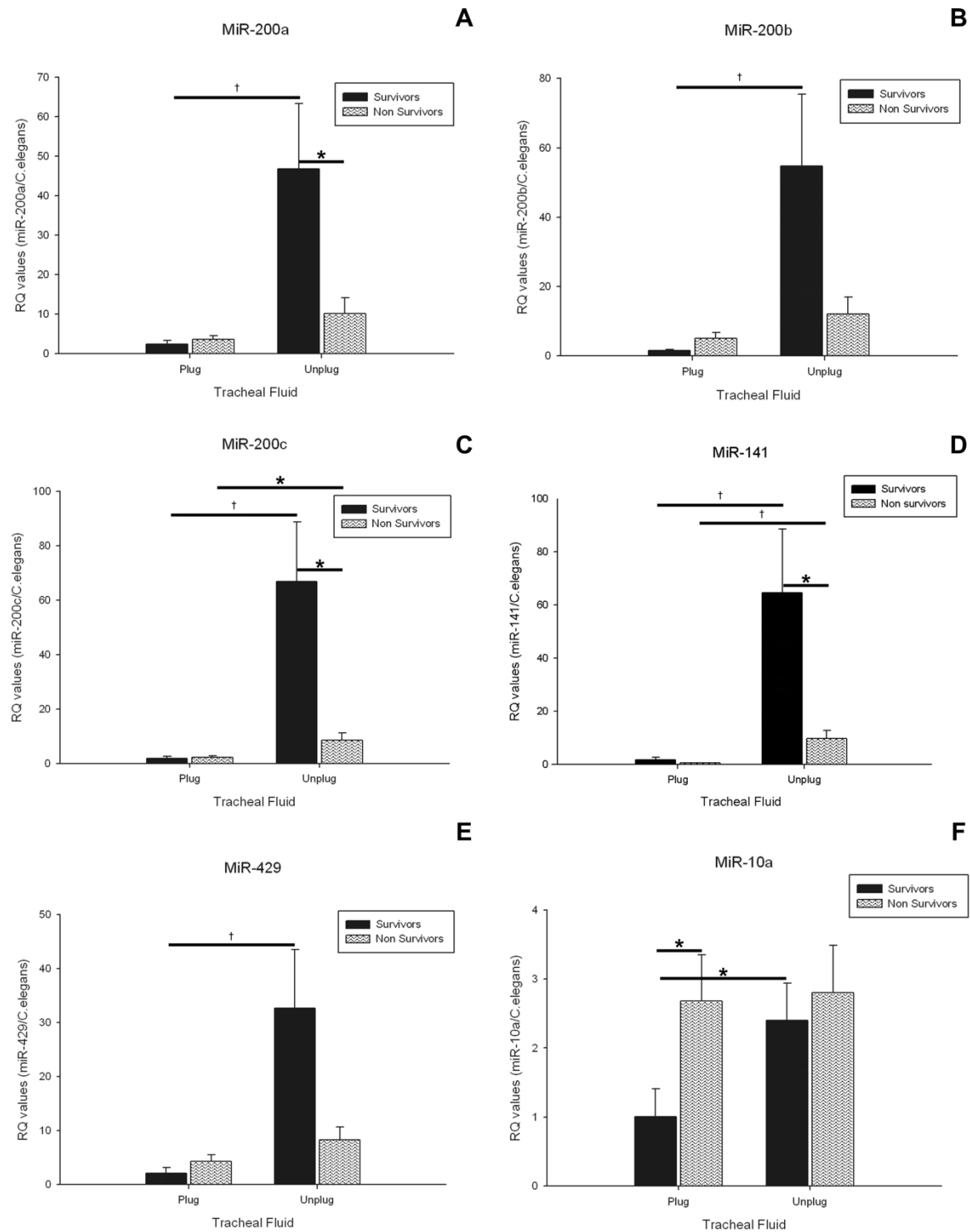


Figure 3-2: MiR-200 family members and miR-10a expression analysis in tracheal fluid samples of surviving and non-surviving patients with CDH undergoing FETO. Expression levels of miR-200a (A), miR-200b (B), miR-200c (C), miR-141 (D), miR-429 (E), and miR-10a (F) in tracheal fluid samples. Expression 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, * $P < 0.05$; † $P < 0.01$.*

Expression of miR-200 family and miR-10a in amniotic fluid decreases after FETO.

Following 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. Since the lungs contribute largely to the amniotic content in the third trimester and were occluded during FETO, this was anticipated (Evrard et al. 1997). The expression profiles of all investigated microRNAs exhibited a similar trend: FETO responders had significantly higher expression than non-responders (Fig. 3-3). More specifically, expression of miR-200a and miR-200c significantly decreased after FETO (unplug) (Fig. 3-3A,C). Expression of miR-200a, miR-200c, miR-141 and miR-10a was higher in amniotic fluid of survivors compared to non-survivors after FETO (unplug) (Fig. 3-3A-F).

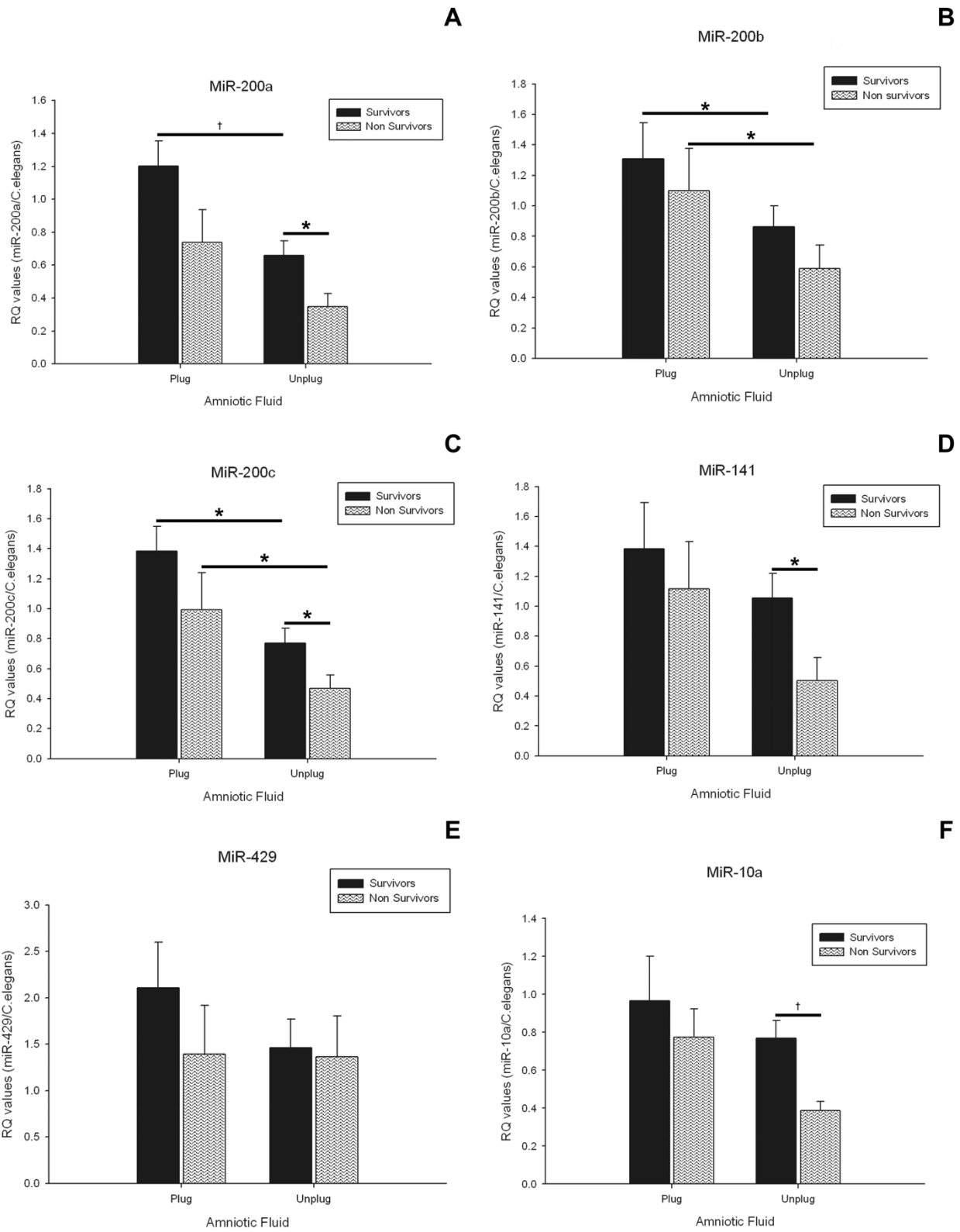


Figure 3-3: MiR-200 family members and miR-10a expression analysis in amniotic fluid samples of surviving and non-surviving patients with CDH undergoing FETO. Amniotic fluid

*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, , * $P < 0.05$; † $P < 0.01$.*

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 non-responders (161.7 (IQR 252.4-140.8) vs. 29.8 (IQR 10.4-50.2); $p < 0.0001$) (Table 3-1). Vascular reactivity could be assessed in 4/10 poor responders and 6/11 good responders. In fetuses with poor response after FETO, we did not observe a change in median pulsatility index (deltaPI) (-2.0% (IQR -10.3-31.0) 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 -1.3-11.9) vs. 23.0% (IQR 3.4-39.8); $p = 0.03$). DeltaPI is the difference in pulsatility index (PI) of the fetal pulmonary artery, before and after exposure to oxygen administration to the mother.

Table 3-1: Demographic information for CDH fetuses undergoing FETO

Base-line characteristics of the embryos			
Characteristics	Responders (n=11)	Non responders (n=10)	P Value
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	7 (m)/4 (f)	4 (m)/5 (f)	0.65
FETO pregnancy data and neonatal outcome			
FETO data/outcome	Responders (n=11)	Non responders (n=10)	P value
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 <24h	1 (9%)	0 (0%)	1.00
PPROM	5 (45%)	3 (33%)	0.67
Gestational Age at	35.0 (35.9-32.6)	35.4 (36.6-29.3)	1.00

PPROM (wk)			
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%)	n.a.	
NICU days	45 (61-30)	n.a.	
Day of neonatal death	n.a.	1 (2-0)	

All median (IQR) or n (%); n.a. not applicable

Expression of miR-200b is higher in the distal part of postnatal hypoplastic lungs.

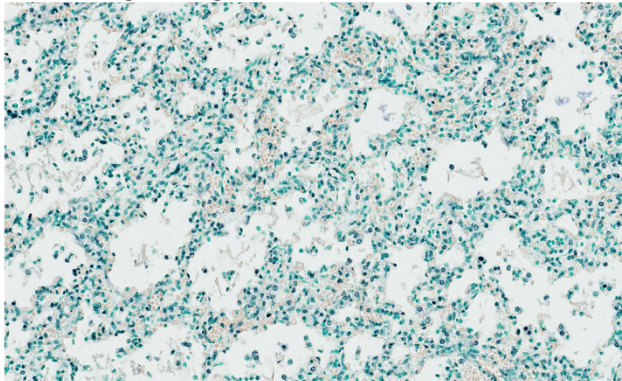
We performed *in situ* hybridization to determine miR-200b expression in a separate set of postnatal lung sections. Hypoplastic CDH lungs were characterized by increased miR-200b expression, particularly in the terminal saccules and alveoli (Fig. 3-4A). We used image analysis software to generate color-coded 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. 3-4B).

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. 3-4C, thick black arrow). In contrast, parabronchial smooth muscle cells were predominantly negative for miR-200b (Fig. 3-4C, 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. 3-4C, black arrowhead). Perivascular smooth muscle cells, on the other hand, were negative (Fig. 3-4C, white arrowhead). Mesothelial cells of the pleura

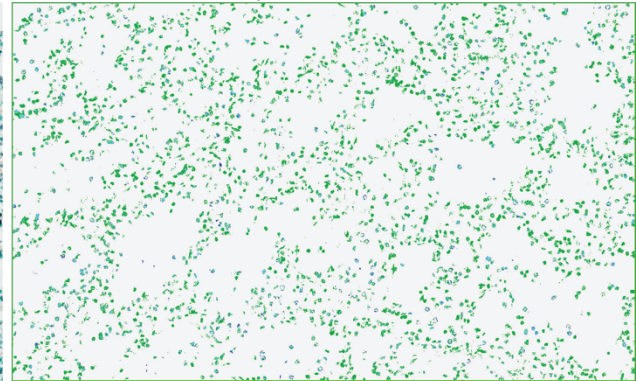
were positive for miR-200b (results not shown).

A

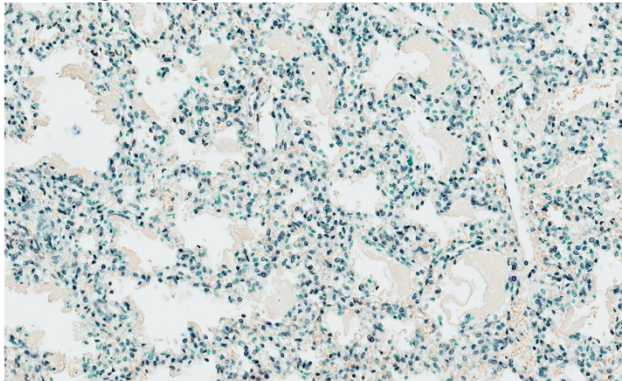
Control - Original Image



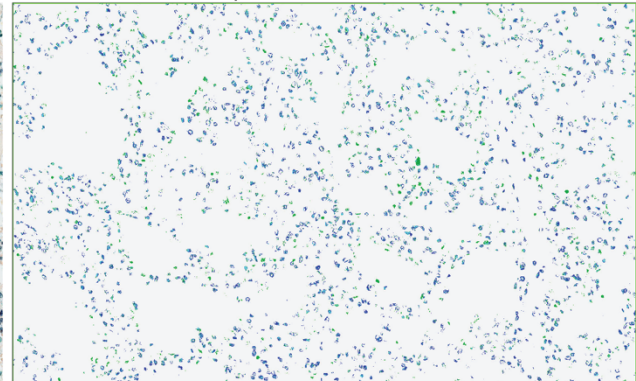
Control - Color-coded Map



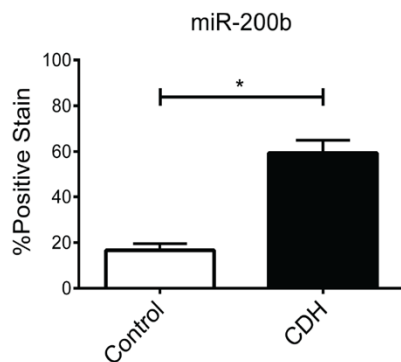
CDH - Original Image



CDH - Color-coded Map



B



C

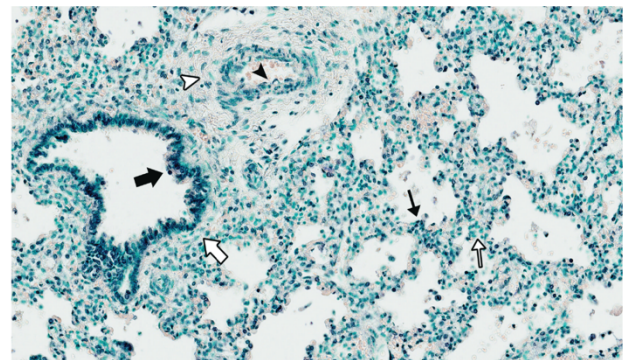


Figure 3-4: In situ hybridization for miR-200b (blue stain) in postnatal lung (methyl green counterstain, 200X magnification). (A) Terminal saccules of control vs. CDH lungs (color-coded maps: blue = positive staining, green = negative staining, aqua = colocalized positive and negative staining). (B) 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.

TGF- β 2, 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 (Burk et al. 2008). We used immunohistochemistry to assess TGF- β 2 expression in postnatal lung tissues. The cellular distribution of TGF- β 2 was very similar to miR-200b, i.e. expressed in bronchial epithelial cells and vascular endothelial cells, but absent from parabronchial and perivascular smooth muscle cells. CDH lungs displayed decreased TGF- β 2 expression in terminal saccules compared to age-matched control lungs (Fig. 3-5A).

MiR-200b down regulates 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 (J. Xu et al. 2009). 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 other hand, addition of inhibitors of miR-200b increased SMAD-luciferase activity by several orders of magnitude (Fig. 3-5B). Collectively, these data suggest that basal TGF- β -induced signaling is tempered by steady state levels of miR-200b, and that reducing miR-200b is

permissive for TGF- β -induced signaling.

In order 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. 3-5C). 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 (J. Xu et al. 2009). 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. 3-5C).

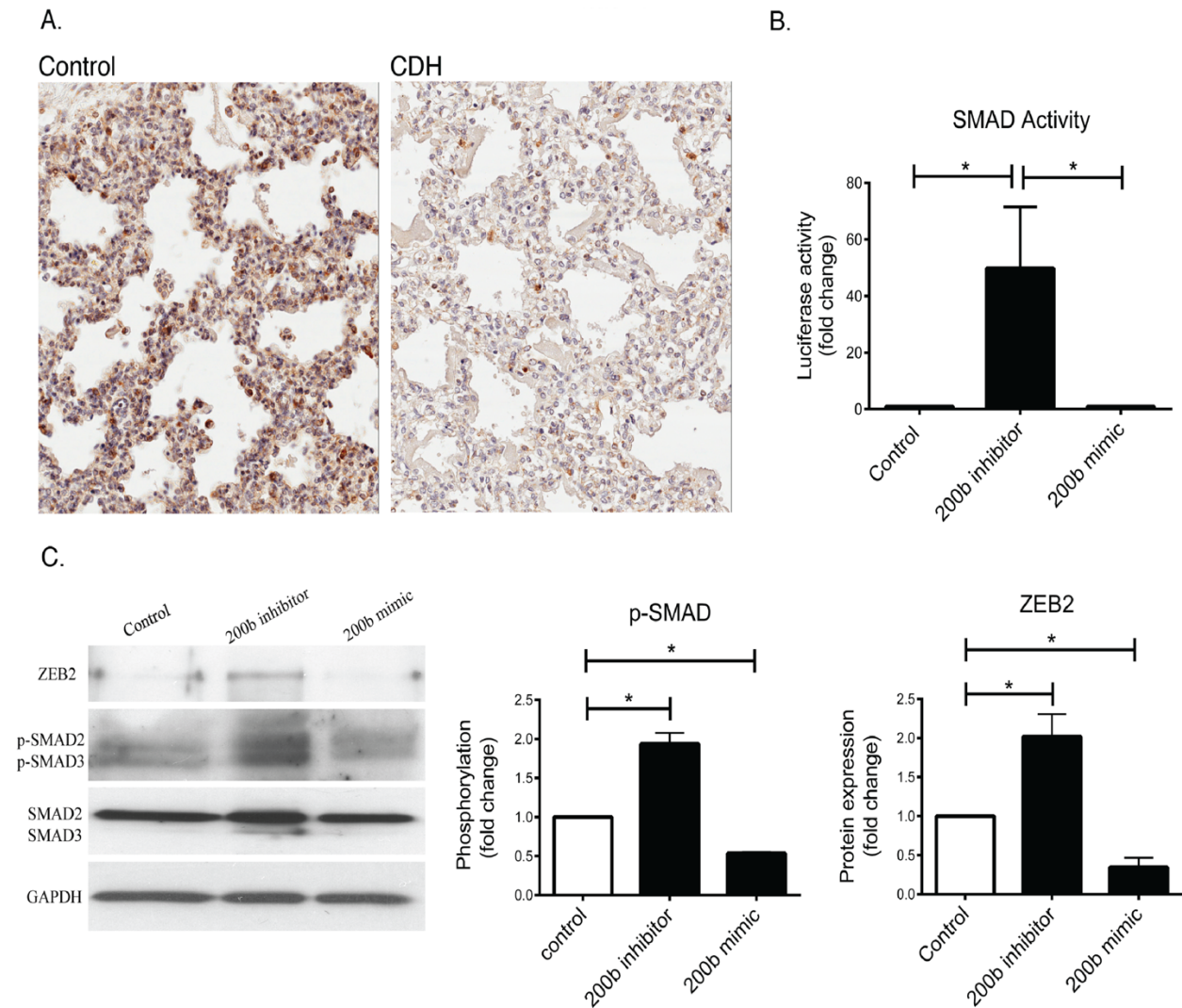


Figure 3-5: MiR-200b Target Gene Expression. (A) Immunohistochemistry for TGF- β 2 (brown stain) in control vs. CDH postnatal lungs (hematoxylin counterstain, 200X magnification). (B) SMAD-luciferase reporter assay in cultures of bronchial epithelial cells. Luciferase activity was measured after a 48-hour 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. (* $P < 0.05$)

3.4. Discussion

The lack of success in identifying a purely 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 overexpression of miR-200b and miR-10a. We then selected tracheal and amniotic fluid samples from fetuses with appropriate or insufficient lung growth following fetal surgery. Responders could be discriminated from non-responders by a significantly higher miR-200 expression in their tracheal fluid, suggesting that stimulated lung development is associated with increased miR-200 expression. Upregulation of miR-200b persists in terminal saccules of CDH patients and is associated with decreased TGF- β 2 expression. *In vitro*, miR-200b inhibits TGF- β /SMAD signaling in bronchial epithelial cells.

Using *in situ* hybridization, we determined that miR-200b is over-expressed 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 (Christoffersen et al. 2007; Philip A. Gregory et al. 2008; Hurteau et al. 2007; Korpai, Esther S Lee, et al. 2008; Sun Mi Park et al. 2008; Burk et al. 2008). These miRNAs have similar sequences and are transcribed in two clusters: miR-200b, miR-200a and miR-429 share a common transcription start site on chromosome 1; while miR-200c and miR-141 are transcribed as a single unit from chromosome 12. There is increasing evidence that 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 (Philip A. Gregory et al. 2008). 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 (Van Loenhout et al. 2012).

The miR-200 family inhibits several genes involved in the TGF- β /SMAD signaling pathway (Gregory et al. 2011; Burk et al. 2008). Increased TGF- β activity enhances miR-200 expression as part of a negative feedback loop (Kato et al. 2011). Others have previously demonstrated in the surgical sheep model of CDH that FETO increased TGF- β 2 expression in hypoplastic lungs (Quinn et al. 1999). In addition, increased TGF- β expression has been observed in hypoplastic lungs in the nitrofen rat model of CDH (Teramoto et al. 2005; Yamataka & Puri 1997). 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 (Greer et al. 2000; Babiuk et al. 2003). Due to the lack 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- β 2 expression, particularly in the terminal saccules. During fetal lung development, endogenous TGF- β blocks hormone-induced type II epithelial cell differentiation (McDevitt et al. 2006). Decreased TGF- β activity in late stages of lung development might explain why human cases of CDH display normal surfactant maturation (Janssen et al. 2003; Boucherat et al. 2007). In addition, exogenous surfactant has been demonstrated to be of no benefit in the treatment of pulmonary

hypoplasia associated with CDH (Van Meurs 2004; Lally et al. 2004).

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 (Pechkovsky et al. 2010; Bragg et al. 2001; Chen et al. 2008). 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 since 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 (Toiyama et al. 2014) and gastric cancer (Valladares-Ayerbes et al. 2012). In contrast, decreased miR-200a expression is associated with poor prognosis and recurrence in ovarian cancer (Hu et al. 2009). 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 (Ohuchida et al. 2012). Furthermore, a combination of miR-10a and miR-200b has recently been reported to be a valuable microRNA signature for metastatic medullary thyroid carcinoma (Santarpia et al. 2013) and bladder cancer (Köhler et al. 2013). We have shown that tracheal fluid expression of miR-200 and miR-10a can serve as a marker of response to FETO.

While 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 (Gallot et al. 2007) and therefore, our three 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 one 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.

In conclusion, 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.

3.5. Material and Methods

MicroRNA screen of lung specimens RNA Isolation

ErasmusMC-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 three fetuses with isolated and severe CDH and three 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, USA).

MicroRNA Profiling

The expression profile of 319 human miRNAs was investigated using a liquid-phase bead-based array according to the manufacturer's instructions. 5µg of total RNA was biotinylated at the 3' end using the FlexmiR MicroRNA labeling kit (Luminex, Austin, TX, USA). The labeled RNA was hybridized to locked nucleic acid (LNA) capture probes. Each probe is bound to a fluorescently dyed xMAP bead. Following washes, the biotinylated miRNAs were detected by reaction with streptavidin-phycoerythrin (SAPE). 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.

Quantitative reverse transcription PCR (RT-qPCR) validation of microRNA profile

We used the miRCURY LNA PCR system according to the manufacturer's instructions (Exiqon, Vedbaek, Denmark). Locked nucleic acid (LNA) primer sets were purchased from Exiqon (Table 3-2). Following reverse transcribing of the isolated RNAs, we used the SYBR Green assay (Bio-Rad, Hercules, CA, USA) for RT-qPCR amplification. We included three 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.

Table 3-2: LNA primer sets (Exiqon) used in real-time quantitative polymerase chain reaction (RT-qPCR)

LNA primer set	microRNA Target Sequence	Description
SNORD38B	TCTCAGTGATGAAAACCTTTGTCCAGTTCTGCTA CTGACAGTAAGTGAAGATAAAGTGTGTCTGAG GAGA	endogenous control
5S rRNA (hsa)	GTCTACGGCCATACCACCCTGAACGCGCCCGA TCTCGTCTGATCTCGGAAGCTAAGCAGGGTCG GGCCTGGTTAGTACTTGGATGGGAGACCGCCT GGGAATACCGGGTGCTGTAGGCTTT	endogenous control
U6 snRNA (hsa, mmu)	GTGCTCGCTTCGGCAGCACATATACTAAAATT GGAACGATACAGAGAAGATTAGCATGGCCCC TGC GCAAGGATGACACGCAAATTCGTGAAGC GTTCCATATTTTT	endogenous control
hsa-miR-10a	UACCCUGUAGA UCCGAAUUUGUG	tested microRNA

hsa-miR-27a	UUCACAGUGGCUAAGUCCGC	tested microRNA
hsa-miR-195	CCAAUUAUUGGCUGUGCUGCUCC	tested microRNA
hsa-let-7a	CUAUACAAUCUACUGUCUUUC	tested microRNA
hsa-miR-1	UGGAAUGUAAAGAAGUAUGUAU	tested microRNA
hsa-miR-200b	UAAUACUGCCUGGUAAGAUGA	tested microRNA

Clinical evaluation of fetuses with CDH

Ultrasound evaluation was performed <48hrs before FETO plug (further referred to as baseline, typically around 26-28 weeks) and <48hrs before balloon removal (typically around 34 weeks). The degree of pulmonary hypoplasia was estimated using 2D ultrasound and was expressed as a lung-to-head ratio (LHR) as described before (Metkus et al. 1996; Jani et al. 2007). 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: $((\text{O/E LHR}_{\text{unplug}} - \text{O/E baseline}) / \text{O/E baseline}) \times 100 \%$. 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 ΔPI : $((\text{baseline PI} - \text{PI}_{\text{O}_2}) / \text{baseline PI}) \times 100 \%$. (Done et al. 2011; Dekoninck et al. 2012).

RNA Isolation of biofluid samples

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 “non-responders” (i.e. poor or absent increase in lung size and eventually neonatal death)(Cannie et al. 2009) (Table 3-1).

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, 1µL of synthetic *Caenorhabditis elegans* miRNA (cel-miR-39) was added to each denatured sample.(Argyropoulos et al. 2013; Mitchell et al. 2008) Small RNAs were then enriched and purified according to the manufacturer’s protocol.

Quantitative reverse transcription PCR (RT-qPCR) for biofluid samples

To optimize RT-qPCR performance, we did a dilution curve to determine what input volume affected RT-qPCR performance. Based on 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 3-3.

Table 3-3: MicroRNA target sequences for RT-qPCR and LNA probes (Exiqon) used for in situ hybridization

Target microRNA	Target Sequence		
hsa-miR-200a-3p	UAACACUGUCUGGUAACGAUGU		
hsa-miR-200b-3p	UAAUACUGCCUGGUAAGAUGA		
hsa-miR-200c-3p	UAAUACUGCCGGGUAAGAUGGA		
hsa-miR-141-3p	UAACACUGUCUGGUAAGAUGG		
hsa-miR-429	UAAUACUGUCUGGUAACAACCGU		
hsa-miR-10a-5p	UACCCUGUAGAUCGAAUUUGUG		
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

The average expression levels of amniotic and tracheal fluid miRNAs were normalized against cel-miR-39 (Kroh et al. 2010; Weber et al. 2010; Argyropoulos et al. 2013). 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**

We obtained post-mortem neonatal lung tissues from three postnatal CDH cases and three age-matched controls, without lung disease (35, 37 and 40 weeks of gestation). All cases died within one hour after birth. *In situ* hybridization was carried out on 5 μ m sections of formalin-fixed tissue as previously described (Jørgensen et al. 2010). 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 produced a very weak signal in postnatal lung tissues. MicroRNA species were hybridized with double-digoxigenin-

labeled LNA probes (Exiqon) for 1 hour (Table 3-3). We detected the hybridized probes with an alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody (1:500) (Roche, Mannheim, Germany). Sections were immunostained with 1-step NBT/BCIP solution, containing 1 mM levamisole (Thermo Scientific, Rockford, IL, USA). The slides were counterstained with methyl green (Sigma-Aldrich, St. Louis, MO, USA).

Microscopy and Image Analysis

Digital microscopy was performed with the ScanScope CS system (Aperio, Vista, CA, USA). Semi-quantitative measurements of the *in situ* hybridization studies were obtained as follows. Images up to 200X 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 staining 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 three 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 three colors. Positive staining was calculated by adding the percentages of blue and aqua (% positive staining).

Immunohistochemistry

We performed immunohistochemistry on neonatal lung tissue using an anti-TGF- β 2 antibody

(1:100) (Abcam, Cambridge, MA, USA) as previously described (Van Loenhout et al. 2012).

Luciferase Assay

We used the Cignal SMAD Reporter kit (SABiosciences, Frederick, MD, USA) to investigate the effects of miR-200b on the TGF- β -induced signal transduction pathway (Leeper et al. 2011). 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, USA), were co-transfected with 0.5 μ g/ml of the SMAD reporter construct and 0.01 μ g/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, USA).

Western Blotting

BEAS-2B cells were transfected with 0.01 μ g/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 μ M β -glycerophosphate, 20 μ M EDTA, 5% SDS, a protease inhibitor cocktail tablet and phosphatase inhibitors (1mM sodium orthovanadate, 2 mM EDTA, 10 mM sodium pyrophosphate, 30 mM sodium chloride). The supernatant protein concentration was determined using RC DCTM Protein Assay (Bio-Rad, Hercules, CA, USA). 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, USA), anti-phospho-SMAD2 (1:750) (Cell Signaling), anti-ZEB2 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 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 $\pm$ SEM or median and IQR where appropriate. Statistical analyses were performed using the statistical software SigmaStat (version 3.5; Systat Software Inc., USA). Statistical comparisons were performed using the unpaired Student's t-test and non-parametric Mann-Whitney-*U* test where appropriate. Paired analysis was performed using a Wilcoxon signed rank test. Differences were considered significant at $P < 0.05$.

3.6. Acknowledgements

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CHAPTER 4. Manuscript II

MicroRNA-200b regulates distal airway development by maintaining epithelial integrity

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(<https://www.nature.com/articles/s41598-017-05412-y>)

4.1. Abstract

miR-200b plays a role in epithelial-to-mesenchymal transition (EMT) in cancer. We recently reported abnormal expression of miR-200b in the context of human pulmonary hypoplasia in congenital diaphragmatic hernia (CDH). Smaller lung size, a lower number of airway generations, and a thicker mesenchyme characterize pulmonary hypoplasia in CDH. The aim of this study was to define the role of miR-200b during lung development. Here we show that miR-200b^{-/-} mice have abnormal lung function due to dysfunctional surfactant, increased fibroblast-like cells and thicker mesenchyme in between the alveolar walls. We profiled the lung transcriptome in miR-200b^{-/-} mice, and, using Gene Ontology analysis, we determined that the most affected biological processes include cell cycle, apoptosis and protein transport. Our results demonstrate that miR-200b regulates distal airway development through maintaining an epithelial cell phenotype. The lung abnormalities observed in miR-200b^{-/-} mice recapitulate lung hypoplasia in CDH.

4.2. Introduction

Every year, over 50,000 children are born with congenital diaphragmatic hernia (CDH) associated with abnormal lung development resulting in lung hypoplasia and persistent pulmonary hypertension(Keijzer & Puri 2010; Kotecha et al. 2012; Greer 2013). CDH occurs as frequently as cystic fibrosis, but its pathogenesis is poorly understood. Smaller lung size, lower number of airway generations and a thicker mesenchyme characterize the abnormal lungs in CDH(Keijzer & Puri 2010). We have previously shown an inherent lung development defect in CDH(Keijzer et al. 2000).

Lung development is a continuous process comprised of five developmental stages: embryonic, pseudo-glandular, canalicular, terminal sacular and alveolar(Cardoso 2000). Functionally, the early two stages are characterized by lung branching morphogenesis. Cell specification, vascularization and reduction of mesenchyme to form thin air-blood interfaces for gas exchange characterize the later stages(Warburton et al. 2000; Kimura & Deutsch 2007).

MicroRNAs (miRNA) are small, non-coding RNAs that regulate gene expression through controlling mRNA stability and translation(Masafumi Inui et al. 2010; Yates et al. 2013; Mohr & Mott 2015). They are essential for development and homeostasis of organs(Schneider 2012; Petri et al. 2014; Vienberg et al. 2017; Bernstein et al. 2003). More than 1800 microRNAs have been identified in human (mirBase 2016). Research focusing on the role of microRNAs in lung development and disease is limited. We recently discovered that miR-200b is elevated in abnormal lungs of human CDH babies. In the same study, we found that higher miR-200b expression in the fetal tracheal fluid of CDH fetus is associated with a better response to fetoscopic endoluminal tracheal occlusion (FETO, a prenatal therapy to promote lung growth) (Pereira-Terra et al. 2015).

MiR-200b belongs to the miR-200 family (miR-141, miR-429, miR-200a, miR-200b and miR-200c) and regulates epithelial-to-mesenchymal transition (EMT) in cancer and organ fibrosis (Junji Kurashige et al. 2012; Chen et al. 2013; Kurashige et al. 2015; Yao et al. 2015). Others have shown that miR-200 is down-regulated in a mouse model of fibrotic lung disease and human patients with idiopathic pulmonary fibrosis (IPF) (Yang et al. 2012). Also, miR-200b can inhibit migration and invasion of non-small cell lung cancer cells (Xiao et al. 2016). The role of miR-200b during normal lung development has yet to be defined.

The goal of this study was to delineate the role of miR-200b during lung development using loss of function models *in vivo*. We generated a miR-200b^{-/-} (KO) mouse to evaluate the functional impact of miR-200b absence on development *in vivo*. miR-200b deficient mice had stiffer lungs due to disturbed distal airway branching, thicker alveolar walls and downregulation of epithelial cell differentiation. Our data suggest that miR-200b is required to achieve the necessary balance in development of lung epithelial cells and fibroblasts to ensure development of a structurally and functionally effective respiratory organ.

4.3. Results

miR-200b is highly expressed during different stages of lung development

We generated miR-200b^{-/-} (KO) mice by replacing the complete miR-200b gene with a LacZ-reporter by targeted homologous recombination in C57Bl/6N mouse embryonic stem cells using the NorCOMM cassette (Fig. 4-1a,b, Supplementary Table. 4-3). MiR-200b^{+/-} mice were intercrossed with the C57Bl/6N mice for at least 8 generations before use in experimental cohort studies. We confirmed complete knockout of miR-200b expression by RT-qPCR in fetal lungs (Fig. 4-2c) and lungs from 8-week old mice (Supplementary Fig.4-13). We showed that mature microRNAs transcribed in the same cluster - miR-200a and miR-429 - were still expressed, albeit lower compared to miR-200b^{+/+} lungs (Fig. 4-2c, Supplementary Fig. 4-13). The expression of miR-200c and miR-141 did not change in the miR-200b^{-/-} lungs compared to miR-200b^{+/+} lungs, suggesting that there were no compensatory effects on other family members (Fig. 4-2c and supplementary Fig. 4-13).

Genotyping data from breeding *miR-200b*^{+/-} x *miR-200b*^{+/-} mice revealed an expected Mendelian distribution, indicating that these mice experience no embryonic lethality, and they were viable, fertile and appeared morphologically normal. In contrast to miR-200b/miR-429^{-/-} mice reported by others (Hasuwa et al. 2013), our *miR-200b*^{-/-} mice carried a lacZ-reporter gene that we exploited to localize miR-200b promoter activity during development (Fig. 4-1c). We observed a high level of expression in the endoderm of lung buds and developing airways during lung development. At E12.5, during branching morphogenesis, we observed lacZ staining mainly in the endodermal cells of the lungs and a faint expression in the mesenchymal cells. Notably, we observed high LacZ-miR-200b expression in other organs where development also hinges on epithelial-mesenchymal interactions and branching morphogenesis. These include the developing

inner ear (Mallo 2001), palate (Yu et al. 2009) and mammary buds (Howard 2012) (Supplementary Fig. 4-11), consistent with prior reports suggesting that miR-200b expression is highly coordinated and associated with control of the development of these organs(Hertzano et al. 2011; Du et al. 2014; Shin et al. 2012).

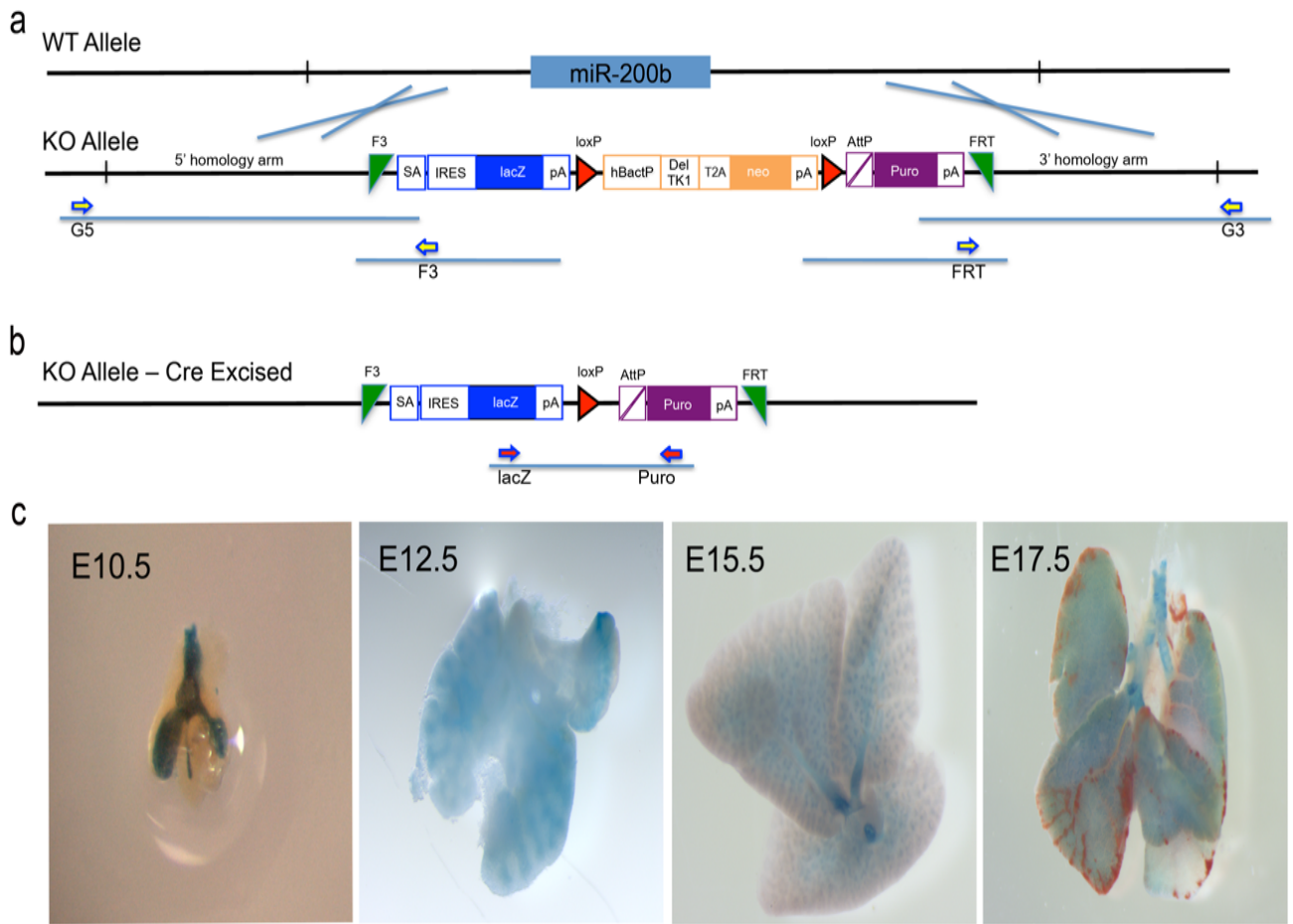


Figure 4-1: Generation and validation of *miR-200b*^{-/-} mice (*miR200b*^{tm1.1(NCOM)MFGC}). (a) Targeted *miR-200b* knockout allele. The complete *miR-200b* non-coding gene (WT allele) was replaced by a NorCOMM cassette via targeted homologous recombination in C57Bl/6N mouse C2 ES cells. The NorCOMM targeting cassette consists of three functional components: a *lacZ* expression reporter (blue boxes); a loxP flanked (red triangles) *hβact* promoter driven Δ TK1-T2A-neomycin selectable marker (orange boxes), which can be subsequently excised by cre-recombinase); and a docking cassette *AttP*-Puro-pA that can be utilized to exchange the entire NorCOMM cassette to any other allele (purple boxes) and once placed, the remaining flanked sequences can be removed by *flpO*-recombinase between *F3* and *FRT* sites (green triangles) in vivo. The length of 5' and 3' homology arms are 2891 bp and 6595 bp in the targeting vector, respectively. The targeted *miR-200b* gene was highlighted in the resulting knockout allele (KO Allele). (b) Targeted *miR-200b* knockout mice. Removal of the *hβact* promoter driven Δ TK1-T2A-neomycin cassette was performed by mating male *miR200b*^{tm1(NCOM)MFGC} mice harboring the *miR-200b* KO allele with female CMV-Cre transgenic mice. Precise cre-excision of the neomycin cassette was determined by PCR and sequence validation, as shown with primers specific to *LacZ* and *Puro* cassette by purple arrows in Panel C. The resultant *miR-200b* cre-excised allele is shown as *miR200b*^{tm1(NCOM)MFGC}. (c) *LacZ* staining of *miR-200b*^{-/-} mice demonstrated the expression in both epithelial and mesenchymal cells during the lung development.

miR-200b absence does not affect proximal airway branching

Based on this high and dynamic expression of miR-200b, we focused first on the role of miR-200b during the early stages of lung development. We cultured mutant lungs from E11.5 mice as described before (Keijzer et al. 2000). Branching patterns between the different lung explants were not affected significantly (Fig. 4-2 a,b). To evaluate if the absence of an effect of miR-200b^{-/-} on proximal lung branching morphogenesis was due to compensatory upregulation of other family members, we assessed the abundance of all family members in fetal lung explants using qPCR (Fig. 4-2 c). We observed that mature microRNAs transcribed in the same cluster - miR-200a and miR-429 - were still expressed, albeit at lower level as compared to miR-200b^{+/+} lungs (wt). The expression of miR-200c and miR-141 did not change in the miR-200b^{-/-} fetal lungs compared to miR-200b^{+/+} lungs, suggesting that there were no upregulation compensatory effects on other family members. Newborn *miR-200b^{+/+}* and *miR-200b^{-/-}* mice did not display any breathing difficulties after birth, and together, these results indicate that proximal airway branching is not influenced by absence of miR-200b during development.

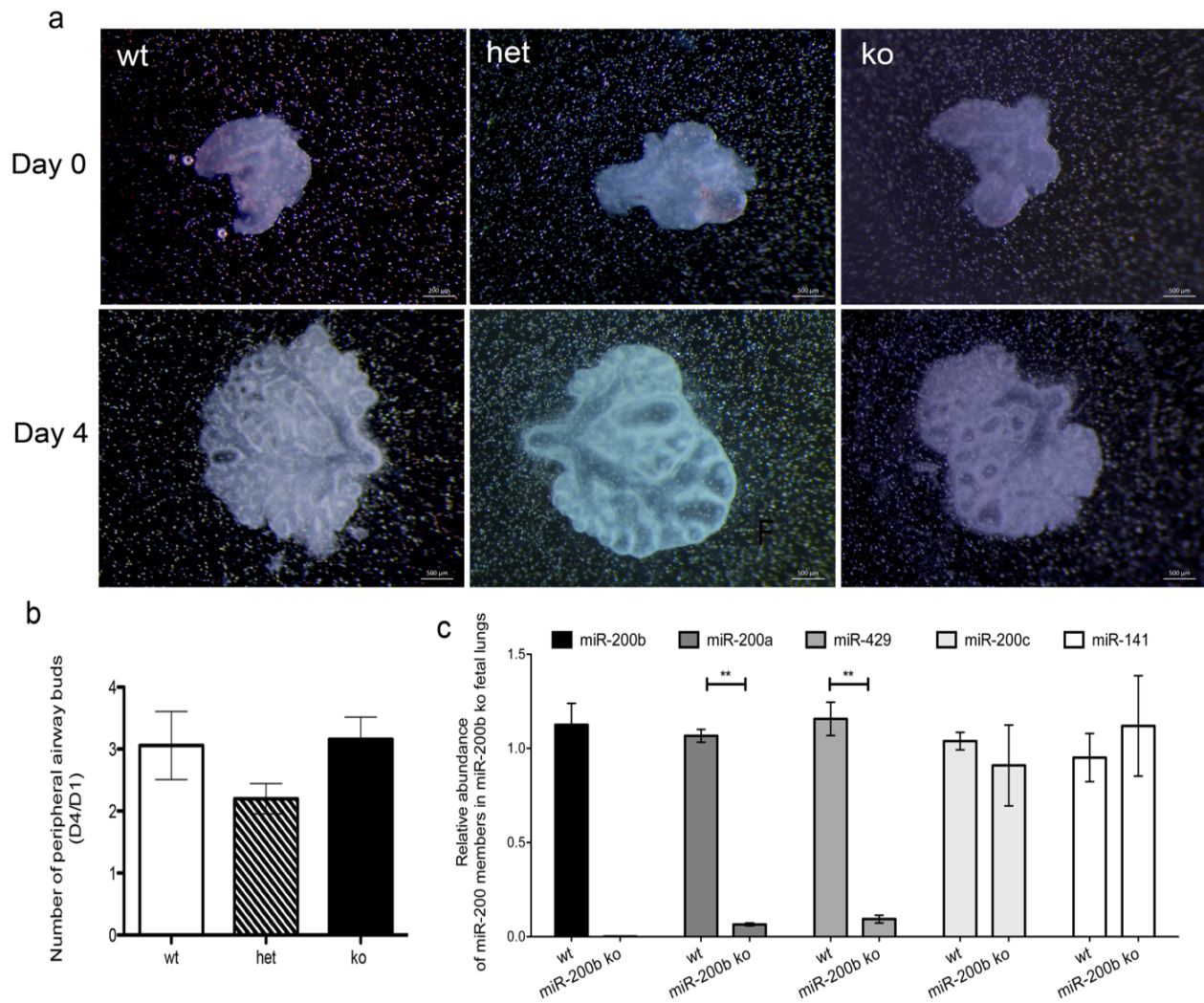


Figure 4-2: MiR-200b absence does not affect proximal airway branching. (a,b) Lung explants culture of E11.5 lung explants did not show significant differences in proximal branching morphogenesis between *miR-200b*^{+/+}, *miR-200b*^{+/-} and *miR-200b*^{-/-} lungs. (c) RT-qPCR for all miR-200 family members on fetal explant lungs mice using LNA primers. *miR-200b* absence was confirmed. *miR-200a* and *miR-429* were significantly downregulated but no changes were observed in abundance of *miR-200c* and *miR-141* ***P* < 0.01, Student's *t*-test, Data represent mean $\pm$ SEM of at least four independent experiments.

***miR-200b*^{-/-} mice have higher lung tissue damping and elastance with lower hysteresivity**

To determine whether lung function is affected in adult *miR-200b*^{-/-} mice, we performed *in vivo* lung mechanics analyses using a *flexiVENT* small animal ventilator in 8-week-old *miR-200b*^{-/-} mice and compared these with studies from *miR-200b*^{+/-} and *miR-200b*^{+/+} mice. Mice were

subjected to increasing doses of nebulized methacholine (MCh) to assess concentration dependent response characteristics of respiratory mechanics. Inhaled methacholine causes constriction of airway smooth muscle cells (bronchoconstriction)(Wagner & Jacoby 1999) At rest, *miR-200b*^{-/-} mice did not exhibit altered peripheral tissue/airway resistance (tissue damping), however after challenge with methacholine (MCh; 6 mg/ml and higher) a substantive increase in tissue damping was revealed (Fig. 4-3a). Consistent with development of a low compliance or “fibrotic” lung, *miR-200b*^{-/-} mice also showed significantly higher tissue elastance upon MCh-challenge (12 mg/ml and higher) (Fig. 4-3b). At baseline, airflow resistance in conducting airways (Newtonian resistance) was not different between different groups of mice, but was significantly higher in *miR-200b*^{-/-} mice challenged with high concentrations of MCh (Fig. 4-3c). Finally, *miR-200b*^{-/-} mice demonstrated significantly lower hysteresivity (elastic hysteresis) in pressure-volume loops obtained before and after MCh challenge, consistent with the increased elastance we observed in these animals (Fig. 4-3d, Supplementary Fig. 4-11). To examine if the absence of miR-200b affect the expression of other family members and therefore contribute to the phenotype we observed in the 8 weeks old *miR-200b*^{-/-} lung, we accessed the abundance of all miR-200b family members in 8 weeks old *miR-200b*^{-/-} lungs. Similar to what we observed in fetal lungs, adult *miR-200b*^{-/-} lungs had lower miR-200ba and miR-429 abundance with no change in miR-200c and miR-141 (Supplementary Fig 4-13). We also observed lower miR-200a and miR-429 in kidney tissues of *miR-200b*^{-/-} mice (Supplementary Fig 4-14).

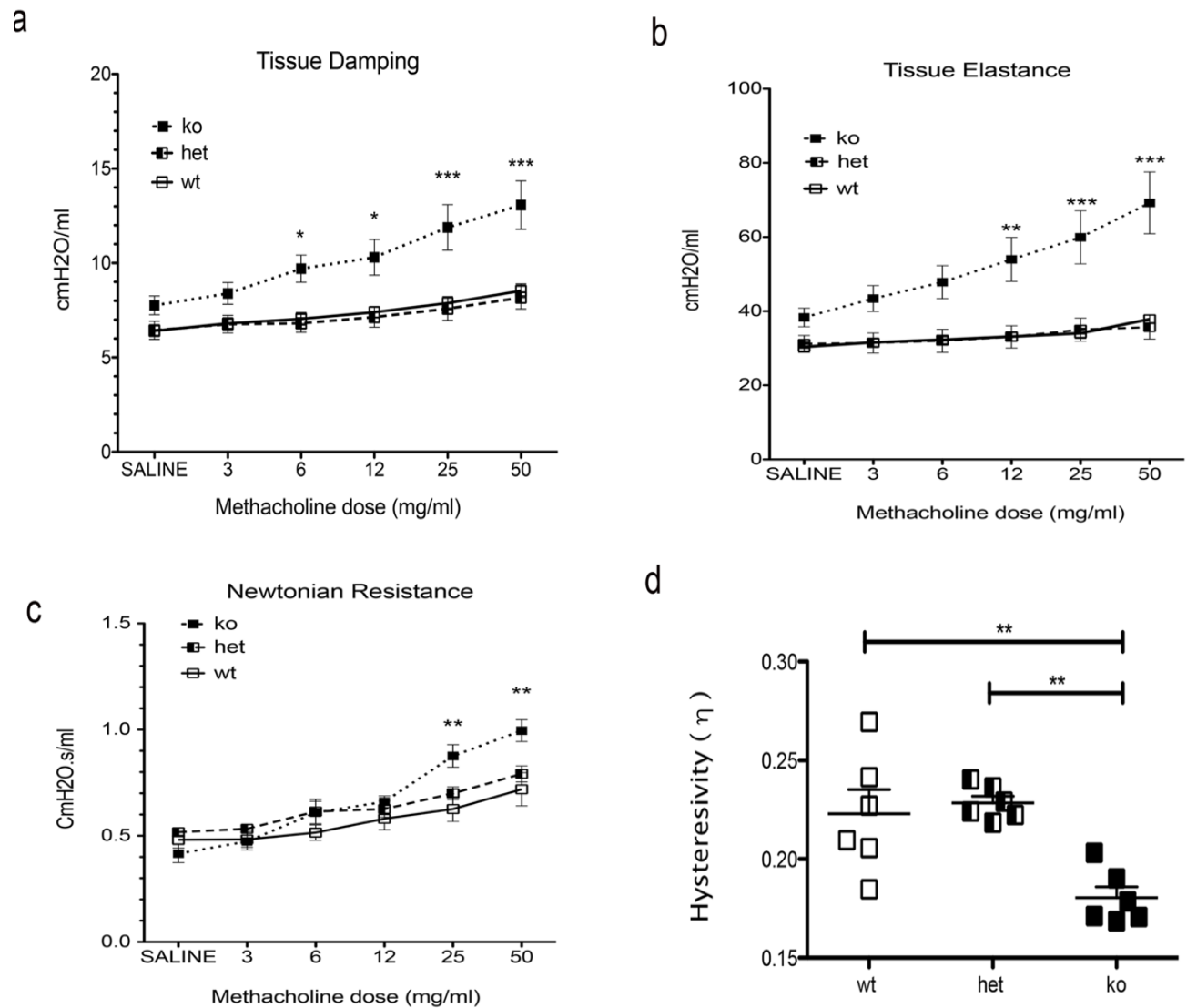


Figure 4-3: MiR-200b^{-/-} lungs have abnormal lung function. (a) Lung mechanics studies performed on 8-week-old mice demonstrated that miR-200b^{-/-} (ko) mice have higher lung tissue resistance (damping) when challenged with 6 mg/ml methacholine or higher (b) miR-200b ko mice have higher lung tissue stiffness (Elastance) at 12, 25 and 50 mg/ml of methacholine (c) and more conducting airway resistance at high concentrations of methacholine. (d) Accessing lung hysteresivity at the time of saline challenge (before methacholine challenge) showed lower hysteresivity in miR-200b ko mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-way ANOVA. Data represent mean $\pm$ SEM of at least six independent experiments.

***MiR-200b*^{-/-} lungs have dysfunctional surfactant and denser parenchyma with more fibroblast- like cells**

Some of the lung function abnormalities in *miR-200*^{-/-} mice could result directly from changes in surfactant function or distal airway branching. Therefore, we assessed the biophysical function of surfactant from *miR-200b*^{-/-} and *miR-200*^{+/+} lungs using capillary surfactometry. We found that the surface tension-reducing capacity of surfactant in *miR-200b*^{-/-} mice was markedly reduced (Fig. 4-4a), a finding that correlated with compromised labeling of pro-Surfactant Protein-C (SP-C) and Surfactant protein-B (SP-B) (Fig. 4-4b-e) in these lung tissues. Our data suggest a functional role for *miR-200b* in development of surfactant properties, resulting in reduced lung compliance in *miR-200b*^{-/-} mice.

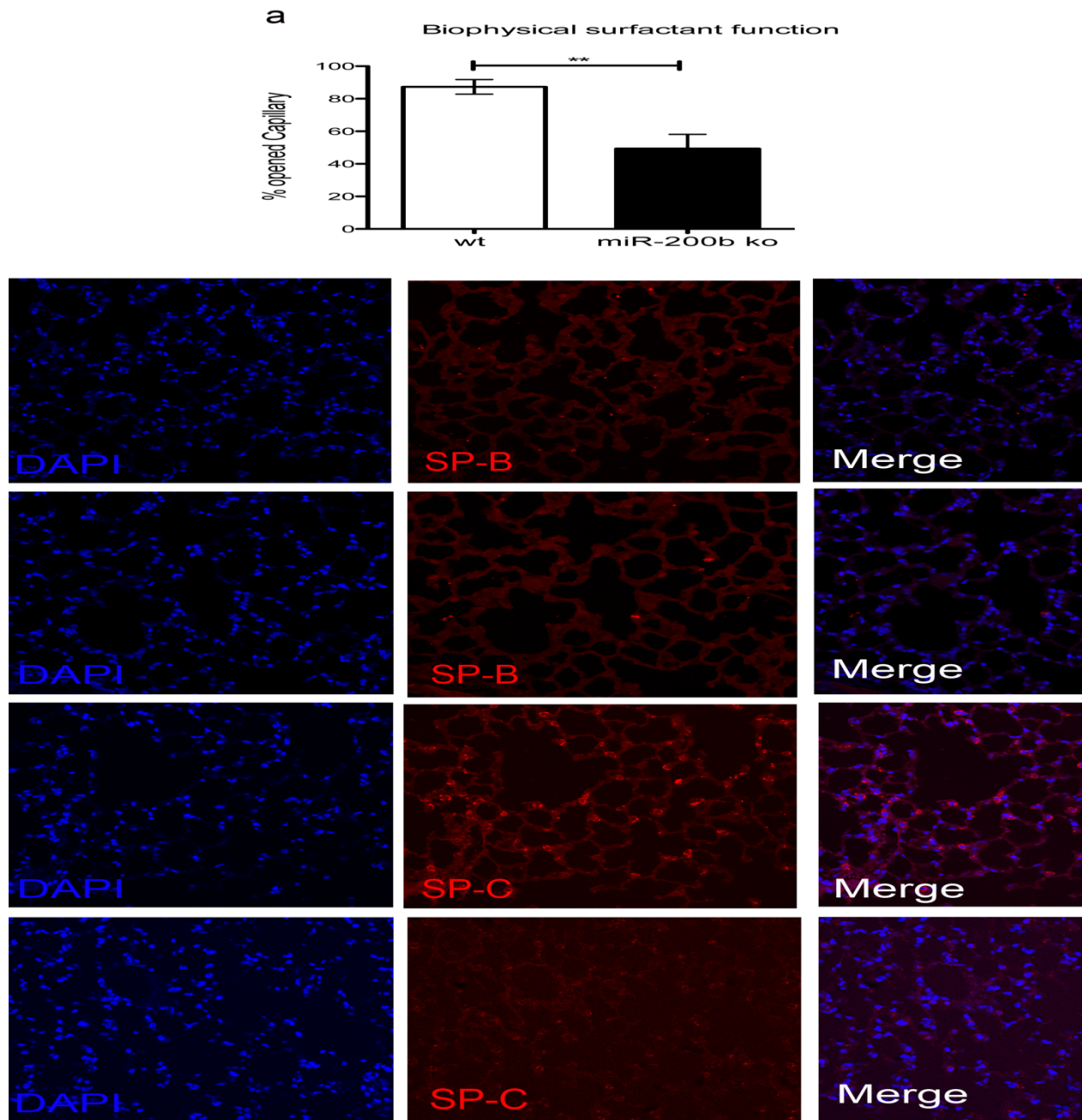


Figure 4-4: MiR-200b^{-/-} lungs have dysfunctional surfactant with lower expression surfactant proteins. (a) Biophysical surfactant function of miR-200b ko lungs (8 weeks old) measured using capillary surfactometry. MiR-200b ^{-/-} mice have decreased surfactant function compared to wt. **P < 0.01, Student's t-test, Data represent mean ± SEM of at least three independent experiments. (b,c) Immunofluorescence of 8 weeks old wt (b) and ko (c) lungs showed decreased abundance of Surfactant Protein-B (SP-B) than wt. (d,e) Immunofluorescence of 8 weeks old wt (d) and ko (e) lungs showed decreased abundance of pro-Surfactant Protein-C (SP-C) than wt.

We evaluated lung morphometry in *miR-200b*^{-/-} mice in a quantitative fashion, using two different, complementary approaches. We first performed *in vivo* high-resolution micro-CT scanning on live animals to eliminate the effects of tissue processing and inflation on the morphometry of the lungs. We made reconstructions of the lungs so that data between mice could be directly compared (i.e. if two images show the same level of grey scale, then they exhibit the same degree of x-ray attenuation). We then set a threshold to segment the data into solid tissue and air (using the same threshold value for all images) and calculated the volume of air in the lungs. To ensure that we used comparable areas of the lungs, we identified the carina in each lung and then moved 3.25 mm above this. We then calculated the air volume from this point to the base of the lungs. Using *in vivo* high-resolution micro-CT scanning, we found that the density of lung parenchyma in *miR-200b*^{-/-} mice is greater (gray area), and by measuring the airspace volume we demonstrated that distal alveoli are less air-filled compared to lungs from wildtype mice (Fig. 4-5a,b). This can result from increased alveolar space collapse or smaller alveolar air volume.

Hematoxylin and eosin (H&E) staining of *miR-200b*^{-/-} lungs that were inflated and embedded in paraffin confirmed that these lungs have fewer septae and thicker alveolar walls when compared to *miR-200*^{+/+} lungs (Fig. 4-5c). We then scanned three complete sections per lung of three *miR-200b*^{+/+} and three *miR-200*^{-/-} lungs using an Axio Scan.Z1 and used the ZEN Image Analysis software module to calculate the airspace area percentage of each lung. We observed lower percentages of airspace in *miR-200*^{-/-} lungs (Fig. 4-5c).

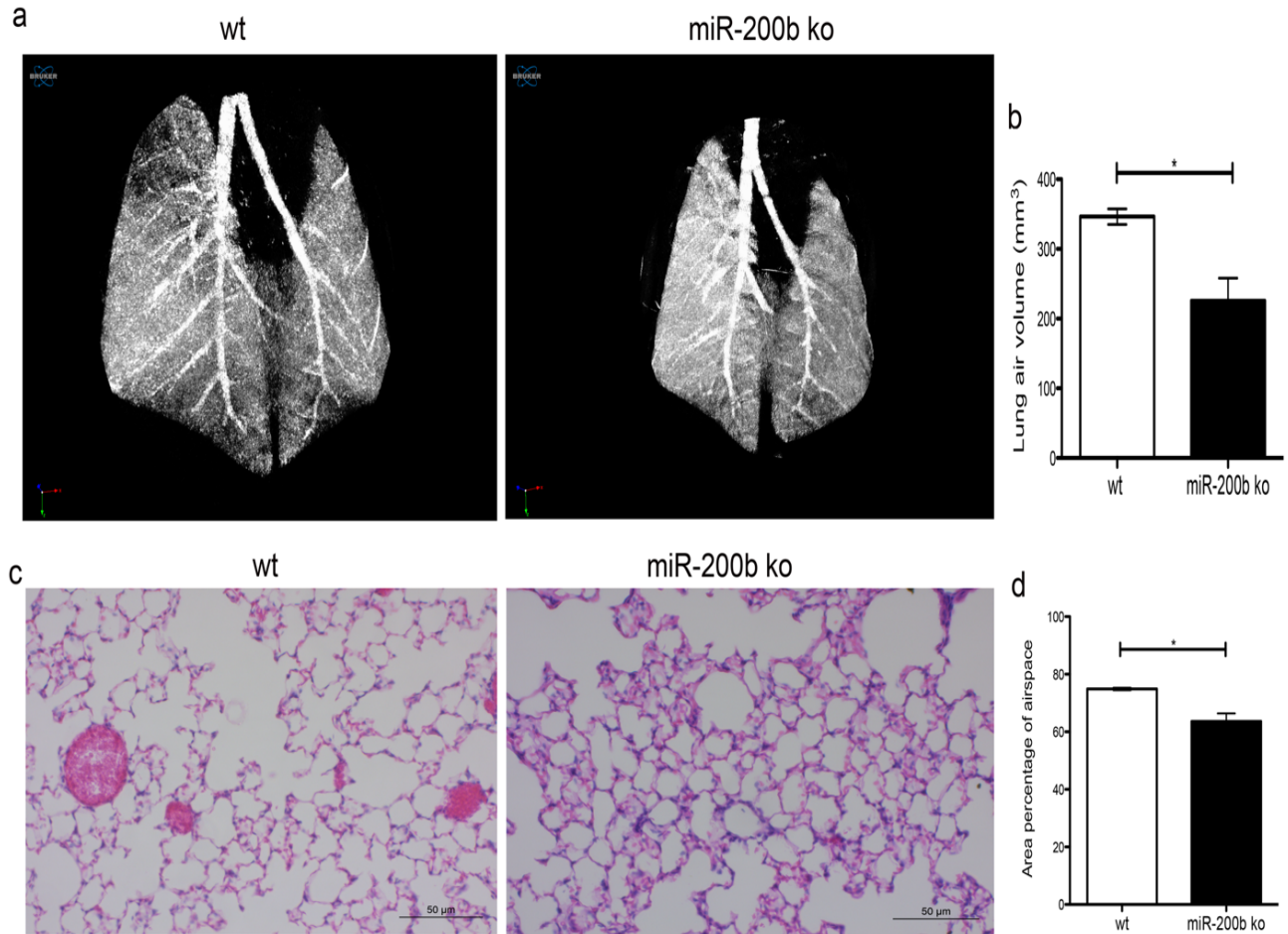


Figure 4-5: MiR-200b knockout mice have denser parenchyma, thickened alveolar walls and lower distal branching (a) In vivo micro-CT scans of 8-week-old mice using the SkyScan 1176 x-ray microtomography system equipped with a large format 11 megapixel x-ray camera. MiR-200b knockout (ko) mice have denser lung parenchyma (gray area in the peripheral area) and a lower number of distal airways (smaller distance between the large airways). (b) miR-200b^{-/-} lungs have significantly lower levels of lung air volume than miR-200b^{+/+}. Lung airspace volume was measured on alive mice using micro-CT scan. *P < 0.05, Student's t-test, Data represent mean ± SEM of at least three independent experiments. (c) Hematoxylin and eosin (H&E) staining of miR-200b^{-/-} lungs confirmed that these lungs have less septation and thicker alveolar walls when compared to wild type lungs. (d) Area percentage of airspace of miR-200b^{-/-} lungs was significantly decreased compare to miR-200b^{+/+} lungs. H&E stained peripheral lung sections were scanned and the percentage of airspace over total lung was measured using ZEN Image Analysis.

Immunofluorescence studies for vimentin showed that *miR-200b*^{-/-} lungs have more vimentin-positive cells, suggesting an increased presence of fibroblasts-like, mesenchymal cells (Fig. 4-6a,b). Also, *miR-200b*^{-/-} lungs showed higher expression of Twist 1 protein – a transcription factor and marker for EMT - compared to *miR-200b*^{+/+} lungs (Fig. 4-6c,d). There were no differences in immunofluorescence patterns for other markers of lung fibroblast cell differentiation (Fibroblast growth factor-10) and epithelial cell differentiation (E-cadherin, cytokeratin, and CC-10) (data not shown). Taken together, these results indicate that even though *miR-200b*^{-/-} mice do not experience obvious breathing difficulties, their lungs display a functional phenotype similar to lung fibrosis and lung hypoplasia observed in children with CDH.

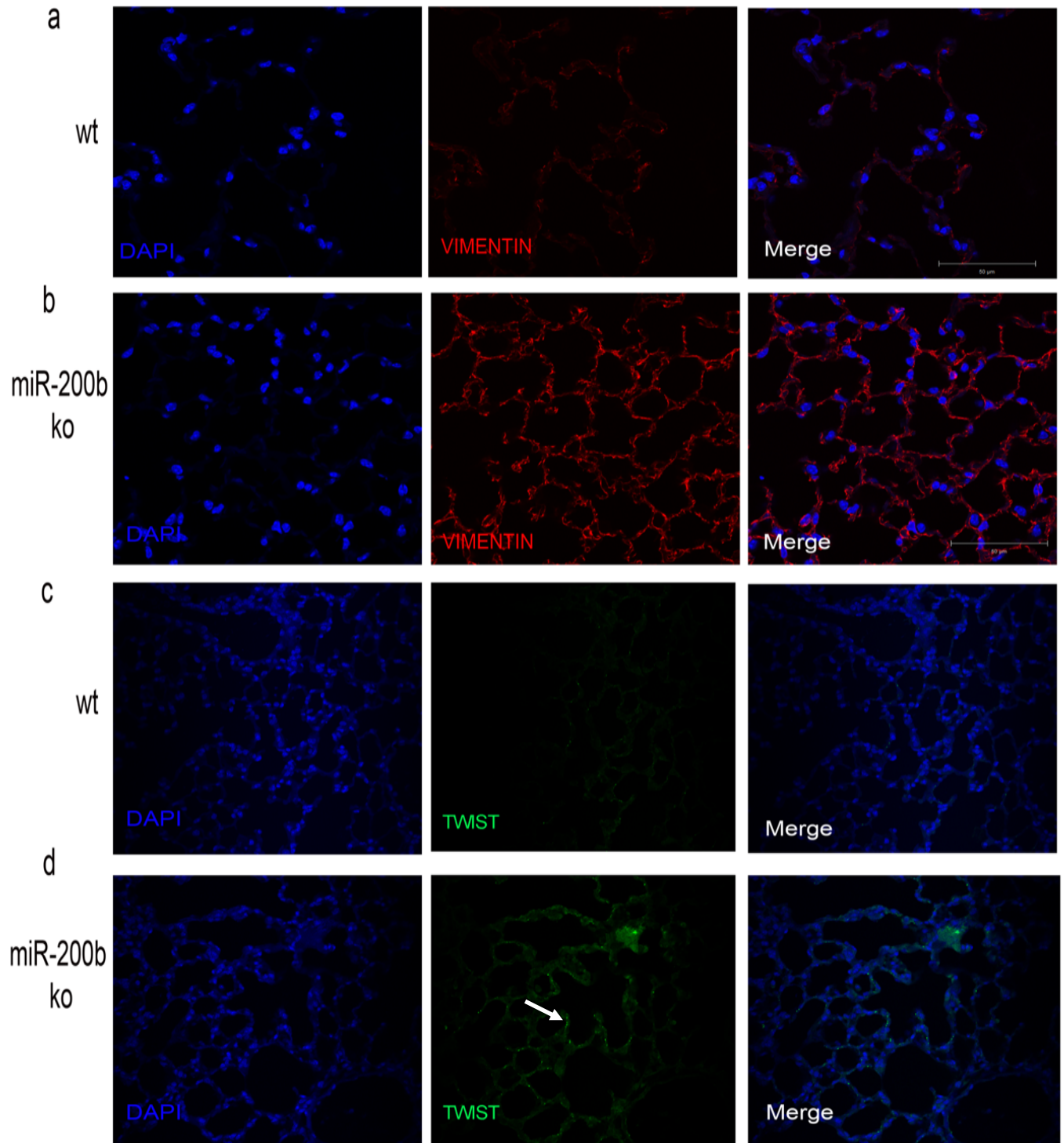


Figure 4-6: MiR-200b knockout mice have more fibroblast like cells and EMT marker. (a,b) Immunostaining for Vimentin on wt (a) and ko (b) lungs demonstrated higher expression of Vimentin protein in ko parenchyma than the wt. (c,d) Immunofluorescence for Twist protein on wt (c) and ko (d) demonstrated higher expression of Twist in the ko than the wt.

MiR-200b maintained human bronchial epithelial phenotype and function

To evaluate the effect of miR-200b on maintaining human bronchial epithelial cell phenotype, we transfected BEAS-2B cells with miR-200b inhibitors and performed double-immunofluorescence with cytokeratin and vimentin to mark epithelial cells and fibroblasts, respectively. We found that miR-200b inhibitors promoted accumulation of fibroblast cell markers following down regulation of miR-200b (Fig. 4-7a). To study if this also resulted in functional mesenchymal properties, we performed a scratch wound-healing assay (Fig. 4-7b,c). We transfected BEAS-2B cells with miR-200b inhibitors and 18h later, we observed 25% scratch wound closure in the control group, whereas, in cultures transfected with miR-200b inhibitors, wound closure was enhanced by 50%.

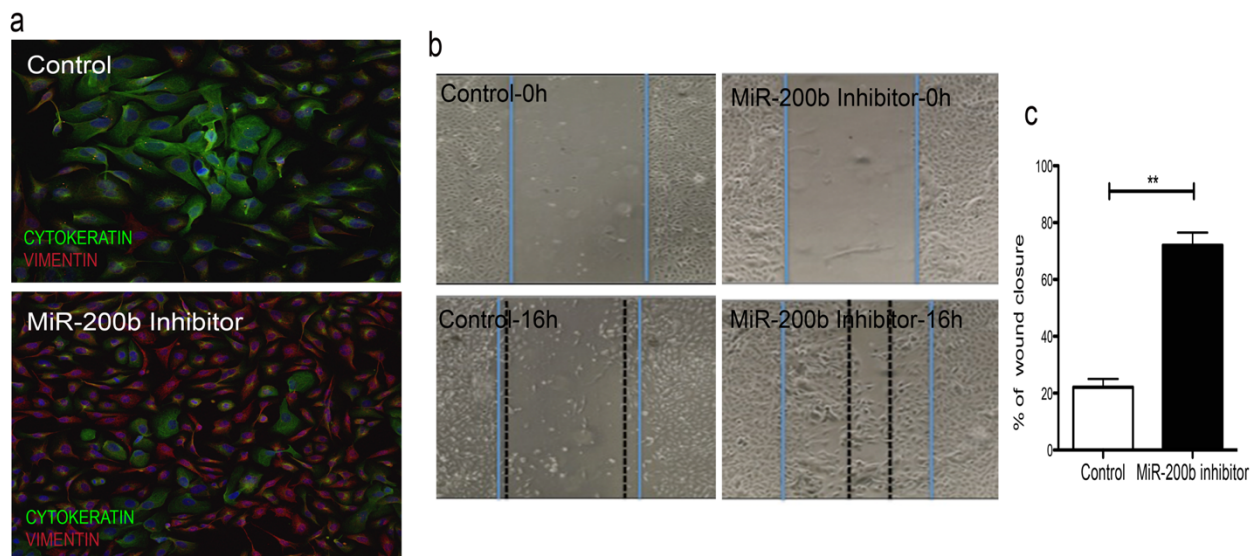


Figure 4-7: MiR-200b maintained human bronchial epithelial phenotype and function (a) Immunostaining of the miR-200b transfected cells after 48h demonstrated higher expression of Vimentin and lower expression of Cytokeratin in these cells compared to cells transfected with a negative control. (b,c) A wound healing (scratch) assay was performed on BEAS-2B or control cells transfected with miR-200b inhibitors (for 18h). Pictures were taken at time 0 h when the scratch was made and 18h later and the migration rates were calculated (the difference between distance from the right to left border at 16h divided by the distance from the right to left border at the start time).

mRNA-Seq whole transcriptome analysis demonstrated that mRNAs of epithelial cell differentiation and surfactant genes are most affected in miR-200b^{-/-} lungs.

In order to evaluate the effect on downstream targets and associated pathways owing to loss of miR-200b on the lung tissue transcriptome, we performed Next Generation Sequencing (NGS) on total RNA samples from lungs of three 8-week-old *miR-200b^{-/-}* mice and three *miR-200^{+/+}* (wt) mice. Heat Map and unsupervised hierarchical clustering by sample and transcripts was performed on all samples passing QC using the top 500 genes that have the largest coefficient of variation based on FPKM counts (Fig. 4-8a). Table 4-1 shows the ten most differentially expressed mRNAs. The full list of differentially expressed transcripts is shown in Supplementary Table 4-6. We used Gene ontology (GO - Gene Ontology Consortium, 2000) enrichment analysis to identify GO terms that are significantly associated with differentially expressed protein coding genes. Using PANTHER Gene Ontology classification system (Mi et al. 2013), we identified Notch and Wnt signalling among the most affected biological pathways and cytoskeletal as well as immunity proteins among the most affected protein class in *miR-200b^{-/-}* lungs compare to *miR-200b^{+/+}* (Fig. 4-8b,c). Among differentially expressed mRNAs, we confirmed the expression of *Plunc*, *Cyp2a5* and *Cdh16* by q-PCR (Fig. 4-8d,e,f).

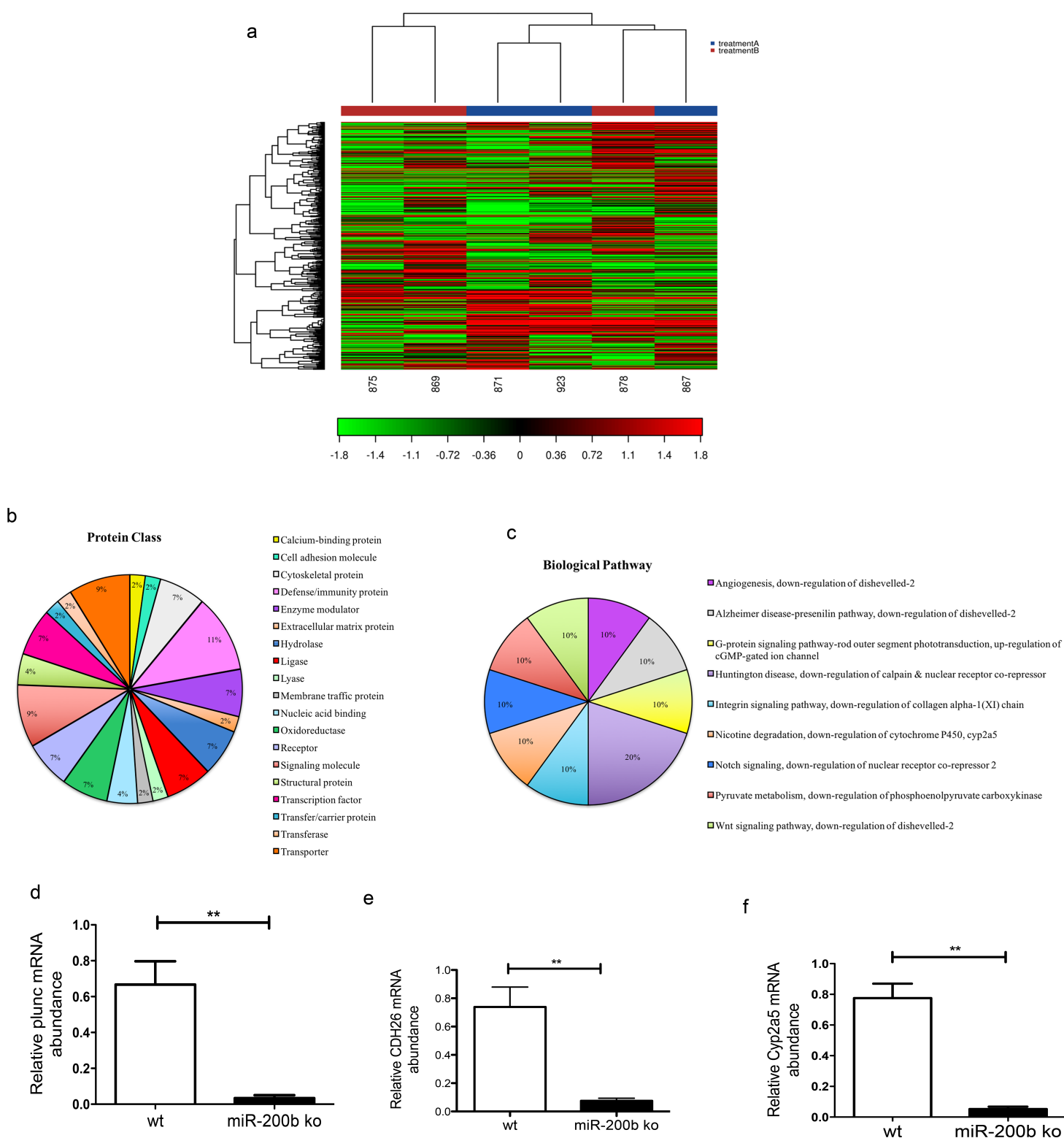


Figure 4-8: Next Generation Sequencing and Gene ontology (GO) showed the most affected pathways in the lungs of *miR-200b*^{-/-} mice. (a) The heat map diagram shows the results of a two-

way hierarchical clustering of RNA transcripts and samples. It includes the 500 genes that have the largest coefficient of variation based on FPKM counts. Each row represents one gene and each column represents one sample. The color represents the relative expression level of a transcript across all samples. The color scale is shown below: red represents an expression level above the mean; green represents an expression level below the mean. (wt samples: 867, 871 and 923; miR-200b ko samples: 875, 878 and 869). (b,c) Pie charts for transcript gene functional analysis conducted for Biological Pathway and Protein class using the PANTHER gene ontology database. (d,e,f) Q-PCR confirmed significantly lower *Plunc*, *Cdh26* and *Cyp2a5* mRNA levels in *miR-200^{-/-}* (ko) lungs than *miR-200^{+/+}* (wt).

We used R package topGO (Anon n.d.) in order to generate Go network for Biological process and evaluate Biological function. Our current findings revealed that the three most affected biological processes in *miR-200b^{-/-}* mice lungs were related to cell cycle, apoptosis and protein transport (Figure 4-9). The most affected Biological Function is shown in Table 4-2.

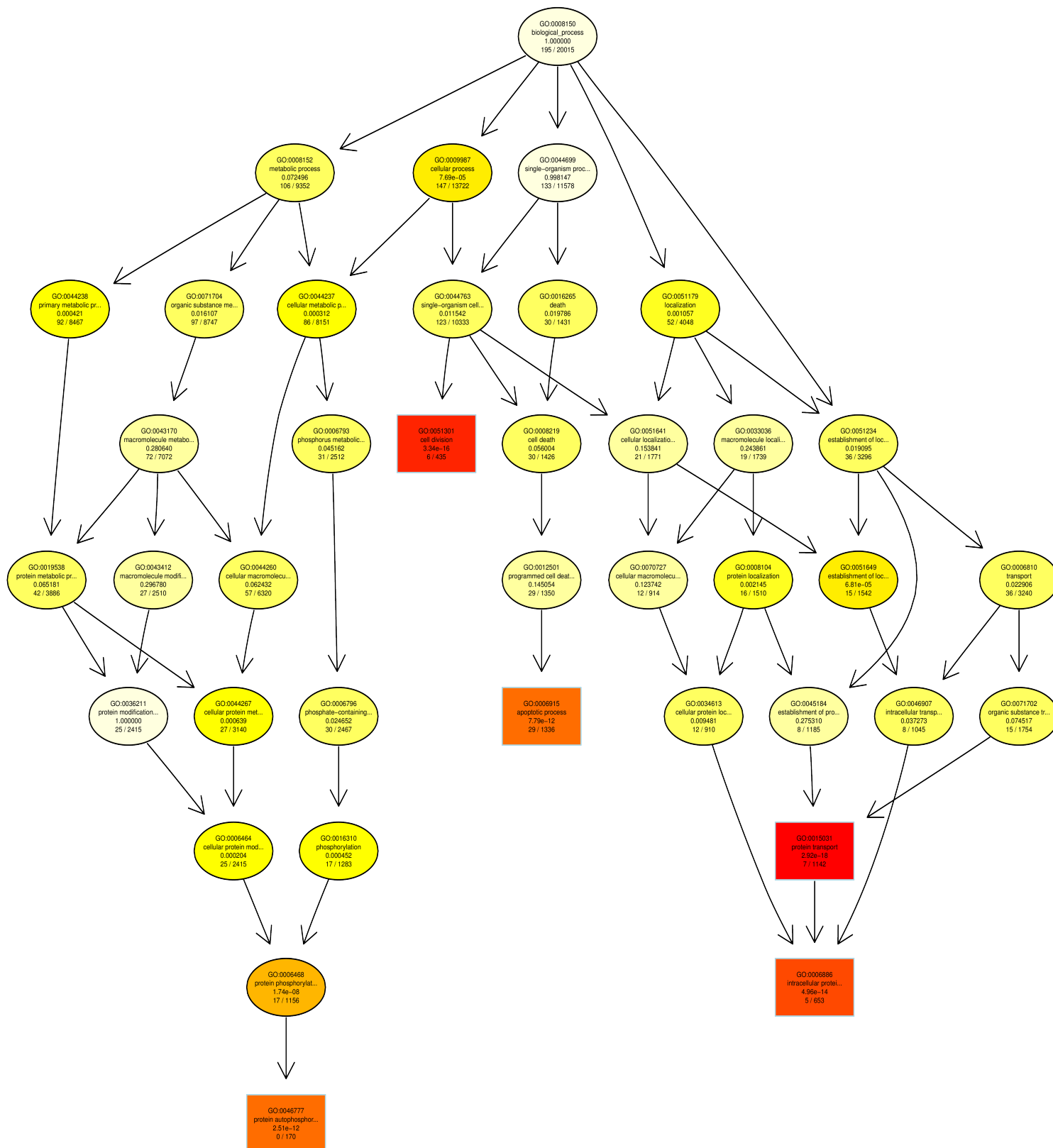


Figure 4-9: Cell cycle, apoptosis and protein transport are the most affected Biological process in *miR-200b*^{-/-} lungs. GO network generated from the GO terms predicted to be enriched for the

Biological process (BP vocabulary). Nodes are colored from red to yellow with the node with the strongest support colored red and nodes with no significant enrichment colored yellow. The five nodes with strongest support are marked with rectangular nodes.

Table 4-1:

Gene_id	Gene	Locus	treatmentA	treatmentB	Log2 fold change	Q_value
XLOC_008125	Lrrtm3	10:63430097 -65003667	0.231458	0.00345736	-6.06493	0.0355741
XLOC_020864	Tcrg-C2	13:19304679 -19311304	50.7836	1.27343	-5.31757	0.00634618
XLOC_044124	Bpifa1 or plunc	2:154142879 -154149219	1353.78	76.2106	-4.15086	0.00634618
XLOC_069330	Slc5a11	7:123214779 -123273253	0.0723419	1.11466	3.94563	0.00634618
XLOC_008308	Trpm2	10:77907721 -77970563	17.5321	1.28117	-3.77447	0.00634618
XLOC_014662	Krt15	11:10013175 7-100135928	6.93844	0.507409	-3.77339	0.00634618
XLOC_022181	Thbs4	13:92751589 -92794818	0.440093	0.033428	-3.71868	0.0340586
XLOC_014413	Spata20	11:94478903 -94486179	0.0590388	0.773681	3.712	0.00634618

XLOC_067530	Cyp2a5	7:26835304- 26952462	282.331	23.1095	-3.61083	0.00634618
XLOC_044638	Cdh26	2:178430530 -178487366	4.61108	0.383838	-3.58654	0.00634618

The top 10 most significantly differentially expressed mRNAs, with log fold change (FPKM Log2_FC) between groups treatmentA (miR-200b^{+/+}) and treatmentB (miR-200b^{-/-}) with Benjamini-Hochberg FDR corrected q-values.

Table 4-2: The significant GO terms for the genes found to be differentially expressed between treatmentA (wt) and treatmentB (ko) their corresponding annotation for Biological Function (BF).

GO.ID	Term	Annotated	Significant	Expected
GO:0015031	protein transport	1142	7	11.13
GO:0051301	cell division	435	6	4.24
GO:0006886	intracellular protein transport	653	5	6.36
GO:0046777	protein autophosphorylation	170	0	1.66
GO:0006915	apoptotic process	1336	29	13.02
GO:0007067	mitosis	306	3	2.98
GO:0006397	mRNA processing	322	2	3.14
GO:0008285	negative regulation of cell proliferation	451	5	4.39
GO:0001701	in utero embryonic development	374	3	3.64
GO:0043065	positive regulation of apoptotic process	295	10	2.87
GO:0000122	negative regulation of transcription from RNA polymerase II promoter	534	5	5.2

GO:0006281	DNA repair	357	0	3.48
GO:0043066	negative regulation of apoptotic process	499	16	4.86
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	715	12	6.97
GO:0009968	negative regulation of signal transduction	692	10	6.74
GO:0045893	positive regulation of transcription, DNA- dependent	991	15	9.66
GO:0007049	cell cycle	1135	10	11.06
GO:0001666	response to hypoxia	225	11	2.19
GO:0006355	regulation of transcription, DNA- dependent	2844	31	27.71
GO:0006468	protein phosphorylation	1156	17	11.26

Annotated: Number of genes associated to the GO term, Significant: Number of significantly differentially expressed ($p \leq 0.05$) genes within the annotated genes, Expected: Number of genes within the annotated genes that are expected to be significantly differentially expressed ($p \leq 0.05$) by random.

4.4. Discussion

We demonstrate for the first time that miR-200b plays a role in peripheral lung development by maintaining an epithelial cell phenotype. Our miR-200b deficient mice have lung function abnormalities, surfactant biophysical dysfunction with compromised pro-Surfactant Protein-C and surfactant protein-B expression. MiR-200b^{-/-} lungs have decreased distal airway branching, a denser lung parenchyma with thicker alveolar walls, a higher number of fibroblast-like cells and over-expression of a marker for EMT: Twist.

We generated *miR-200b*^{-/-} mice by targeted deletion of miR-200b in ES cells. We evaluated mature forms of all miR-200 family members to determine if there are any compensatory effects between the family members. Although miR-200a and miR-429 were expressed lower in miR-200b^{-/-} lungs, their expression was not undetectable like miR-200b. Based on our studies, we cannot exclude that downregulation of miR-200a and miR-429 contributed to the observed lung phenotype in miR-200b deficient mice. Moreover, miR-200c and miR-141, which are transcribed independently, are expressed normally suggesting that there are no compensatory effects.

Our *miR-200b*^{-/-} mice did not show any obvious breathing problems after birth, but lung function studies demonstrated severe peripheral airway obstruction in 8-week old *miR-200b*^{-/-} mice. These observed lung function abnormalities can be due to lung surfactant deficiency or lung fibrosis. Using human fetal lung cultures, Benlhabib, *et al.* showed previously that miR-200 family members regulate epithelial type II cell differentiation and function (Benlhabib et al. 2015). They found that miR-200 family inhibitors down-regulated pro-SP-C and SP-B expression. Here we show that miR-200b^{-/-} lungs have dysfunctional surfactant and compromised expression of these two proteins.

Using *in vivo* micro-CT scanning on alive animals, we found higher parenchymal density with significantly less air-filled distal alveoli in *miR-200b*^{-/-} mice. Our lung morphometry studies corroborated these results by showing that the percentage of airspace in *miR-200b*^{-/-} lungs was lower than in *miR-200b*^{+/+} lungs. Thus, both studies suggest that the observed peripheral airway obstruction might be due to thicker alveolar walls reducing the airspace inside *miR-200b*^{-/-} lungs. Our immunofluorescence studies indicate that Vimentin and Twist expression are upregulated in the peripheral lung tissues of *miR-200b*^{-/-} mice. Interestingly, this finding was not confirmed in our NGS studies of *miR-200b*^{-/-} lungs. It has been shown that regulation of vimentin and twist expression are mainly at the translational level (Zong et al. 1999; Evdokimova et al. 2009). Our data suggests that *miR-200b* is involved in the translation regulation of these genes in the lung. Taken together, the observed lung function abnormalities in *miR-200b*^{-/-} mice can be due to a mesenchymal-skewed, “fibrosis”-like lung phenotype. Others have suggested a role for *miR-200b* in lung fibrosis before (Yang et al. 2012).

Using NGS analysis, we identified changes in the transcriptome in *miR-200b*^{-/-} lungs. Of note, we did not observe upregulation of the primary direct targets of the *miR-200* family in the transcriptome analysis of *miR-200b*^{-/-} lungs. We hypothesize that this can be explained by the fact that most microRNAs directly regulate gene expression at the translational level. Therefore, changes in proteins of the direct gene targets of the *miR-200* family might not be reflected in the transcriptome of *miR-200b*^{-/-} lungs. Gene ontology analysis showed that different signaling pathways are affected by *miR-200b* absence.

Cyp2a5 (cytochrome P450, family 2, subfamily a, polypeptide 5) mRNA is one of the most down-regulated mRNAs in *miR-200b*^{-/-} lungs. Cytochrome P450s comprise a superfamily of enzymes crucial for metabolism of a diverse group of compounds, drugs and environmental

pollutants. These enzymes are mainly present in the liver but lower levels have been detected in the lung (Nemery et al. 2001). Down-regulation of CYP2A5 suggesting the involvement of miR-200b in metabolism in the lung.

Palate lung and nasal epithelial clone (*Plunc*) is one of the mRNAs that was down-regulated more than four times in miR-200b^{-/-} lung. PLUNC is the most abundant secretory protein in the lung and is expressed in nasal, oropharyngeal, and lung epithelial cells. Plunc acts as airway surfactant and plays a role in pulmonary host defence (Bartlett et al. 2011; Liu et al. 2013). It is essential for maintaining normal airway surface liquid homeostasis (Gaillard et al. 2010; Garcia-Caballero et al. 2009). Lower levels of *Plunc* can explain the increased elastance and airway resistance observed in the miR-200b^{-/-} lungs.

Cadherin-26 (CDH26) is another significantly down-regulated mRNA in the miR-200b^{-/-} lungs. Recently, others showed that CDH26 is involved in regulating lung epithelial cell polarity and differentiation. Knockdown of CDH26 results in a lack of epithelial cell polarity and differentiation (Lachowicz-Scroggins et al. 2016). Down-regulation of CDH26 in our knockout lungs can explain the fibroblast-like phenotype of the lungs in our knockout mice and the role of miR-200b in maintaining an epithelial cell phenotype.

Interestingly, these lungs still express the epithelial marker cytokeratin normally. These findings suggest a “partial EMT” in the lung parenchyma of miR-200b deficient mice. Others have shown that patients with idiopathic pulmonary fibrosis (IPF) have epithelial basal cells with a partial mesenchymal phenotype surrounding the fibroblastic foci (Jonsdottir et al. 2015). Members of the miR-200 family are down-regulated in the lungs of patients with IPF and a mouse model of lung fibrosis (Yang et al. 2012). We have previously discovered that lung hypoplasia in CDH lungs

is characterized by reduced airway branching and a thickened interstitial mesenchymal cell layer recapitulating a fibrotic lung phenotype (Van Loenhout et al. 2012; Keijzer et al. 2000).

Knocking down miR-200b in our mice resulted in down-regulation of mature miR-200a and miR-429. These two miR-200 family members share the same transcript with miR-200b. This down-regulation can result from the removal of the miR-200b gene on the promoter activity or can be a post transcriptional effect of miR-200b on processing of the other microRNAs. Although miR-200c has the exact seed sequence as miR-200b, the abundance of this microRNA along with miR141 was not changed in lungs and kidneys of 8-week old *miR-200b*^{-/-} mice suggesting the absence of any compensatory effects of these two miR-200 family members in miR-200b deficient mice. However, it is unclear at this point what the influence of downregulation of miR-200a and miR-429 on the lung phenotype of miR-200b deficient mice is.

Our *in vivo* studies demonstrate a new role for miR-200b in distal lung airway development by regulating epithelial and fibroblast cell differentiation.

4.5. Materials and Methods

Ethics and animal work

Mice were maintained in accordance with the guidelines of “*Guide to the Care and Use of Experimental Animals*”. The *in vivo* experiments were approved by the Animal Research Review Committee at the University of Manitoba.

Generation of C57BL/6; miR-200b^{tm1.1(NCOM)MFGC} mice

Vector design and construction: The mouse miR-200 locus is comprised of three microRNA genes, mmu-mir-200b, mmu-mir-200a and mmu-mir-429, in the first intron of the *Ttll10* gene of mouse chromosome 4. The targeting vector was designed strategically to target the deletion of only miR-200b (ENSMUSG00000065549) within the cluster and to avoid any changes to miR-200a, miR-429 or any other conservative regions within the locus. The miR-200b targeting vector was constructed utilizing the pGOHANU vector, which contains the F3/FRT flanked NORCOMM targeting cassette comprised of three functional units: the SA-IRES-LacZ-pA genetrap reporter, a loxP-flanked hβactP-ΔTK1-T2A-Neo-pA selection cassette, and an AttP-Puro-pA docking cassette (Austin et al. 2004; Bradley et al. 2012) (Fig. 1a). The docking cassette was designed to facilitate docking applications with ΦC31 integrase in ES cells to modify a given targeted allele (Monetti et al. 2011) and was not used in this study. Assembly of the final targeting vector included an 11 kb mus musculus genomic region containing miR-200b on chromosome 4 (155429790 – 155429859/ negative strand) using the NORCOMM recombining strategy modified from that developed by EUCOMM (Skarnes et al. 2011). Briefly, the identified C57BL/6 BAC clones, RP23-118E21, RP23-382A8, RP23-350D2, RP23-139J18, were used to assemble intermediate and final targeting vectors according to the Sanger Design ID #373799. The region was designed

such that the 2891 bp 5' homology arm (155429953 – 155432844) and the 6595 bp 3' homology arm (155423096 – 155429691) define the points of homologous recombination (Fig. 1b). In the process, intervening sequences contain miR-200b genomic sequences were replaced by the NORCOMM cassette (F3-SA-IRES-LacZ-pA-loxP-hβactP-ΔTK1-T2A-Neo-pA-loxP-AttP-Puro-pA-FRT). The final targeting vector was created by use of a three-way gateway (Invitrogen) mediated *in vitro* recombination sub-cloning strategy. Final targeting vectors were sequence validated across junction points of subcloned arms, and across the site-specific recombination elements, loxP, F3, FRT and AttP-docking sites.

ES cell targeting and PCR genotyping of targeted miR-200b allele: The miR-200b knock out mice were generated by replacement of the endogenous miR-200b with the NorCOMM cassette using a homologous recombination targeting strategy (Fig. 1b) (Gertsenstein et al. 2010). Briefly, 10⁷ C2 embryonic stem (ES) cells (C57BL/6NTac), were electroporated using 2.5 µg of linearized targeting vector with AsiSI restriction enzyme. Electroporated ES cells were cultured ten days in G418 (neo) selection (100 µg/ml) over neomycin resistant mouse embryo fibroblast feeder cells derived from TgN(DR4)1Jae/J mice. 32 healthy neomycin resistant ES colonies were picked and cloned cells were expanded in 96 well plate format for either genomic DNA isolation or cryopreservation. Correctly targeted ES cells were identified by long range PCR screening of the 5' and 3' homology arms using primers spanning from the NorCOMM cassette to a site beyond the end of the respective homology arms. ES clones that had undergone correct recombination would have a 5' homology arm PCR product of 3356 bp (primers GH2871 and GH717, Fig. 1b G5). Four 5' homology arm sequence validated clones were next screened to identify the integrity of the 3' homology arm by producing a correct PCR product size of 6979 bp (primers GH1300 and GH2892, Fig. 1b G3). Both amplified PCR products were subjected to sequencing across the ends of the

homology arms to validate correct and precise recombination events. PCR-sequencing was also used to validate the integrity of the F3, FRT and loxP sites in the NorCOMM cassette to ensure downstream applications of *in vivo* site-specific recombination (Fig. 1b). Three of four ES clones (A9, B9, F9) were fully sequence validated and validated by Southern blotting (data not shown).

Generation of miR-200b knock out transgenic mice: MiR-200b knock out F9 ES clone was used to generate chimeras (F0) by ES cell injection into B6(Cg)Tyr^{c-2j}/J blastocysts, as previously described (Hicks et al. 2000), resulting in chimeras with greater than 80% black coat color chimerism. Male chimeras were mated with C57BL/6N females to derive mice with germ line transmission (GLT) of the miR-200b KO allele. This line was designated as miR-200b^{tm1(NCOM)MFGC}. Removal of the hβact promoter driven ΔTK1-T2A-neomycin cassette was performed *in vivo* by mating male miR-200b^{tm1(NCOM)MFGC} mice harboring the miR-200b KO allele with female B6.C-TG(CMV-Cre)1Cgn/J transgenic mice, in which Cre recombinase is constitutively expressed (The Jackson Laboratory, #006054). The resultant miR-200b Cre-excised allele, miR200b^{tm1.1(NCOM)MFGC} (Fig. 1c), was sequence validated following PCR analysis across the remaining loxP site (primers GH3617 and GH3618). Loss of the neomycin selection cassette was also verified by PCR (primers GH3619 and GH3620). Elimination of the transgenic Cre-recombinase allele was selected for during routine breeding and genotyping. The resulting miR-200b^{tm1.1(NCOM)MFGC} mice on a homogenous C57BL/6N background were used for all experiments in the present study. Mice were genotyped from ear biopsies using a multiplex PCR strategy (primers GH3369 and GH3370 for 494 bp wild type allele product, and primers GH3369 and GH717 for 390 bp knock out allele product) under the following conditions: 98°C 2 min, 98°C 10 sec, 64°C 30 sec, 72°C 45 sec, Cycle to Step 2 another 32 times, 72°C for 5 min, 8°C to Hold (Supplementary Table 4-3).

LacZ staining

Whole mouse embryos are stained for β -galactosidase (lacZ) activity using X-gal as described before (Nagy et al. 2007). For E14.5 and older, we used a razor blade to section the embryos in half to facilitate penetration.

RNA extraction and qPCR

lungs were isolated in icecold PBS, snap frozen in liquid nitrogen and stored at -80°C until processed. Total RNA was extracted using the miRCURY™ Isolation Kit (Ambion), according to the manufacturer's instructions. For microRNA analysis, cDNA was randomly primed from 20 ng total RNA using the Exiqon cDNA synthesis kit. RT-qPCR was subsequently performed using locked nucleotide acid (LNA) primers for miR-200 family members and miR-103 (as an endogenous control). All primer sequences are provided in the Supplementary Information (Supplementary Table 4-4). We used the miRCURY LNA™ Universal RT microRNA PCR protocol (Exiqon) in a total reaction volume of 20 μ l. Briefly, RT-qPCR was performed in triplicate with a 1:80 dilution of cDNA using the SYBR green PCR system on an ABI 7500 Real-Time PCR machine (Applied Biosciences). Data were collected and analyzed using ABI 7500 v1.4.0 software (Applied Biosciences). MicroRNA expression levels were determined using the relative quantification feature of the ABI 7500 v1.4.0 software.

Lung mechanics

Eight-week-old male miR-200b^{+/+}(wt) or *miR-200b*^{-/-} (ko) mice (at least six mice for each group) were anesthetized with intra-peritoneal sodium pentobarbital (90 mg/kg). The trachea was

dissected using fine dissection scissors and a 20-gauge polyethylene catheter was inserted which was further connected to a flexiVent small animal ventilator (Scireq Inc. Montreal). Mice were ventilated with a tidal volume of 10 ml/kg body weight, 150 times per minute. A positive end expiratory pressure (PEEP) of 3 cmH₂O was used for all studies. Mice were subjected to an increased dose of nebulized methacholine (MCh) challenge protocol to assess concentration response characteristics of respiratory mechanics. For MCh challenge, ~ 30μL of saline containing from 0 to 50 mg/ml MCh was delivered over 10 seconds using an in-line ultrasonic nebulizer. To assess the effects of MCh challenge on respiratory mechanics we used low frequency forced oscillations (1-20 Hz). Respiratory mechanical input impedance (Z_{rs}) was derived from the displacement of the ventilator's piston and the pressure in its cylinder. Correction for gas compressibility, and resistive and accelerative losses in ventilator, tubing and catheter were performed per the manufacturer instructions, using dynamic calibration data obtained from snapshot perturbation applied to the system in an open and closed configuration. By fitting Z_{rs} to the constant phase model and Prime-3 perturbation flexiVent software calculated conducting airway resistance known as Newtonian resistance (R_n), peripheral tissue/airway resistance known as tissue damping (G) and tissue elastance or stiffness (H). Values for each parameter were calculated as the mean of all 12 perturbation cycles performed after each MCh challenge.

Surfactant biophysical properties

Bronchoalveolar lavage fluid (BALF) was collected with 4 repeated washes of excised lungs using 2 ml saline in total. The fresh supernatant was used for assessing biophysical surfactant function using a capillary surfactometer according to the manufacture's protocol (Calmia Medical, Inc.).

We used 3 *miR-200b*^{-/-} male mice and 3 *miR-200b*^{+/+} for this measurement and each sample was measured 5 times.

Micro-CT scans

Three *miR-200b*^{-/-} and three *miR-200b*^{+/+} male mice were anesthetized in an anesthetic chamber with 5% isoflurane and scanned for 32 minutes, while the mice were breathing normally under an anesthetic mask, using the SkyScan 1176 x-ray microtomography system equipped with a large format 11 megapixel x-ray camera (Small animal model imaging core facility, University of Manitoba) . Images were acquired at 18 µm resolution with an exposure time of 310 ms and 0.5° rotation step using a 0.5 mm aluminum filter and source current and voltage of 500 µA and 50 kV respectively. Images were reconstructed using NRecon (Bruker MicroCT, Kontich, Belgium) with the dynamic range set to the same values for each mouse (0-0.08) and a beam hardening correction of 30%. Reconstructed images were processed using CTan (Bruker MicroCT, Kontich, Belgium) to visualize airways. To ensure that the same region of tissue was used for comparison between mice, the slice showing initial branching of the trachea into bronchi was found and the first slice for analysis was set to be 3.25 mm (181 slices) above this slice. The last slice included in the analysis was set to the slice corresponding to the base of the lungs (i.e. the first slice showing only diaphragm and no lung tissue). Image size was reduced by drawing a region of interest around the lung tissue, excluding muscle and bone, and saving the reduced volume within the region of interest between the upper and lower slice limits. Slices were then loaded in CTVox (Bruker MicroCT, Kontich, Belgium) as minimum intensity projection images. The reduced data set was further processed in CTan to allow calculation of airspace volume and structure thickness. The 8-bit images were binarised using a lower threshold of 15 and an upper threshold of 255 to segment

tissue containing air from surrounding tissues. An ROI shrink-wrap was performed in 2D followed by the bitwise operation $\text{image} = (\text{image}) \text{ XOR } (\text{ROI})$ to produce an image of the air-containing tissue. This image was then de-speckled to remove white speckles smaller than 15 voxels. The volume of the segmented regions was calculated to produce an estimate of the volume of air in the lungs.

Measuring area percentage of airspace

Three *miR-200b*^{-/-} and three *miR-200b*^{+/+} 8-week-old lungs were inflation-fixated by formalin using constant pressure and embedded in paraffin. After performing H&E staining on three sections per lungs, we scanned the whole sections using an Axio Scan.Z1 microscope from Zeiss. We measured area percentage of the airspace using ZEN Image Analysis software and a module based on color coding the tissue and empty areas (airspace). Using the software, we eliminated the large airways from the calculation (supplementary Fig. 4-15).

Tissue immunofluorescent staining

Three 8-week-old *miR-200b*^{-/-} or *miR-200b*^{+/+} lungs were inflated intratracheally with 1ml of 10% formalin via the cannula by gentle infusion. All tissues were fixed in 10% formalin for overnight. Lungs were embedded in paraffin after dehydration in a graded ethanol series followed by xylene. Immunofluorescence was performed on 5µm sections using vimentin (Abcam; ab92547; 1:800), pro-Surfactant Protein-C (Abcam; ab40879; 1:800), pro+ mature Surfactant Protein-B (Abcam; ab40876, 1:50) and twist (Abcam; ab50887; 1:200) antibodies as previously described (Chi & Chandy 2007).

Cell immunofluorescent staining

Human bronchial epithelial cells, BEAS-2B (ATCC, Manassas, VA, USA), cells were transfected with 0.01 µg/ml of LNA-hsa-miR200b inhibitor or negative control oligonucleotides (Exiqon, Denmark). After 48h cells were fixed with 4% PFA and immunofluorescence was performed using vimentin (Abcam; ab92547; 1:800) and cytokeratin (BioLegend, 628602, 1:200).

Fetal lung explant culture

For mice lung explant culture, lungs were isolated from E11.5 embryos (offspring from a miR-200b +/- cross) and transferred to porous membranes (Isopore™) filters with dimensions of 1 mm x 1.5 mm pore size (Millipore, USA) in a 12-well plate for a semidry floating explant culture and cultured for four days in a 1:1 mixture of DMEM and Ham's F-12 Nutrient supplemented with 100 µg/ml streptomycin, 100 units/ml penicillin, 0.25 mg/ml ascorbic acid. Branching morphogenesis and epithelial perimeter length were monitored daily in all groups by stereomicroscopy, photographs taken and measurements performed using ImageJ software. The difference between day 0 (D0: 0 hours) and day 4 (D4: 96 hours) of culture, were expressed as D4/D0 ratio.

Scratch (wound healing) assay

The scratch (wound healing) or migration assay was performed as previously described by others (Liang et al. 2007). Briefly, BEAS-2B cells were plated in 12 well plates and transfected with 0.01 µg/ml of LNA-hsa-miR200b inhibitors or negative control oligonucleotides. After 12 hours, we scraped the cell monolayer in a straight line with a 200-µl pipette tip. The cell cultures were washed with PBS to remove the debris followed by transfection with miR-200b inhibitors.

Cell migration was monitored and photographed over time. Distance travelled from the initial scratch site was measured after 18h and the migration distance was quantified with ImageJ software.

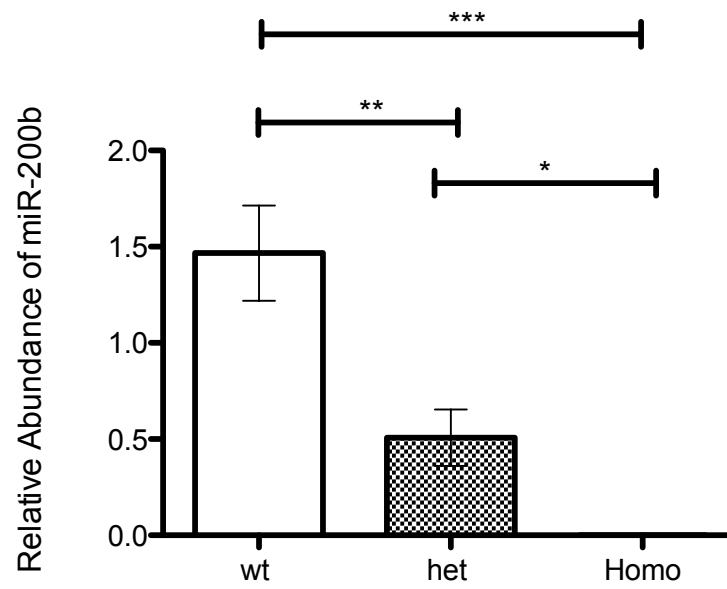
Library preparation and next generation sequencing

The library preparation was done using the TruSeq® Stranded mRNA Sample preparation kit (Illumina inc). The starting material of total RNA (100 ng) was mRNA enriched using the oligodT bead system. The isolated mRNA was subsequently fragmented using enzymatic fragmentation. Then first strand synthesis and second strand synthesis were performed and the double stranded cDNA was purified (AMPure XP, Beckman Coulter). The cDNA was end repaired, 3' adenylated and Illumina sequencing adaptors were ligated onto the fragment ends, and the library was purified (AMPure XP). The mRNA stranded libraries were pre-amplified with PCR and purified (AMPure XP). The libraries size distribution was validated and quality inspected on a Bioanalyzer high sensitivity DNA chip (Agilent Technologies). High quality libraries were quantified using qPCR, the concentration normalized and the samples pooled according to the project specification (number of reads). The library pool(s) were re-quantified with qPCR and optimal concentration of the library pool used to generate the clusters on the surface of a flowcell before sequencing using Nextseq500/ High Output sequencing kit (51 cycles according to the manufacturer instructions (Illumina Inc.)). All experiments were conducted at Exiqon Services, Denmark.

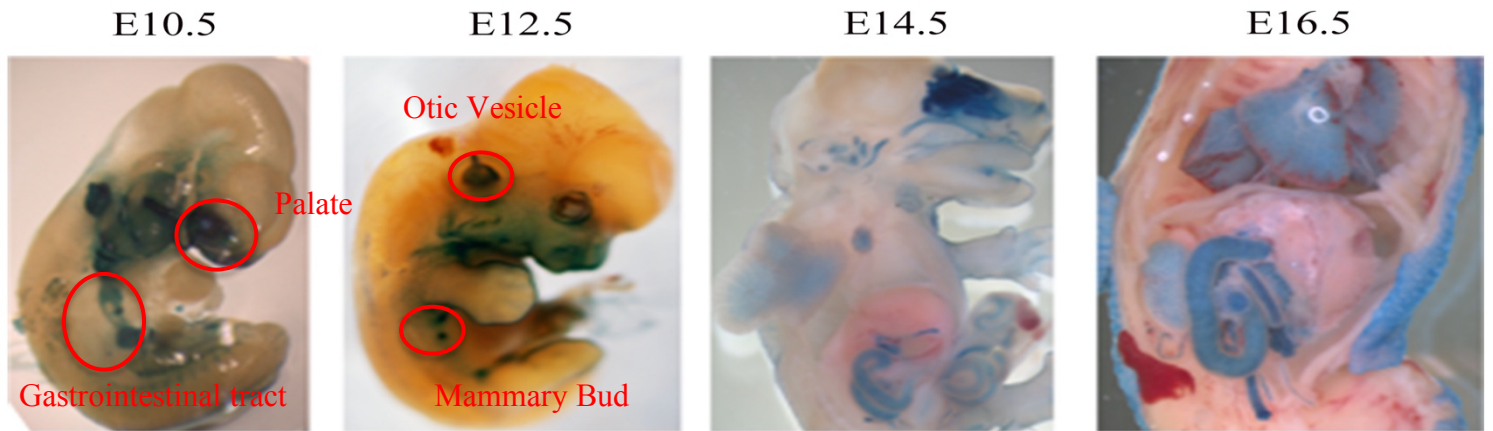
Statistical analysis

All data are presented as mean $\pm$ standard error of mean, from a minimum of three independent experiments. Statistical significance was determined by two-way ANOVA or by *student's t*-test as indicated in the figure legends. A p-value ≤ 0.05 was considered significant.

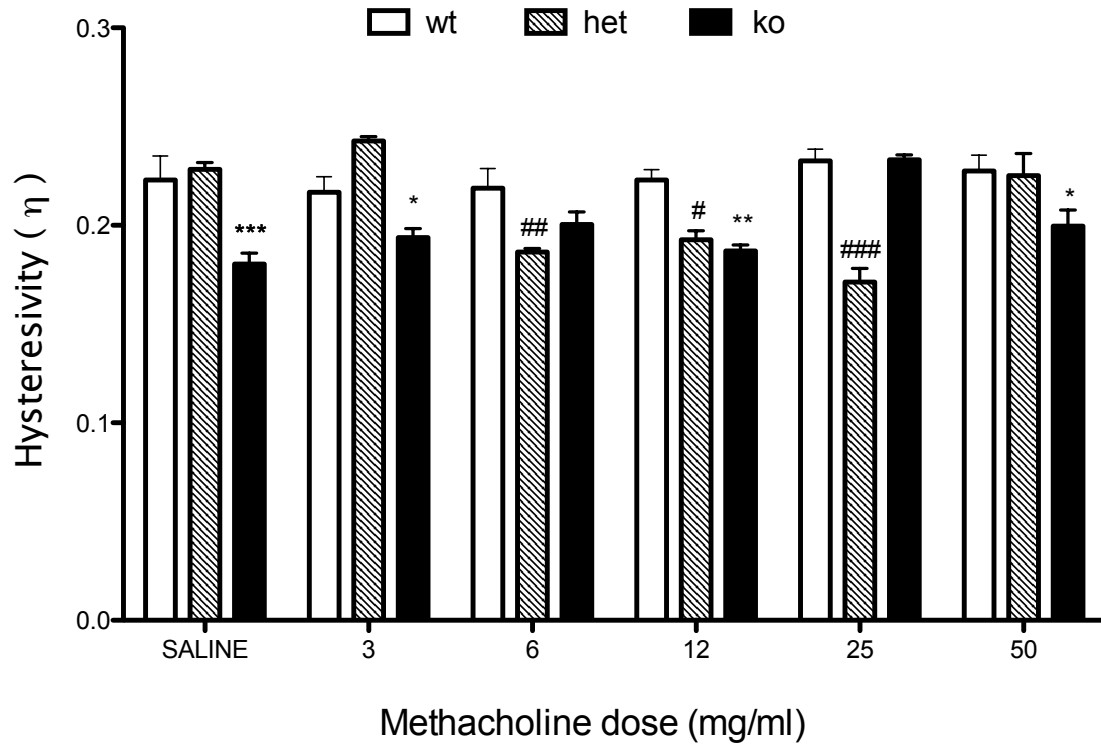
4.6. Supplementary Results



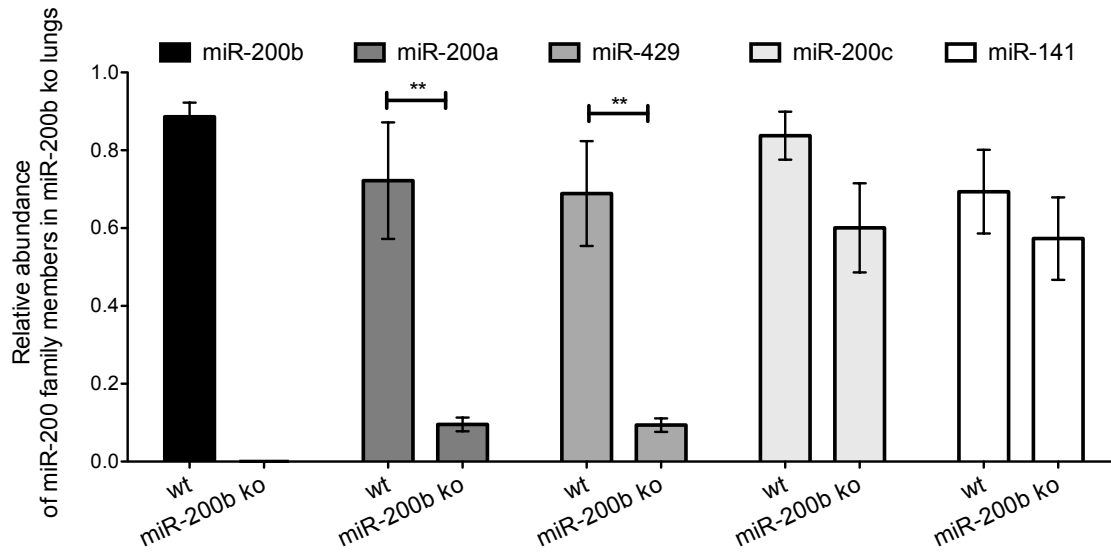
Supplementary Figure 4-10: Absence of miR-200b expression in miR-200b knockout lung explants was confirmed using RT-qPCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA. Data represent the mean of three independent experiments.



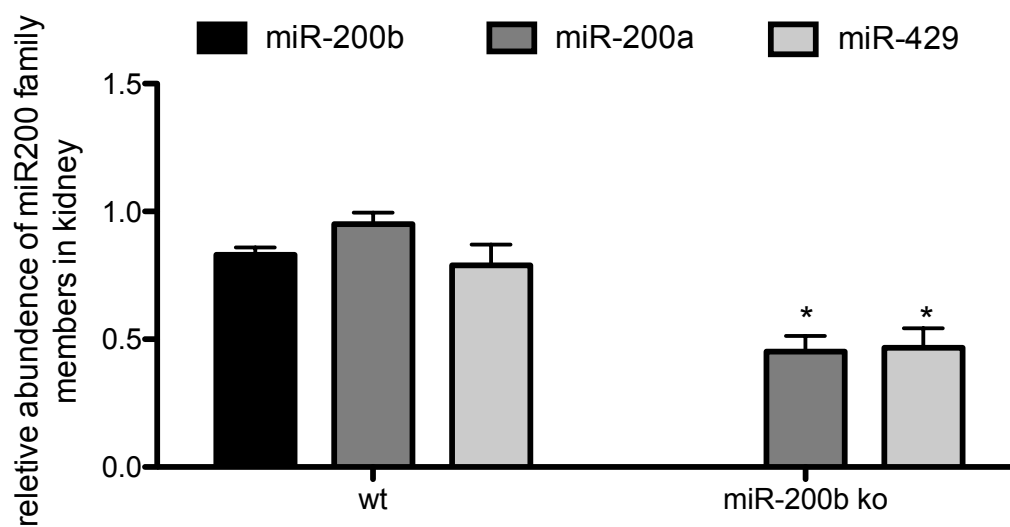
Supplementary Figure 4-11: Whole mount lacZ expression in miR-200b ko embryos at E10.5, E12.5 and E14.5. LacZ staining (blue) is observed in the lungs, palate, otic vesicle and mammary buds.



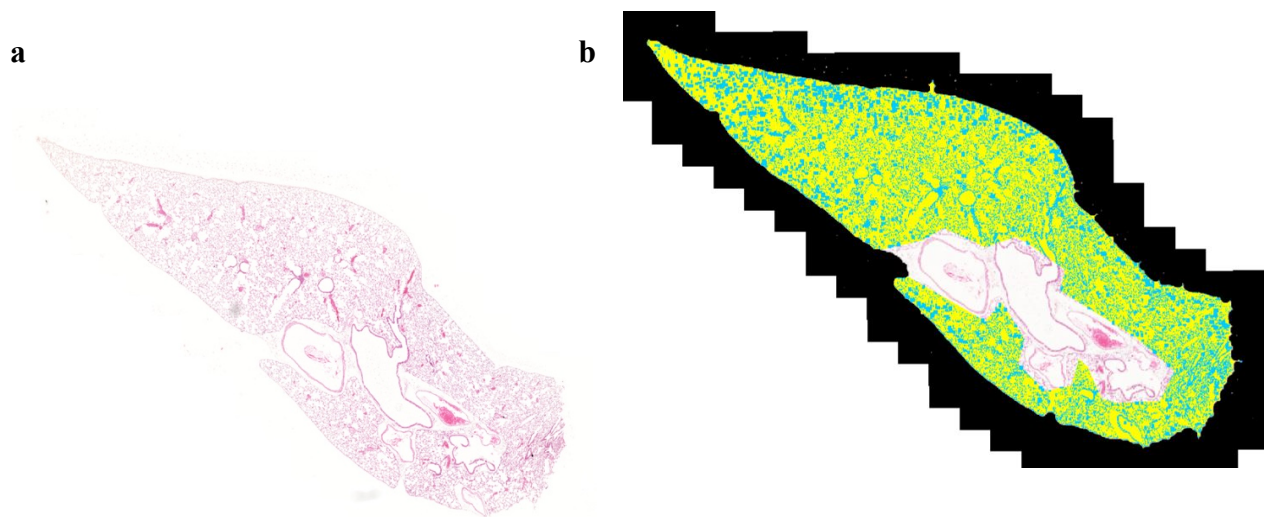
Supplementary Figure 4-12: *MiR-200b ko mice have significantly lower Hysteresivity before methacholine challenge, at 3, 12 and 50 mg/ml of MCh. Also, miR-200b +/- mice have lower hysteresivity at 6, 12 and 25 mg/ml of MCh as well. Comparisons were made to wildtype. #P < 0.05, ##P < 0.01, ###P < 0.001, *P < 0.05, **P < 0.01, ***P < 0.001, two-way ANOVA. Data represent the mean of at least 6 independent experiments.*



Supplementary Figure 4-13: RT-qPCR for all miR-200 family members on lungs from 8-week-old mice using LNA primers. *miR-200b* absence was confirmed. *miR-200a* and *miR-429* were significantly downregulated but no changes were observed in abundance of *miR-200c* and *miR-141* ** $P < 0.01$, Student's *t*-test, Data represent mean $\pm$ SEM of at least four independent experiments.



Supplementary Figure 4-14: Absence of miR-200b expression and lower expression of miR-200a and miR-429 in kidneys of miR-200b knockout mice was confirmed using RT-qPCR.



Supplementary Figure 4-15: Area percentage of airspace measurement. Example of tissue scanned by Zeiss Laser Scanning Microscope (a) and We measured area percentage of the airspace using ZEN Image Analysis software and module (base on color coding the tissue and empty areas (airspace)). (b) Using the software, we could eliminate the large airways from the calculation

4.7. Supplementary Tables

Supplementary Table 4-3: PCR & Sequence Validation.

	miR-200b Targeted Clones		
	A9	B9	F9
G5 – Confirms 5' Arm Recombination	+	+	+
G3 – Confirms 5' Arm Recombination	+	+	+
F3 – Confirms F3 Cassette element	+	+	+
FRT- Confirms FRT Cassette element	+	+	+

Following gene targeting, neomycin-resistant ES cell clones were selected with G418 and subjected to rigorous PCR and sequenced-based analysis to confirm the correct targeting of miR-200b. Correct sized long range PCR products spanning from gene-specific primers outside the homologous arms of the targeting vector to vector-specific primers within the NorCOMM cassette confirmed correct targeting of miR-200b. Sequence analysis of long range PCR products across the genomic junction spanning the ES cell genomic loci and the genomic region of the homology arms within the original targeting vector definitively confirm miR-200b targeting (PCR products – blue lines in panel A; sequencing primers G5 and G3 site relative primers are indicated by blue arrows). Similarly, sequence analysis of PCR product spanning the junction between the genomic homology arms and the integrity of the functional elements of the targeting vector (F3 and FRT sequencing primers highlighted) were confirmed with Southern Blot. Summary of data shows three miR-200b knockout ES clones are fully validated and utilized for generation of miR200b^{tm1(NCOM)MFGC} of germ line transmitted mice.

Supplementary Table 4-4: Primers used for genotyping or Sequencing of miR-200b ^{+/+}, ^{-/+} or ^{-/-}

Primer	Assay	Primer type	5' to 3' sequence	Product size
GH2871	5' homology arm validation	Forward	CTTAAAGCAGCCACTGCTGTTCC	3356 bp
GH717	5' homology arm validation	Reverse	CACCGACGCCAATCACAAACAC	
G5 (GH2883)	5' homology arm sequencing	Sequencing	GGGCATGAAGATCTCGTCTCTGT	–
GH1300	3' homology arm validation	Forward	TCTTATCATGTCTGCTCGAAGC	6979bp
GH2892	3' homology arm validation	Reverse	TGAAGGTCAAAGAAGCTCCAAGC	
G3 (GH2976)	3' homology arm sequencing	Sequencing	ATTGGCCTTTCTTTGCTGTCAG	–
GH1343	F3 validation	Forward	CGCATAACGATACCACGATATCAAC	1402bp
GH1177	F3 validation	Reverse	ACAGTATCGGCCTCAGGAAGATC	
F3 (GH681)	F3 Sequencing	Sequencing	GTAAGTCGATATGTTTATTCTTC	–
GH1353	FRT validation	Forward	TGAATGGAAGGATTGGAGCTACG	1490bp
GH1345	FRT validation	Reverse	TACTGCGACTATAGAGATATCAAC	

FRT (GH809)	FRT sequencing	Sequencing	CTGCATTCTAGTTGTGGTTTGTCC	–
GH3617	Δ TK-T2A-Neo cassette excision	Forward	CCCGTCAGTATCGGCGGAAT	659 bp
GH3618	Δ TK-T2A-Neo cassette excision	Reverse	ACCCACACCTTGCCGATGTC	
GH3619	check neo specific loss	Forward	GGAAGGGACTGGCTGCTATTGG	521 bp
GH3620	check neo specific loss	Reverse	TCAAGAAGGCGATAGAAGGCGAT G	
GH3370	Genotyping WT allele	Reverse	CCCATAGCCCTACCTTGGATAAGG	494 bp
GH717	Genotyping Mut allele	Reverse	CACCGACGCCAATCACAAACAC	390 bp
GH3369	Genotyping common	Forward	AGGGGAACTTGTCTATGGCCATG	

Supplementary Table 4-5: *Locked nucleic acid (LNA) primer sets used in quantitative reverse transcription PCR (RT-qPCR)*

LNA microRNA primer set	Description
U6 snRNA (hsa, rno,mmu)	endogenous control
miR-103	endogenous control
hsa-miR-200b	tested microRNA
hsa-miR-200a	tested microRNA
hsa-miR-429	tested microRNA

Supplementary Table 4-6: Table of full list of significantly differentially expressed mRNAs, with log fold change (FPKM Log₂_FC) between groups treatment (wt) and treatment (miR-200b^{-/-}) with Benjamini-Hochberg FDR corrected q-values.

Gene_id	Gene	Locus	Treatment A	Treatment B	Log2_fold_change	q_value
XLOC_07937 5	-	9:18558358-18571556	3.54387	279.112	6.29938	0.00634618
XLOC_00812 5	Lrrtm3	10:63430097- 65003667	0.231458	0.00345736	-6.06493	0.0355741
XLOC_04632 0	Gm10800	2:98666546-98667301	31.0653	0.467457	-6.05432	0.00634618
XLOC_02086 4	Tcrg-C2	13:19304679- 19311304	50.7836	1.27343	-5.31757	0.00634618
XLOC_02349 9	Trac	14:54187894- 54224201	1272.67	35.2599	-5.17369	0.00634618
XLOC_08074 1	AC163666.1, SNORD50	9:88595233-88599516	0.913589	29.5595	5.01593	0.00634618
XLOC_04743 4	Gm14221	2:160568378- 160619973	2.88636	55.1965	4.25725	0.00634618
XLOC_04412 4	Bpifa1	2:154142879- 154149219	1353.78	76.2106	-4.15086	0.00634618
XLOC_06933 0	Slc5a11	7:123214779- 123273253	0.0723419	1.11466	3.94563	0.00634618
XLOC_06180 0	-	5:137178738- 137180434	1.87136	0.124166	-3.91375	0.00634618
XLOC_00830 8	Trpm2	10:77907721- 77970563	17.5321	1.28117	-3.77447	0.00634618

XLOC_01466		11:100131757-				
2	Krt15	100135928	6.93844	0.507409	-3.77339	0.00634618
XLOC_02218		13:92751589-				
1	Thbs4	92794818	0.440093	0.033428	-3.71868	0.0340586
XLOC_01441		11:94478903-				
3	Spata20	94486179	0.0590388	0.773681	3.712	0.00634618
XLOC_06941		7:126847415-				
8	Doc2a	126865377	0.542942	0.0442615	-3.61667	0.00874121
XLOC_06753						
0	Cyp2a5	7:26835304-26952462	282.331	23.1095	-3.61083	0.00634618
XLOC_04463		2:178430530-				
8	Cdh26	178487366	4.61108	0.383838	-3.58654	0.00634618
XLOC_03536	1110038B12R ik,SNORD48, Snord52,snR7	17:34950237-				
9	8	34952471	151.769	13.8386	-3.45511	0.00634618
XLOC_02739		15:99875641-				
3	-	99879598	8.49763	0.803171	-3.40328	0.00634618
XLOC_07713						
7	Taf1d	9:15283336-15316913	134.224	14.274	-3.23319	0.00634618
XLOC_00654		10:88885991-				
1	Slc5a8	88929505	1.14442	0.123307	-3.21428	0.00634618
XLOC_06369						
8	Mitf	6:97807057-98021349	3.20994	26.4442	3.04234	0.00634618
XLOC_08059						
0	7SK	9:78175302-78175633	21.3643	2.62084	-3.0271	0.00634618
XLOC_01102		11:70000594-				
	Dvl2		54.2064	6.88511	-2.97691	0.00634618

9		70015411				
XLOC_00189		1:162639149-				
8	Myoc	162658173	1.38254	0.197349	-2.8085	0.00634618
XLOC_06565						
2	Reg3g	6:78466268-78468872	214.689	31.3487	-2.77577	0.00634618
XLOC_01524		11:121053457-				
8	Sectm1b	121063569	0.868546	0.127885	-2.76375	0.0233719
XLOC_05727		4:148000721-				
3	Gm13054	148004014	6.76491	1.06767	-2.6636	0.00686653
XLOC_00444		1:162916550-				
9	Fmo6	162937225	1.52488	0.257978	-2.56337	0.00634618
XLOC_05378		4:115411623-				
1	Cyp4a12b	115439034	2.13431	0.381192	-2.48518	0.00634618
XLOC_06072						
3	Cnga1	5:72603695-72642752	0.311571	1.65699	2.41093	0.00634618
XLOC_04968		3:114030539-				
9	Col11a1	114220718	0.562002	0.109728	-2.35664	0.0187494
XLOC_06992						
0	Sbk2	7:4957080-4964348	2.42296	0.479149	-2.33822	0.00791765
XLOC_07794						
0	Cyp11a1	9:57697602-57703823	51.9639	10.7954	-2.2671	0.00634618
XLOC_03429		17:80896864-				
9	-	80928497	12.6891	61.0005	2.26523	0.00634618
XLOC_06656		6:128539821-				
5	BC048546	128581606	9.52031	2.0065	-2.24633	0.00634618
XLOC_04365		2:130274429-				
4	Nop56	130284547	62.102	284.344	2.19493	0.00634618

XLOC_03750		18:67321208-				
0	Cidea	67367794	3.7549	0.823973	-2.18811	0.00634618
XLOC_03568		17:45630203-				
6	Capn11	45659325	0.941383	0.207637	-2.18071	0.00634618
XLOC_01682	Serpina3k,Ser	12:104338485-				
2	pina3m	104394257	1.956	0.45524	-2.10321	0.00634618
XLOC_05398		4:123787873-				
6	Rhbd12	123830013	1.48329	0.357287	-2.05365	0.00634618
XLOC_01060		11:54303797-				
2	Acsl6	54364756	0.402241	0.0997272	-2.012	0.0408845
XLOC_07348						
9	Ank1	8:22974843-23150497	6.75914	1.68162	-2.00699	0.00634618
XLOC_06305						
8	AC140385.1	6:67896176-67896642	4.02257	15.6759	1.96236	0.00634618
XLOC_04453		2:173153047-				
3	Pck1	173159273	9.55376	2.45353	-1.96121	0.0210639
XLOC_03075		16:90738323-				
4	Mrap	90749785	3.03823	0.813923	-1.90027	0.00634618
XLOC_06089						
1	Sult1d1	5:87554644-87569027	26.7668	7.17198	-1.9	0.00634618
XLOC_05956		5:137981520-				
5	Azgp1	137990233	3.74742	1.00488	-1.89887	0.00634618
XLOC_05746		4:156034608-				
4	Ttll10	156059802	5.55515	1.51785	-1.8718	0.00634618
XLOC_02520		14:56059743-				
5	Mcpt4	56062310	1.996	0.554963	-1.84665	0.0117784

Supplementary Table 4-7: Important parameters used in SkyScan1176 for the in vivo lung micro-CT scans

Parameters	Description
Source Voltage	50 kV
Source Current	500 μ A
Image Pixel Size	9 μ m
Filter	0.5 mm Al
Exposure	1240 ms
Rotation Step	0.500 deg
Frame Averaging	ON (3)
Scan duration	00:32:25

4.8. Acknowledgements

We would like to thank Songyan Liu for bioinformatics analysis and Luke DeLange for assistance with vector construction. This work was supported by the Canadian Institutes of Health Research; the Manitoba Medical Services Foundation; the Children's Hospital Research Institute of Manitoba; the Molly Towell Perinatal Research Foundation; Department of Surgery GFT Surgeons; the Thorlakson Foundation; RK is the recipient of a Career Development Award from the Canadian Child Health Clinician Scientist Program and a New Investigator Salary Award from the Canadian Institutes of Health Research, Manitoba Lung Association and the Children's Hospital Research Institute of Manitoba. Canadian Institutes of Health Research and the Manitoba Research Innovation Fund supports GGH. AJH is supported by the Canada Research Chair Program.

CHAPTER 5. Manuscript III

Prenatal microRNA miR-200b therapy improves nitrofen-induced pulmonary hypoplasia associated with the congenital diaphragmatic hernia.

Naghmeh Khoshgoo, Ramin Kholdebarin, Patricia Pereira-Terra, Thomas H. Mahood, Landon Falk, Chelsea A. Day, Barbara M. Iwasiow, Fuqin Zhu, Drew Mulhall, Carly Fraser, Jorge Correia-Pinto, Richard Keijzer

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(https://journals.lww.com/annalsofsurgery/Abstract/publishahead/Prenatal_microRNA_miR_200b_Therapy_Improves.95830.aspx).

5.1. Abstract

Objective: We aimed to evaluate the use of miR-200b as a prenatal transplacental therapy in the nitrofen rat model of abnormal lung development and congenital diaphragmatic hernia (CDH).

Background: Pulmonary hypoplasia and pulmonary hypertension determine mortality and morbidity in CDH babies. There is no safe medical prenatal treatment available for this disorder. We previously discovered that higher miR-200b is associated with better survival in CDH babies. Here, we investigate the role of miR-200b in the nitrofen rat model of pulmonary hypoplasia and CDH and evaluated its use as an *in vivo* prenatal therapy.

Methods: We profiled miR-200b expression during nitrofen-induced pulmonary hypoplasia using RT-qPCR and *in situ* hybridization in the nitrofen rat model of pulmonary hypoplasia and CDH. The effects of nitrofen on downstream miR-200b targets were studied in bronchial lung epithelial cells using a SMAD luciferase assay, Western blotting and Immunohistochemistry. We evaluated miR-200b as a lung growth promoting therapy *ex vivo* and *in vivo* using lung explant culture and transplacental prenatal therapy in the nitrofen rat model.

Results: We show that late lung hypoplasia in CDH is associated with (compensatory) upregulation of miR-200b in less hypoplastic lungs. Increasing miR-200b abundance with mimics early after nitrofen treatment decreases SMAD-driven TGF- β signaling and rescues lung hypoplasia both *in vitro* and *in vivo*. Also, prenatal miR-200b therapy decreases the observed incidence of CDH.

Conclusions: Our data indicate that miR-200b improves pulmonary hypoplasia and decreases the incidence of CDH. Future studies will further exploit this newly discovered prenatal therapy for lung hypoplasia and CDH.

5.2. Introduction

Congenital Diaphragmatic Hernia (CDH) is characterized by the incomplete formation of the posterolateral diaphragm, resulting in herniation of the abdominal viscera into the chest cavity (Veenma et al. 2012; Longoni et al. 2014). The incidence of CDH is 1 in 2000 to 3000 live births (Langham Jr. et al. 1996; Wynn et al. 2013). Substantial morbidity and mortality result from the associated pulmonary hypoplasia (PH) and persistent pulmonary hypertension (PPH) (Gischler et al. 2009; Van Loenhout et al. 2009).

The genetic cause of ~85% of patients with CDH is unknown (Pober et al. 2005a; Pober 2008), suggesting that epigenetics are involved. MicroRNAs are important epigenetic regulators (Kluth et al. 1990; M Inui et al. 2010). In 2015, we were the first to report that miR-200b is increased during late abnormal lung development in infants with CDH. In addition, we demonstrated that CDH babies with increased survival after a prenatal lung growth-promoting therapy – fetoscopic endoluminal tracheal occlusion (FETO) – have a higher miR-200b expression in their tracheal fluid compared to babies who did not survive (Pereira-Terra et al. 2015).

The miR-200 family is comprised of five members including miR-200b (Brabletz & Brabletz 2010). MiR-200 family members are considered epithelial markers and can revert a process called epithelial-mesenchymal transition (EMT) in cancer and pulmonary fibrosis by directly targeting two transcription factors, ZEB1 and ZEB2 (Howe et al. 2012; S M Park et al. 2008; J Kurashige et al. 2012; Yang et al. 2012). TGF- β /SMAD signaling promotes EMT by activating transcription factors including ZEB1 and ZEB2 (P A Gregory et al. 2008).

We recently published a meta-analysis that showed that FETO improves survival in CDH (Al-Maary et al. 2016). However, this invasive surgical therapy has adverse effects, such as preterm premature rupture of the membranes (Jani et al. 2009). Here, we explored a novel prenatal,

non-surgical treatment using miR-200b to improve abnormal lung development in a rat model of CDH. It is impossible to study early lung development in humans with CDH, therefore, we resorted to the nitrofen rat model of CDH (Keijzer et al. 2000). In this study, the spatiotemporal expression pattern of miR-200b during normal and nitrofen-induced abnormal lung development due to CDH shows higher expression of miR-200b in less hypoplastic lungs from non-CDH pups just before birth. Nitrofen-induced inhibition of miR-200b increases SMAD-driven TGF- β signaling in lung bronchial epithelial cells. More importantly, nitrofen-induced abnormal branching morphogenesis can be rescued both *ex vivo* and *in vivo*. Finally, we demonstrate that prenatal transplacental miR-200b therapy reduces the incidence of CDH and improves lung morphometry in the nitrofen rat model of CDH.

5.3. Results

MiR-200b expression is higher in less hypoplastic lungs from rats without CDH just before birth.

We recently reported the full transcriptomes of embryonic day 13 (E13) lungs from control and nitrofen-induced hypoplastic lungs (Mahood et al. 2016). Analysis of the primary data set for the abundance of all members of the miR-200 family revealed that miR-200b-3p ($p = 1.78\text{E-}28$) and miR-200b-5p ($p = 1.89\text{E-}11$) were down-regulated significantly when compared to control lung tissues at this early stage of lung development (E13) (Fig. 5-1a). Based on these findings we used Integrated Pathway Analysis and identified several signaling pathways of interest in hypoplastic lungs, including TGF- β 1 ($p = 1.15\text{E-}4$); FOXL-2 ($p = 1.31\text{E-}4$); and, FGF-2 ($p = 1.87\text{E-}4$) (Fig. 5-1b).

We performed RT-qPCR in normal and hypoplastic lungs during lung development at E13, E15, E18 and E21 to evaluate the temporal expression of miR-200b-3p (Fig. 5-1c). Expression of miR-200b was not significantly different at the early stages of lung development when normal closure of the diaphragm has not yet occurred and discrimination between CDH and non-CDH is impossible. When we compared miR-200b expression in nitrofen-induced hypoplastic lungs with and without CDH at later stages of lung development (E21), the abundance of miR-200b was significantly higher in hypoplastic lungs without CDH (moderate hypoplasia) compared to control lungs and hypoplastic lungs with CDH (severe hypoplasia) ($p = 0.0118$) (Fig. 5-1d).

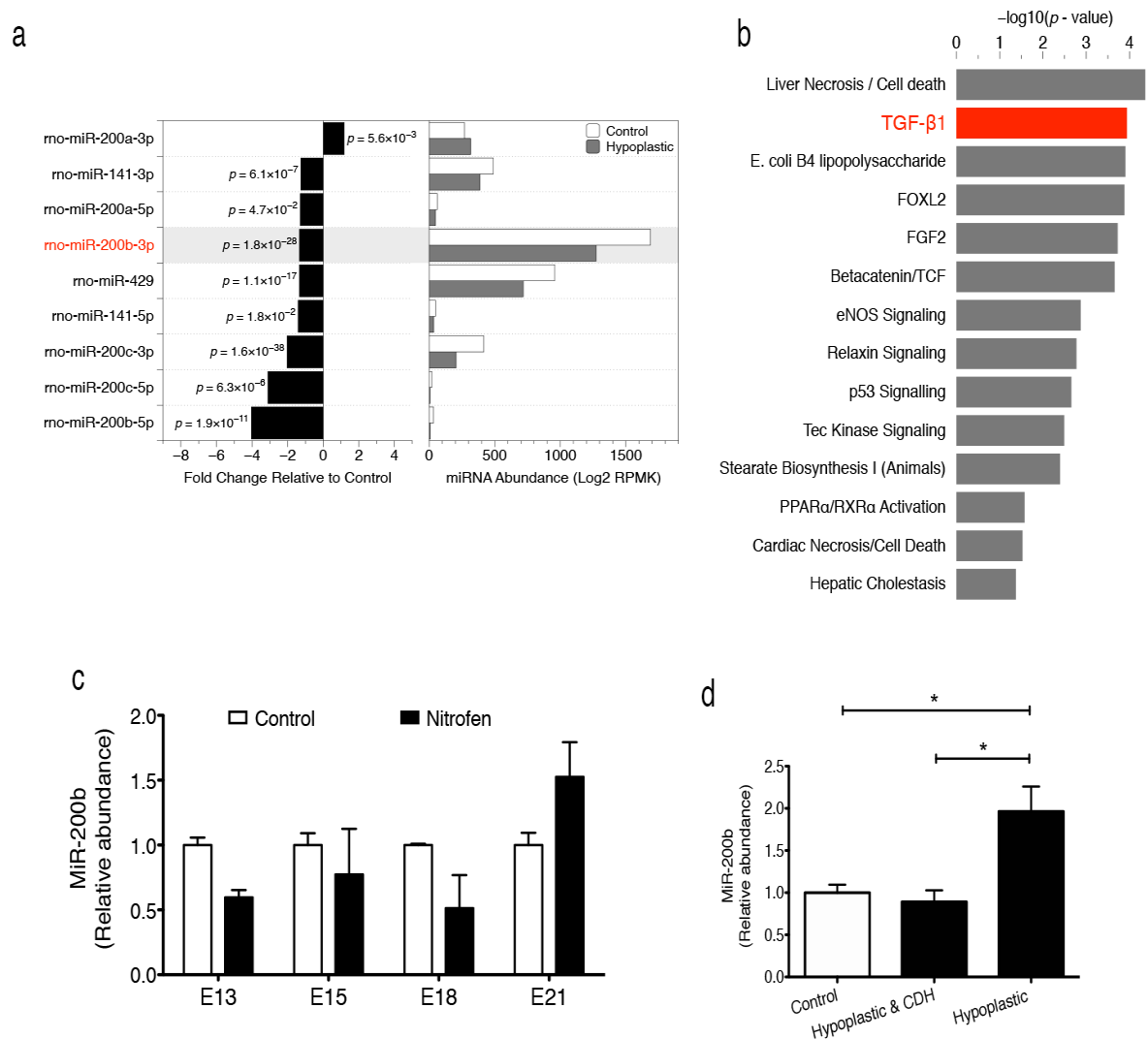


Figure 5-1: MiR-200b expression is dependent on the developmental stage and degree of lung hypoplasia in the nitrofen rat model of CDH. (a) MiR-200 family members abundance and fold change using miRNA sequencing on control or hypoplastic E13 lungs. All miRNAs shown, except miR-200a, are down-regulated in the hypoplastic lungs. miR-200b-5p ($p = 1.89 \times 10^{-11}$) and miR-200b-3p ($p = 1.78 \times 10^{-28}$) were down-regulated significantly when compared to control lung tissues. miR-200b has the highest abundance among all members (b) Integrated pathway analysis of mRNA transcripts identified the strongest pathway relationships in hypoplastic lungs compared to control lungs. (c) RT-qPCR for miR-200b expression on lungs from controls and nitrofen-induced hypoplastic lungs at embryonic day (E) 13, 15, 18 and 21. The expression of miR-200b was decreased in hypoplastic lungs compared to control in E13, 15 and 18. The expression increased at E21, but the difference was not significant. Selection of lungs was regardless of herniation. (d) RT-qPCR on E21 lungs considering herniation and severity of lung hypoplasia. Expression of miR-200b is upregulated in hypoplastic lungs with no herniation, but there is no change in expression of miR-200b in hypoplastic lungs with CDH. * $P < 0.05$ one-way ANOVA, all $n = 4$ per group.

Distribution of miR-200b expression is dynamic during lung development and disturbed in nitrofen-induced lung hypoplasia

We performed *in situ* hybridization to evaluate the tissue distribution of miR-200b in normal and hypoplastic lungs (Fig. 5-2). At E13 and E15, the parabronchial mesenchyme displayed high miR-200b expression surrounding the elongating branching airway tips (Fig. 5-2a, b). Hypoplastic lungs had lower miR-200b expression. This was more pronounced in the pulmonary mesenchyme, particularly in the mesenchyme surrounding the distal epithelium at E13 and E15. We have previously shown that the pulmonary mesenchyme is probably responsible for the observed abnormal lung development in the nitrofen rat model of CDH (Van Loenhout et al. 2012). During the canalicular stage of lung development (E18), the expression of miR-200b in the distal airway tips remained high but decreased in the mesenchymal layer relative to E15. We observed lower miR-200b expression in both the mesenchyme and epithelium of hypoplastic lungs (Fig. 5-2c). At E21, during the saccular stage, we observed higher expression of miR-200b in the proximal epithelial lining of the large airways as compared to the epithelial cells in the distal terminal saccules. There was no difference in the distribution of miR-200b between control and hypoplastic lungs with CDH (Fig. 5-2d).

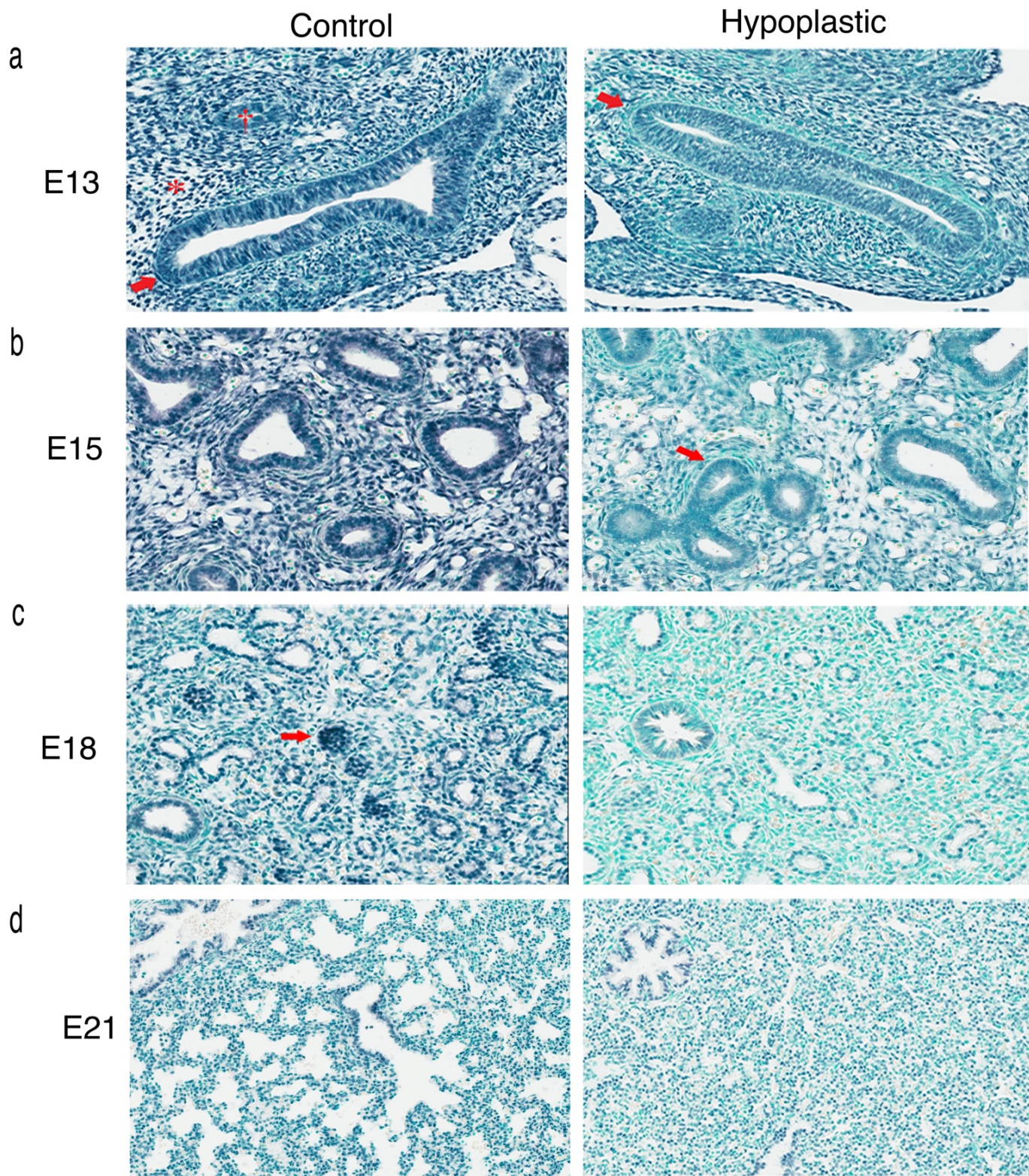


Figure 5-2: MiR-200b spatial expression is disturbed during abnormal lung development in nitrofen-induced hypoplastic lungs. In situ hybridization on normal and nitrofen-induced hypoplastic lungs showed spatial expression of miR-200b in control and hypoplastic lungs (200X magnification) (a-d). blue = miR-200b signal, green = background stain). (a) At E13, in normal lung, undifferentiated splanchnic mesenchyme (*) is positive for miR-200b. Also, distal parabronchial cells (arrow) are positive for miR-200b, while proximal parabronchial cells are

negative. In nitrofen-induced hypoplastic lungs at E13, both distal and proximal parabronchial cells are negative for miR-200b. ((†) indicates esophagus). (b) At E15, in control lungs, most cells are positive for miR-200b, but nitrofen-induced hypoplastic lungs have lower expression of miR-200b, especially in the parabronchial mesenchyme (arrow). (c) At E18, nitrofen-induced hypoplastic lungs have lower expression of miR-200b and contain fewer tip structures. (d) E21 control lungs and E21 nitrofe-induced hypoplastic lungs show no difference in expression of miR-200b.

The parabronchial mesenchymal cells in hypoplastic lungs with lower miR-200b expression lack adipose differentiation-related protein

To define the cell type of the parabronchial mesenchymal cells that had lower miR-200b expression in hypoplastic lungs, we combined *in situ* hybridization with immunohistochemistry. Combined detection of miR-200b and α -smooth muscle actin (α -SMA) demonstrated no differences in expression of α -SMA expression in the parabronchial cells with decreased miR-200b expression (Fig. 5-3a). When we combined *in situ* hybridization with immunohistochemistry for adipose differentiation-related protein (ADRP, a marker for lipofibroblasts (Ahlbrecht & McGowan 2014)), we found that ADRP expression is disturbed in this cell layer (Fig. 5-3b).

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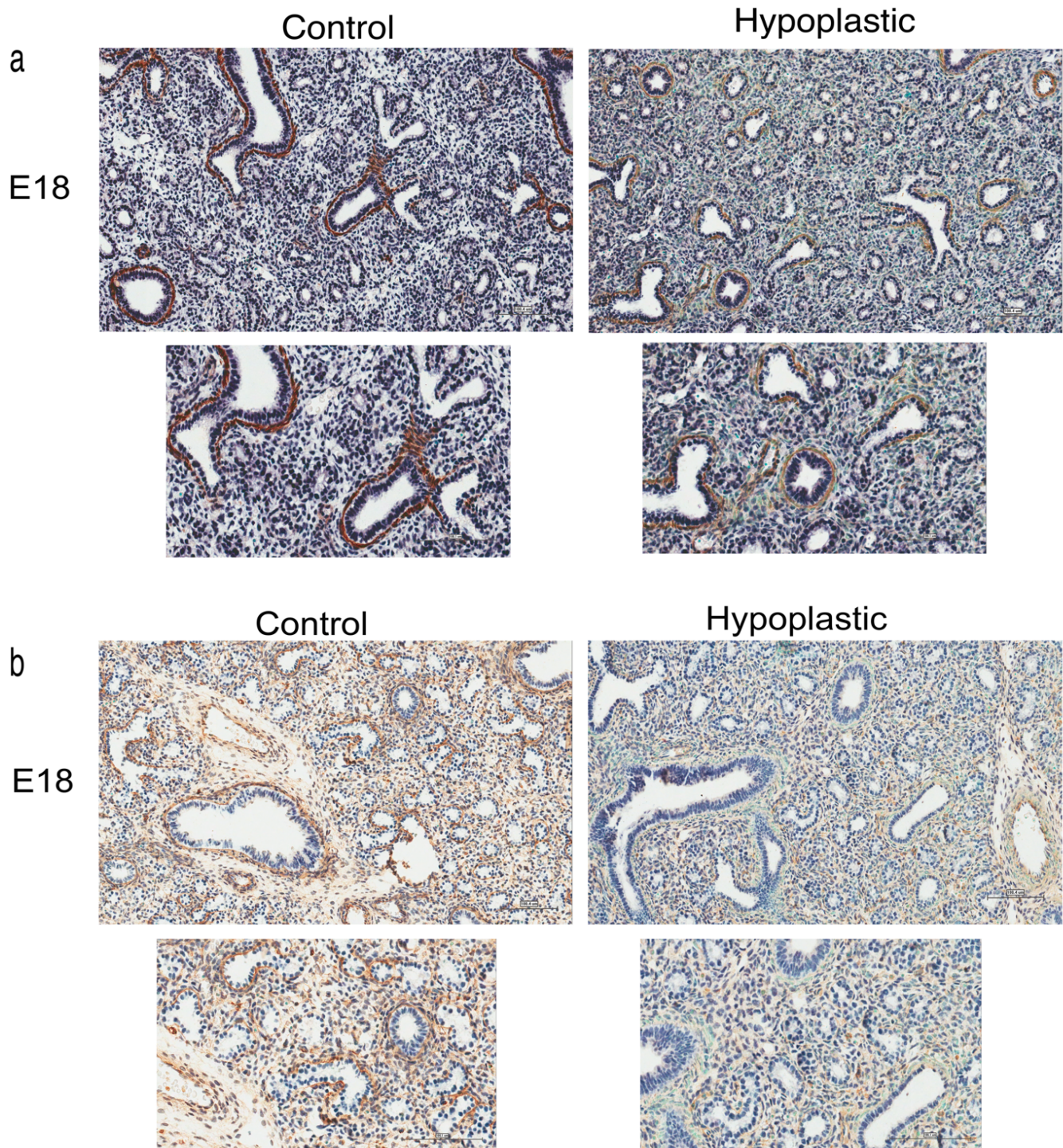


Figure 5-3: The parabronchial mesenchymal cells in hypoplastic lack adipose differentiation-related protein. Combined in situ hybridization/immunohistochemistry for miR-200b and alpha-SMA (a) or ADRP (b) was performed on control and nitrofen-induced hypoplastic lungs at E18. (a) We observed no differences in alpha-SMA protein expression in parabronchial mesenchymal cells between control and nitrofen-induced hypoplastic lungs. (b) Both miR-200b and ADRP levels were lower in nitrofen-induced hypoplastic lungs in the cells surrounding the large airways compared to control. Immunohistochemistry signal: brown; miR-200b expression: blue.

Nitrofen-induced inhibition of miR-200b increases SMAD-driven TGF- β signaling in lung bronchial epithelial cells

To understand the interaction between miR-200b and SMAD-driven TGF- β signaling, we next exposed human bronchial epithelial cells, (BEAS-2B) to nitrofen *in vitro* and found that miR-200b expression was down-regulated ($p = 0.0481$) (Fig. 5-4a). We showed previously that SMAD-luciferase activity increases in response to miR-200b inhibition using oligonucleotides (Pereira-Terra et al. 2015). Here, we show that nitrofen-induced inhibition of miR-200b directly increases SMAD-luciferase activity. Using miR-200b mimics to increase miR-200b levels in nitrofen-treated cells abrogated nitrofen-induced SMAD luciferase activation (Fig. 5-4b).

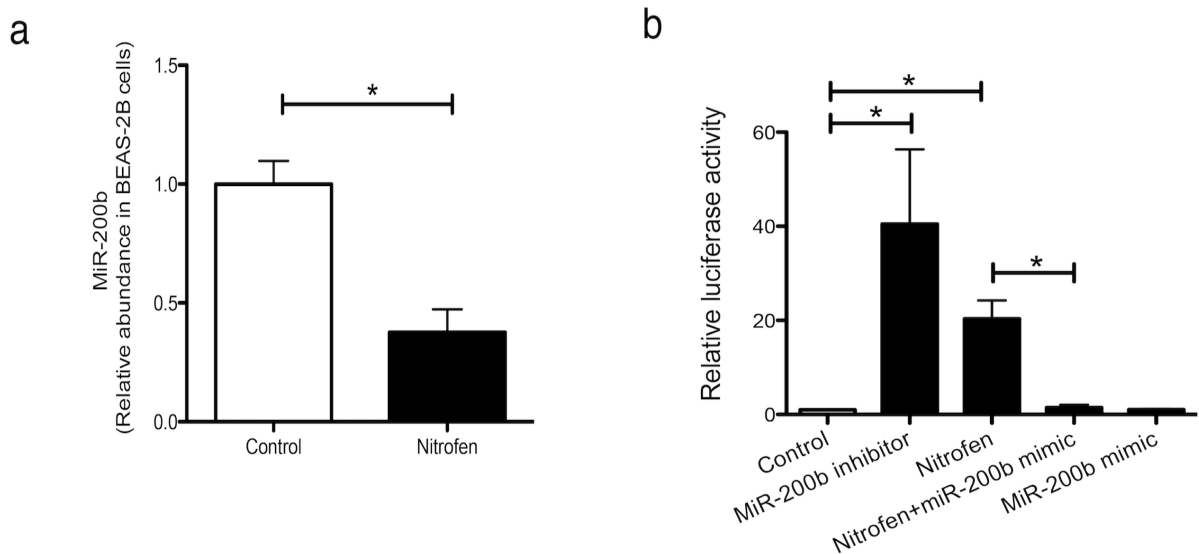


Figure 5-4: MiR-200b downregulation by nitrofen increased SMAD-driven TGF- β signaling. (A) Expression of miR-200b is significantly decreased in BEAS-2B cells treated with nitrofen for 48 hours. * $P < 0.05$ student's *t*-test, all $n = 3$ per group. (B) Effect of miR-200b and nitrofen on SMAD2/3 signaling as measured by a dual luciferase assay. Bronchial epithelial cells treated with nitrofen stimulate smad2/3 signaling. miR-200b mimics (overexpression of miR-200b) decrease the effect of nitrofen on smad2/3 signaling. * $P < 0.05$, one-way ANOVA, all $n = 4$ per group.

We validated these luciferase assays using Western blotting for SMAD2 and p-SMAD2 (the activated form of SMAD2). We confirmed that both SMAD2 and p-SMAD2 were increased in the nitrofen-treated human bronchial epithelial cells compared to control cells (Fig. 5-5a,b). To evaluate the impact of nitrofen on known targets of miR-200b, we measured TGF- β 2, ZEB1 and ZEB2 abundance using Western blotting in nitrofen-treated cells. The abundance of ZEB2 was higher in these cells (Fig. 5-5c,d), but ZEB1 and TGF- β 2 were not affected by nitrofen (Supplementary Fig. 5-8 and Supplementary Fig. 5-9). Immunohistochemistry for ZEB2 in hypoplastic rat lungs at E18 and E21 showed a higher abundance of ZEB2 in both epithelial and mesenchymal cells (Fig. 5-5e).

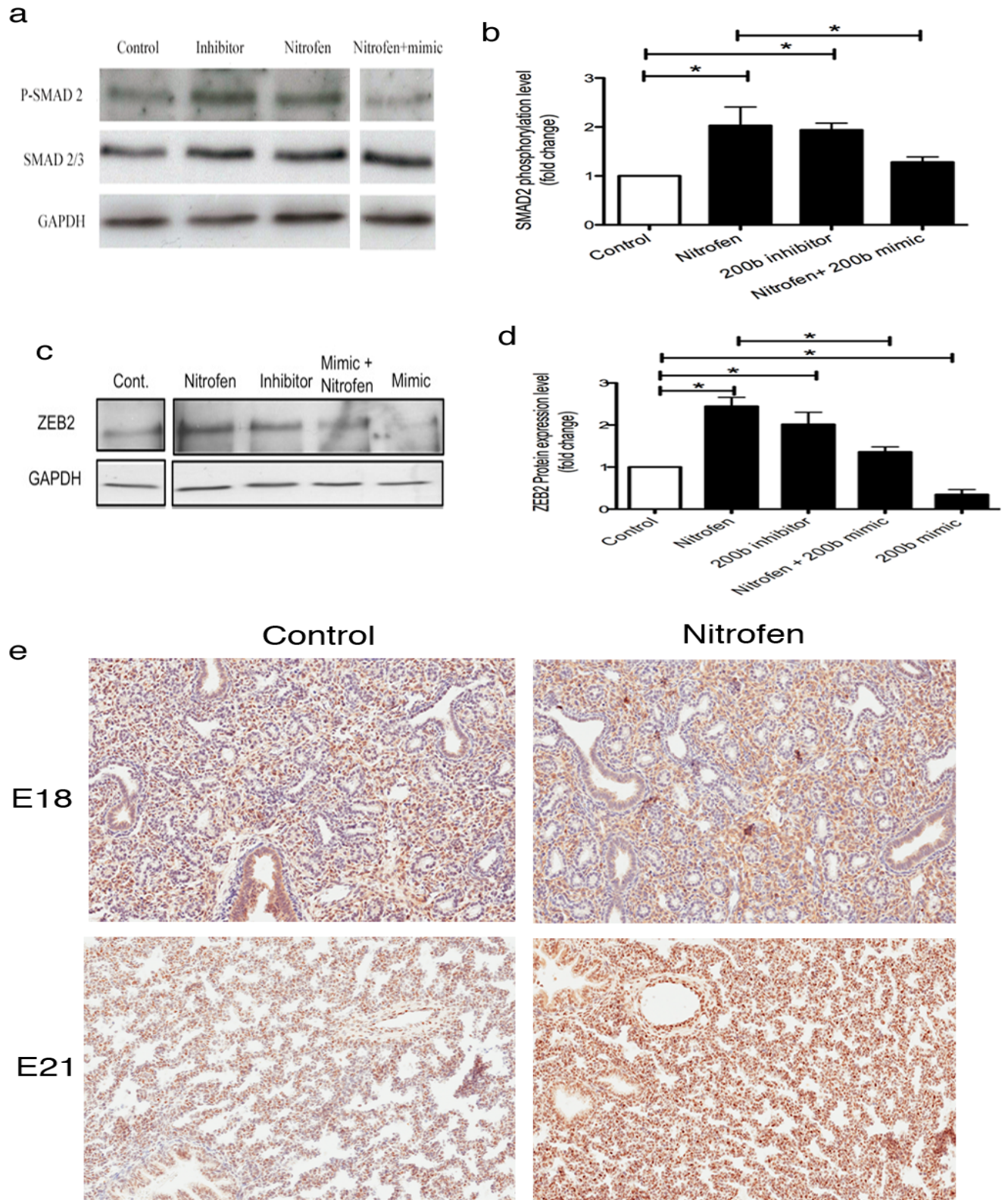


Figure 5-5: MiR-200b can normalize the TFG- β overexpression in nitrofen treated cells (a) Nitrofen promotes SMAD2 phosphorylation as measured by Western blot. In BEAS2B cells,

*nitrofen stimulate smad2 phosphorylation, and miR-200b mimic (overexpression of miR-200b) decrease the effect of nitrofen on smad2 phosphorylation. *P < 0.05, one-way ANOVA, all n=3 per group. (a,b) Nitrofen can promote ZEB2 expression and overexpression of miR-200b can reduce it. *P < 0.05 one-way ANOVA, all n=3 per group. (b) The ZEB2 expression is higher in nitrofen-induced hypoplastic lungs. Immunohistochemistry on control and nitrofen lung sections at E18 and E21.*

Reintroducing miR-200b corrects nitrofen-induced abnormal branching morphogenesis in *ex vivo* lung explants

In situ hybridization of serial sections of lungs at the pseudoglandular stage of normal lung development (E15) demonstrated that the tips of the elongating airways have the highest miR-200b expression, suggesting an important role for miR-200b in branching morphogenesis (Fig. 5-6a). We observed fewer tip structures expressing miR-200b in hypoplastic lungs compared to control lungs during the canalicular stage (Fig. 5-2c). To explore if miR-200b can directly affect lung branching morphogenesis in normal or nitrofen lungs, we used an *ex vivo* lung explant culture system. We dissected control rat lungs at E13 and incubated them for one hour with 200nM of either negative control miRNA, scrambled nucleotide oligomers, or miR-200b inhibitors. Peripheral airway buds were counted at day 0 (D0) and day 4 (D4). MiR-200b inhibitors decreased the ratio between D4 and D0 of the total number of peripheral airway buds demonstrating a direct inhibitory effect of reduced miR-200b expression on airway branching (Fig. 5-6b,d). Hypoplastic E13 lungs from nitrofen-exposed rats were incubated with 200nM of miR-200b mimics, or negative control scrambled nucleotide oligomers. Treatment of nitrofen-induced hypoplastic lung explants with miR-200b mimics promoted the formation of peripheral airway buds to normal levels (Fig. 5-6e,g). Changes in total miR-200b expression after lung explant cultures were verified by RT-qPCR to ensure transfection efficiency (Fig. 5-6c,f)

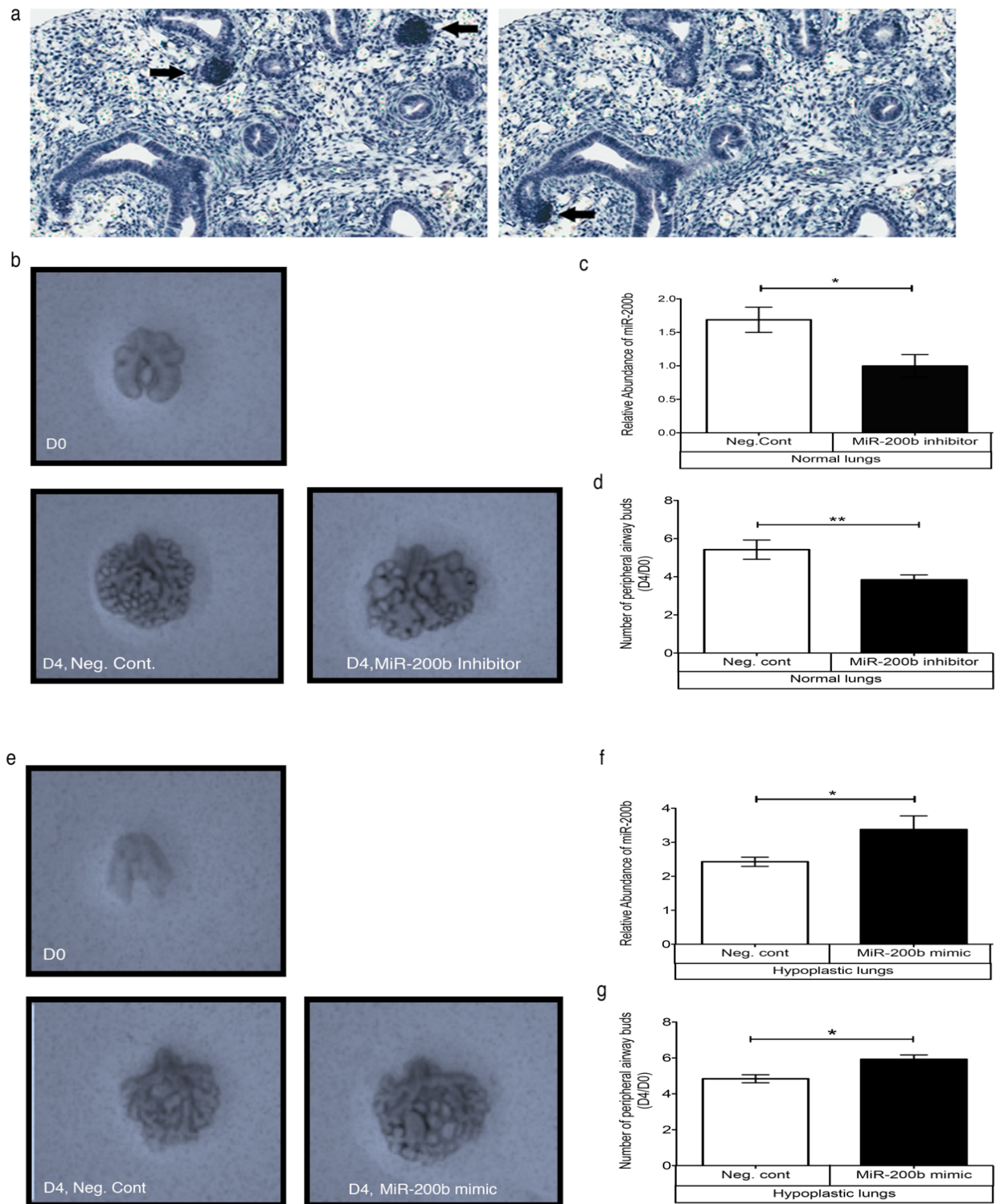


Figure 5-6: MiR-200b is required for normal branching morphogenesis. (a) *In situ* hybridization for miR-200b on serial sections of normal rat lung at E15. miR-200b is expressed in both epithelial

and mesenchymal cell layers. The highest expression is on elongating tip structure of the developing branching airways (black arrows). (b) Lung explant culture of normal lungs treated with miR-200b inhibitors. Upper panel is representative of lung explants at Day Zero Bottom panel represents lung explants treated with the miR-200b inhibitor for four days. (c) MiR-200b expression is significantly down-regulated in lungs treated with the miR-200b inhibitor (d) The number of peripheral airway buds is significantly decreased in lungs transfected with miR-200b inhibitors. (e) Lung explant culture of nitrofen-induced hypoplastic lungs treated with miR-200b mimics. Upper panel is representative of hypoplastic lung explants at Day Zero Bottom panel represents hypoplastic lung explants treated with miR-200b mimic for four days. (f) Up-regulation of miR-200b was confirmed by qPCR in lungs treated with MiR-200b mimic. (g) Re-introducing miR-200b can normalize nitrofen-induced abnormal branching morphogenesis. (D0: Day Zero) (D4: Day Four) * $P < 0.05$ ** $P < 0.01$, Student's *t*-test, $n = 9$ lungs per group.

Prenatal transplacental miR-200b *in vivo* therapy reduces the incidence of CDH and improves lung morphology.

Next, we examined a role for miR-200b as a prenatal transplacental therapy in lung hypoplasia and CDH *in vivo*. We administered stabilized miR-200b mimic or cel-miR-239b (negative control nucleotide oligomer) to dams intravenously after nitrofen gavaging at E9 of gestation. Others have shown previously that microRNAs can pass the placenta and regulate fetal genes (Li et al. 2015).

We observed that 90% of the pups in the miR-200b prenatal therapy group were more active and pink up till 1 hour after birth (predefined end-point of study). In contrast, 70% of the pups in the control group died within the first 10 minutes after birth (Fig. 5-7a). The incidence of CDH in the miR-200b prenatal therapy group was lower than in the control group (26.4% vs. 60%, $p < 0.0001$) (Fig. 5-7b, Supplementary table 5-1). We evaluated lung morphometry by measuring the mean linear intercept, representing the size of the air sacs, and surface tissue density on randomly selected H&E stained sections of E21 lungs with or without CDH. Hypoplastic lungs from rats without CDH in the miR-200b prenatal therapy group had a higher mean linear intercept and lower surface tissue density compared to the control group (Fig. 5-7c-e). The mean linear

intercept of lungs with CDH in the miR-200b prenatal therapy group was higher than in the control group, but the difference was not significant (Supplementary Fig. 5-11).

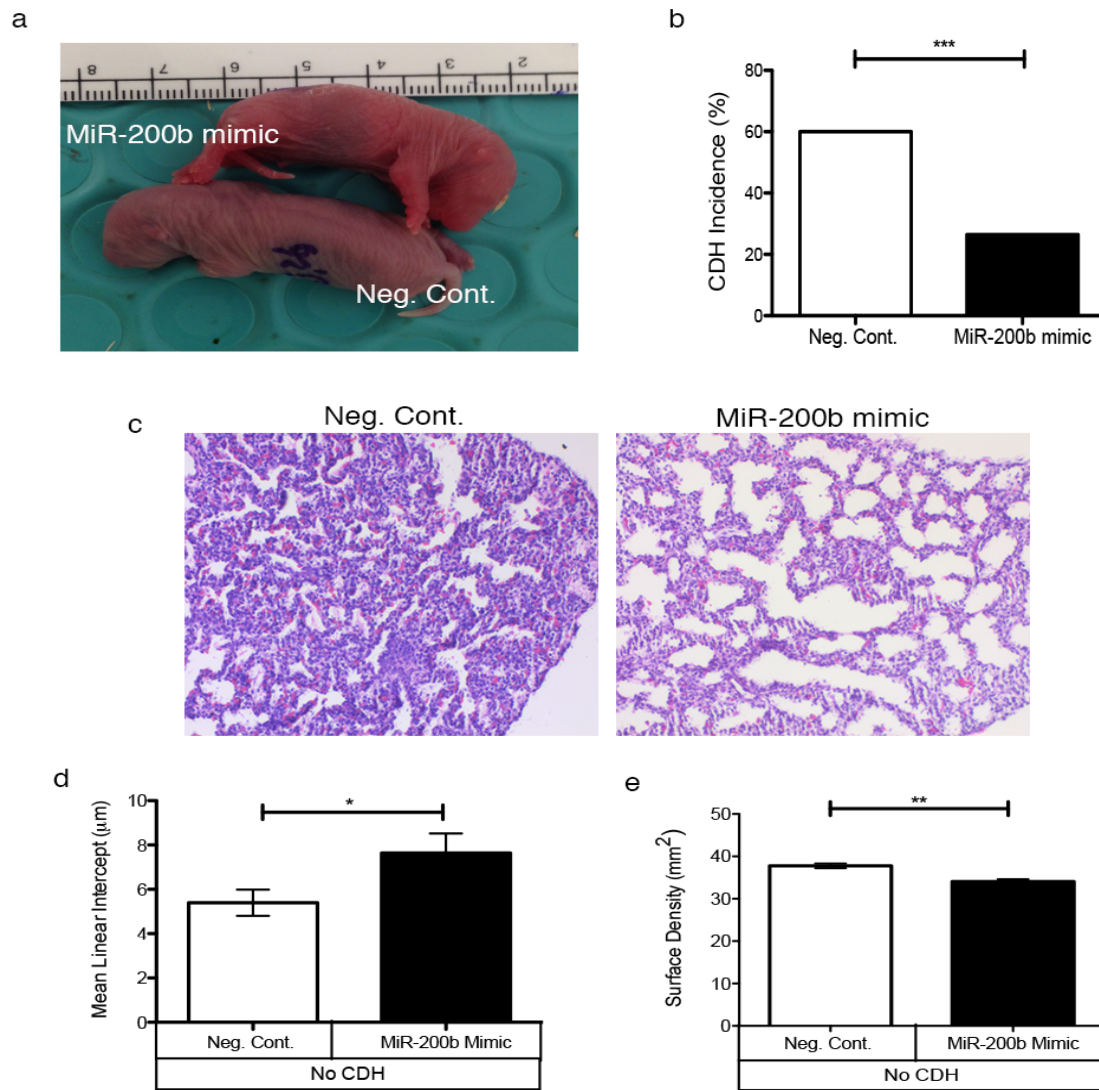


Figure 5-7: Maternal administration of miR-200b significantly reduces the incidence of CDH and improves lung morphology. MiR-200b mimic or oligo negative control was injected intravenously to the dams (6 and 4 rats respectively) at E9 of gestation. The outcome of the treatments was evaluated on P1 exactly after delivery for 1 hour. (a) The pups in miR-200b group were active and pink during the observation time. (b) Comparing the incidence of CDH using Chi-square analysis showed the very significant relationship between treatments (miR-200b vs. negative control) and the outcomes (CDH vs. non-CDH). *** $P < 0.001$, $n = 121$ lungs for miR-200b

group, $n = 60$ lungs for negative control group. (c) H&E staining of the lungs from the E21 embryos without CDH showed improved in lung hypoplasia in miR-200b group. (d) MiR-200b group has significantly higher lung mean linear intercept compare to negative control group in E21 embryos without CDH. (e) MiR-200b group has significantly lower lung surface tissue density compare to negative control in pups without CDH. $*P < 0.05$ $**P < 0.01$. Student's t -test, $n = 3$ lungs per group (total fields of 30 per lung used for mean linear intercept and surface density).

5.4. Discussion

We are the first to describe that prenatal miR-200b therapy reduces the incidence of CDH in the nitrofen rat model of CDH and improves nitrofen-induced lung hypoplasia in pups with no CDH. We also demonstrate that the level of miR-200b expression is negatively correlated with the severity of lung hypoplasia in the nitrofen rat model at later stages of lung development (E21). When miR-200b expression is decreased by nitrofen *in vitro*, TGF- β /SMAD signaling is induced and the expression of miR-200b target ZEB-2 increases.

Our *in situ* hybridization studies show that the highest miR-200b expression is observed in the tips of the elongating airways, while differentiated proximal airway cells have very low expression of miR-200b. Nitrofen-induced hypoplastic lungs have fewer numbers of these elongating buds with lower expression of miR-200b at the tips and the mesenchymal tissue surrounding the airways. Rawlins *et al.* have shown that these distal tip cells are comprised of epithelial progenitors that give rise to the more differentiated proximal cells (Rawlins et al. 2009). In addition, Wang *et al.* have demonstrated that the miR-200/ZEB2 axis can induce pluripotent stem cell generation (G. Wang et al. 2013). Therefore, future studies should evaluate if miR-200b is involved in maintaining the pluripotent features of the distal tip cells in the tips of the elongating airways.

At the earlier stages of lung development - before closure of the diaphragm normally occurs - we cannot determine the severity of lung hypoplasia in relation to abnormal diaphragm closure. Therefore, miR-200b abundance in the hypoplastic lungs was measured without considering the degree of lung hypoplasia. In contrast, we can determine the severity of lung hypoplasia in relation to a diaphragmatic defect at the later stages of lung development (E21). Interestingly, miR-200b expression was higher in the less hypoplastic lungs from fetuses without CDH. This validates our

previous studies in human CDH in which we observed higher miR-200b abundance in the tracheal fluid of CDH babies with less hypoplastic lungs and improved survival (Pereira-Terra et al. 2015).

Friedmacher *et al.* demonstrated that ADRP - a cell marker for lipofibroblasts - abundance is decreased at both the mRNA and protein level in nitrofen-induced hypoplastic lungs (Friedmacher et al. 2014). Our combined *in situ* hybridization/immunohistochemistry studies show that the cells with disturbed ADRP protein abundance are the distal parabronchial mesenchymal cells. This suggests that lipofibroblast differentiation around the developing airways is disrupted. Interestingly, these cells have the lowest miR-200b expression, as well, suggesting a role for miR-200b in ADRP production. Others have shown that lipofibroblasts and ADRP have a critical role in surfactant synthesis (Torday & Rehan 2011). Surfactant deficiency is observed in both human and rat CDH (Watanabe et al. 2013; Cogo et al. 2013), although some studies failed to show a difference in the CDH sheep model and human CDH lungs (Boucherat et al. 2007). Also, we recently showed that miR-200b knockout mice have impaired surfactant function and decreased surfactant protein B and C expression, indicating a role for miR-200b in surfactant production and function (Khoshgoo et al. 2017). Therefore, the molecular mechanisms to understand the role of miR-200b in ADRP and surfactant protein production and function should be the objective of future studies.

Mechanistically, we demonstrate that nitrofen has an inhibitory effect on the expression of miR-200b in human bronchial epithelial cells. Lower abundance of miR-200b promotes SMAD-driven TGF- β signaling at two levels: p-SMAD and ZEB2. The increase in SMAD-driven TGF- β signaling can be abrogated by reintroducing miR-200b with mimics. These data demonstrate that nitrofen promotes TGF- β /SMAD signaling by decreasing miR-200b expression and that miR-200b negatively regulates SMAD2/3 signaling. ZEB1 and ZEB2 are two well-known targets of miR-

200b (Xiong et al. 2012) and others have shown that abrogation of SMAD2, SMAD3 or SMAD4 expression increases murine embryonic lung branching in lung explant cultures (Zhao et al. 1998). Therefore, our data suggest that miR-200b promotes lung branching in nitrofen-induced hypoplastic lungs by negatively regulating SMAD2/3 signaling.

Our studies show that decreasing miR-200b expression in fetal lung explants with inhibitors or nitrofen treatment results in reduced branching morphogenesis. Increasing miR-200b abundance with mimics rescues this nitrofen-induced abnormal branching morphogenesis *ex vitro*. Taken together, these data demonstrate an important role for miR-200b in branching morphogenesis of the lung. Most importantly, we demonstrate that prenatal transplacental miR-200b therapy reduces the incidence of CDH from 60% to 23% in rats and improves nitrofen-induced lung hypoplasia in the pups with no CDH. According to the “dual-hit hypothesis”, pulmonary hypoplasia in CDH can be explained by two insults, one affecting both lungs before the development of the diaphragm starts and one affecting the ipsilateral lung only after the defect in the diaphragm occurs (Keijzer et al. 2000). Here, we show that prenatal transplacental miR-200b therapy not only prevents the second hit from happening by reducing the occurrence of diaphragmatic defects, but it can also significantly improve lung hypoplasia after the first hit. The remaining pups with CDH did not show significant improvement in lung hypoplasia. These data suggest that prenatal miR-200b therapy targets two aspects of CDH independently: CDH incidence is reduced and lung hypoplasia in pups without CDH is improved. Thus, we observed most beneficial effects of prenatal miR-200b therapy on the “first hit” of nitrofen-induced abnormal lung development in CDH.

Despite significant improvement in lung hypoplasia by prenatal therapy with miR-200b in the nitrofen rat model of CDH, the possible side effects and potential off-target effects are the

subjects of our future studies. One of the potential challenges with restoring the level of a down-regulated miRNA is the introduction of supraphysiological levels of the miRNA. Therefore, we will determine the optimal dosing and timing of the miR-200b prenatal therapy. In some types of cancer, like ovarian cancer, miR-200 appears to have a dynamic role and expression profile (higher at an earlier stage and lower at the later stages) during tumor progression (Bendoraitė et al. 2010). Therefore, targeted delivery of miR-200b to the cells that need it in the hypoplastic lung is important to prevent unwanted side effects of this therapeutic approach.

The results of our studies provide us with new insights into the pathogenesis and the role of miR-200b in nitrofen-induced lung hypoplasia and CDH. We show that prenatal transplacental miR-200b therapy should be pursued as a novel prenatal medical therapy to improve abnormal lung development in CDH before babies are even born with this devastating disease.

5.5. Materials & Methods

Ethical approval

Our experiments were reviewed and approved by the Animal Research Review Committee at the University of Manitoba. Sprague-Dawley rats were maintained in accordance with the guidelines of “*Guide to the Care and Use of Experimental Animals*”. The animals were mated during the night cycle and the next day after confirming vaginal plug, was considered as embryonic day zero (E0).

Cell culture

BEAS-2B cells (human bronchial epithelial cells) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). These cells were cultured in coated tissue culture dishes with bronchial epithelial cell growth medium (BEGM, Lonza, CA). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂. Cells were detached from dishes using 0.25% Trypsin - 0.53mM EDTA solution containing 0.5% polyvinylpyrrolidone (PVP) during passaging.

Nitrofen rat model of lung hypoplasia and CDH

To induce CDH and lung hypoplasia in fetal rats, 100 mg of nitrofen was dissolved in 1 ml olive oil and given to the dams intragastrically on day 9 (Kluth et al. 1990). CDH and severe lung hypoplasia are normally observed in the nitrofen model in up to 70% of the offspring and moderate lung hypoplasia in the remaining 30%. Animals in the control group received only the vehicle olive oil.

RNA extraction and RT-qPCR

We used the miRCURY™ RNA Isolation Kit (Ambion), and miRCURY LNA™ Universal RT microRNA PCR kit (Exiqon, Denmark) per the manufacturer's instructions. Lungs from Embryonic day (E) 13, 15, 18 and 21 pups were isolated in ice cold PBS, snap-frozen in liquid nitrogen and stored at -80°C until processed. BEAS-2B cells were scraped after the experiment and snap frozen in liquid nitrogen and stored at -80°C until processed. Total RNA from embryonic lung tissues or BEAS-2B cells was extracted using the miRCURY™ Isolation Kit (Ambion), per the manufacturer's instructions. For microRNA analysis, cDNA was randomly primed from 20 ng total RNA using the Exiqon cDNA synthesis kit. RT-qPCR was subsequently performed using locked nucleotide acid (LNA) primers for rno-miR-200b, U6 and miR-103 (as endogenous controls). All primer sequences are provided in the Supplementary Information (Supplementary Table 5-1). We used the miRCURY LNA™ Universal RT microRNA PCR kit (Exiqon) in a total reaction volume of 20µl. Briefly, RT-qPCR was performed in triplicate with 1:80 dilution of cDNA using the SYBR green PCR system on a ABI 7500 Real-Time PCR machine (Applied Biosciences). Data were collected and analyzed using ABI 7500 v1.4.0 software (Applied Biosciences). MicroRNA expression levels were determined using the relative quantification method of the ABI 7500 v1.4.0 software.

Rno-LNA-miR-200b was hybridized with double-digoxigenin-labeled LNA probes (Exiqon, supplementary Table 5-2) for 1 hour at 52°C (30 °C below the calculated RNA melting temperature). The probes were detected with an alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody (1:500) (Roche, Mannheim, Germany). Sections were immunostained with 1-step NBT/BCIP solution containing 1 mM levamisole (Thermo Scientific, Rockford, IL, USA). The slides were then counterstained with methylgreen (Sigma-Aldrich, St. Louis, MO, USA). Digital microscopy was performed using the ScanScope CS system (Aperio, Vista, CA, USA).

***In situ* hybridization**

Lung tissues from the mentioned stages of lung development were isolated and embedded in paraffin. *In situ* hybridization was carried out on 5 µm tissue sections as previously described in section 3-5.

Immunohistochemistry and combined *in situ* hybridization/immunohistochemistry

Immunohistochemistry was performed on rat lung tissue sections using an anti-ZEB2 antibody (1:250, Sigma-Aldrich, St. Louis, MO, USA) (Chi & Chandy 2007). For combined *in situ* hybridization-immunohistochemistry experiments, at the end of the *in situ* hybridization protocol, the hybridized lung tissue sections were exposed to blocking solution. Anti-alpha-SMA (1:1000, CEDARLANE, CANADA) and Anti-ADRP (1:200, Santa Cruz Biotechnology, USA) were used as primary antibodies.

Loss- and gain-of-function studies and SMAD luciferase activity assays

To identify the best primary lung cell line to perform loss- and gain-of-function studies, we examined the expression of miR-200b in several lung cell lines (data not shown). We observed the highest miR-200b expression in BEAS-2B cells (ATCC, Manassas, VA, USA). We treated these cells with 10µM of nitrofen. After one hour of incubation, treated or untreated cells were co-transfected with 0.5 µg/ml of the SMAD reporter construct and 0.01 µg/ml of LNA-hsa-miR-200b inhibitor, mimic or negative control as previously described (Pereira-Terra et al. 2015). briefly, We treated these cells with 10µM of nitrofen. After one hour of incubation, treated or untreated cells were co-transfected with 0.5 µg/ml of the SMAD reporter construct (Signal SMAD Reporter

kit, SABiosciences, Frederick, MD, USA) and 0.01 µg/ml of LNA-hsa-miR-200b inhibitor, mimic or negative control (Exiqon)—using the X-tremeGENE siRNA Transfection Reagent (Roche). SMAD luciferase activity measured as previously described (Pereira-Terra et al. 2015). Briefly, after 48h, the luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Treated or untreated cells were lysed using 100 µl of passive lysis buffer of the kit and put on a shaker for 15 min, and the supernatant of the lysates were collected after centrifugation. 20µl of the supernatants was used for luciferase activity measurement using a (core BMG POLARstar OPTIMA) Microplate Reader. Luciferase activity between the treated groups was compared after normalization with Renilla luciferase activity.

Western blotting

Nitrofen-treated or untreated BEAS-2B cells were transfected with 0.01 µg/ml of LNA-hsa-miR-200b inhibitor, mimic or negative control. After 48h the cells were washed and protein extracted for Western blotting. Specific proteins were detected with the following antibodies: anti-SMAD2/3 (Cell Signaling Technology, Danvers, MA, USA), anti-phospho-SMAD2 (Cell Signaling Technology), anti-ZEB1 and anti-ZEB2 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TGF-β2 antibody (1:200, Abcam, Cambridge, MA, USA) anti-GAPDH (1:6000; Abcam, MA, USA), HRP-conjugated goat–anti-rabbit secondary antibody (Bio-Rad) and HRP-conjugated goat–anti-mouse secondary antibody (Bio-Rad). Exposed films were scanned and densitometry was performed on unmodified output images after background subtraction using ImageJ software (Wayne Rasband, NIH, USA) in a blinded fashion. Densitometric values were normalized against the corresponding GAPDH values.

Fetal lung explant cultures

Nitrofen-induced hypoplastic and control lungs were isolated from E13 embryos using microsurgical techniques and a stereomicroscope (Leica MZ6, Switzerland). Control lungs were treated with oligo negative control or miR-200b inhibitor and cultured for four days. Nitrofen-induced hypoplastic lungs were treated with 200nM negative control scrambled nucleotide oligomers, or 200nM miR-200b mimics (Exiqon, Denmark) and cultured for four days at 37°C in a humidified atmosphere of 5% CO₂. Each group were dipped into 200 µl of a 1:1 mixture of DMEM and Ham's F-12 Nutrient supplemented with 100 µg/ml streptomycin, 100 units/ml penicillin, 0.25 mg/ml ascorbic acid (Sigma-Aldrich, USA) containing 200nM mentioned oligomers, for one hour. Media and lungs were transferred to porous membranes (Isopore™ membrane filters with dimensions of 1 mm x 1.5 mm pore size, Millipore, USA) in 12 well plates for semi-dry floating lung explant cultures. Branching morphogenesis and epithelial perimeter length were monitored daily in all groups using a stereomicroscope and photographs were taken and measurements performed using ImageJ software. The difference between day 0 (D0: 0 hours) and day 4 (D4: 96 hours) of culture, were expressed as the D4/D0 ratio. We cultured a total of 36 lung tissues at E13 in four experimental groups (nine lungs for each group from three different litters).

***In vivo* prenatal transplacental miR-200b therapy**

We obtained custom-made *in vivo* stabilized miRIDIAN miR-200b mimics and cel-miR-239b (negative control nucleotide oligomers) from GE Dharmacon (Mississauga, Canada). miRIDIAN mimics have modifications for additional stability in nuclease-rich environments. At E9 of gestation, we first treated ten dams with nitrofen as described above and we then injected six dams

with 5mg/kg of rno-miR-200b mimics and four with cel-miR-239b intravenously. We evaluated the outcomes after prenatal transplacental therapy at two time points: E21, and postnatal day 1 (P1). The time of delivery and the time of death were recorded for all the pups at P1 and all pups alive after one hour were euthanized as requested by the Animal Research Review Committee at the University of Manitoba.

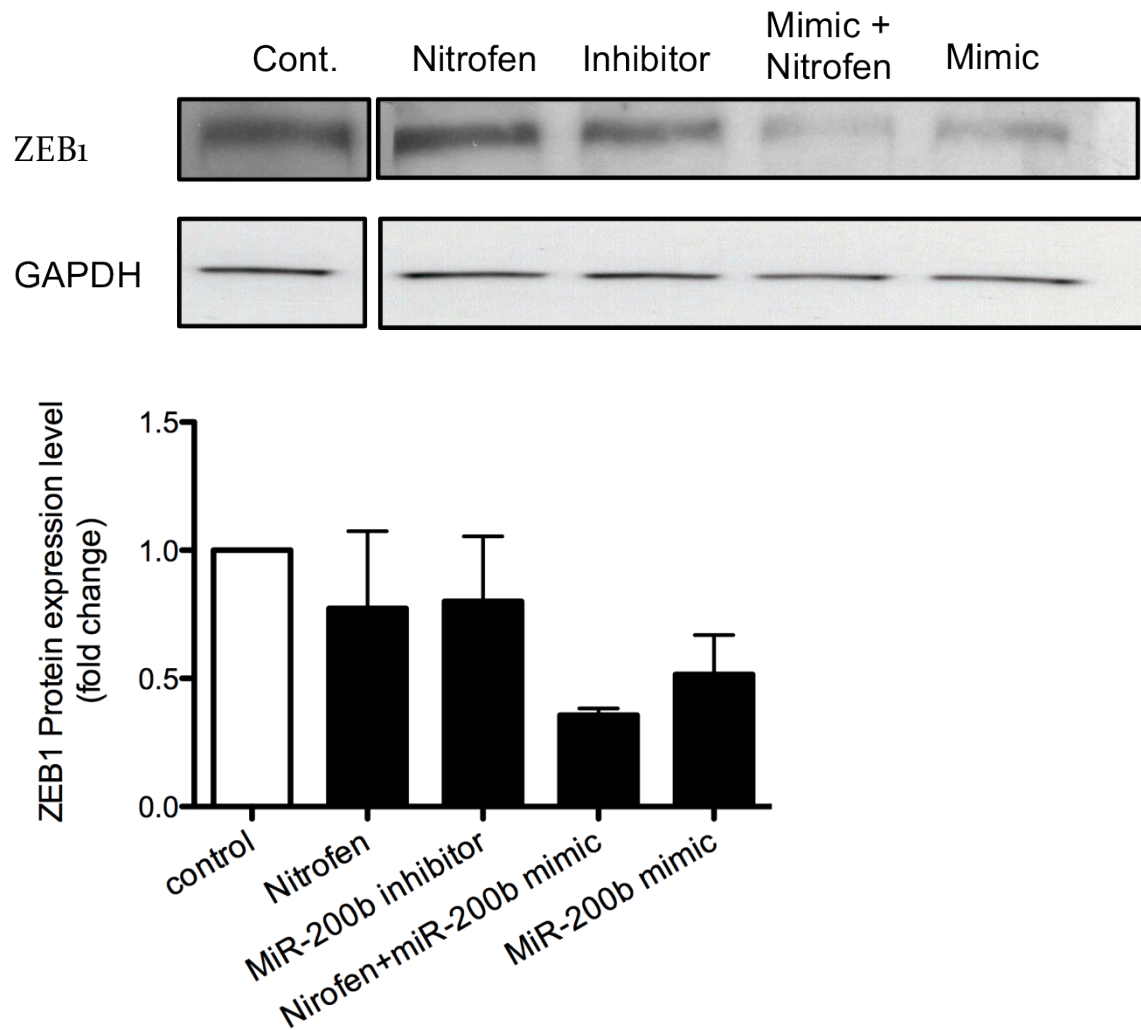
Assessment of Lung morphometry

An independent investigator, blinded for the experimental treatment group, performed lung morphometry on E21 embryos, by determining the mean linear intercept (MLI) and the surface tissue density on hematoxylin and eosin (H&E) stained sections. We randomly selected three hypoplastic lungs from rats with CDH and three hypoplastic lungs from rats without CDH from both the prenatal transplacental miR-200b therapy group and the negative control scrambled nucleotide oligomers group (total of twelve lungs in four groups: miR-200b with CDH, miR-200b without CDH, Neg. cont. with CDH and Neg. Cont. without CDH). Serial sections with 0.5µm thickness were made from the entire lungs. We divided the sections into three equal proximal, medial and distal zones and randomly selected five slides from each zone. H&E staining was performed on all selected slides. We randomly selected two fields per slide to measure mean linear intercept and surface tissue density (total fields of 30 per lung) using a Zeiss microscope and software as previously described (Knudsen et al. 2010; Roubliova et al. 2010).

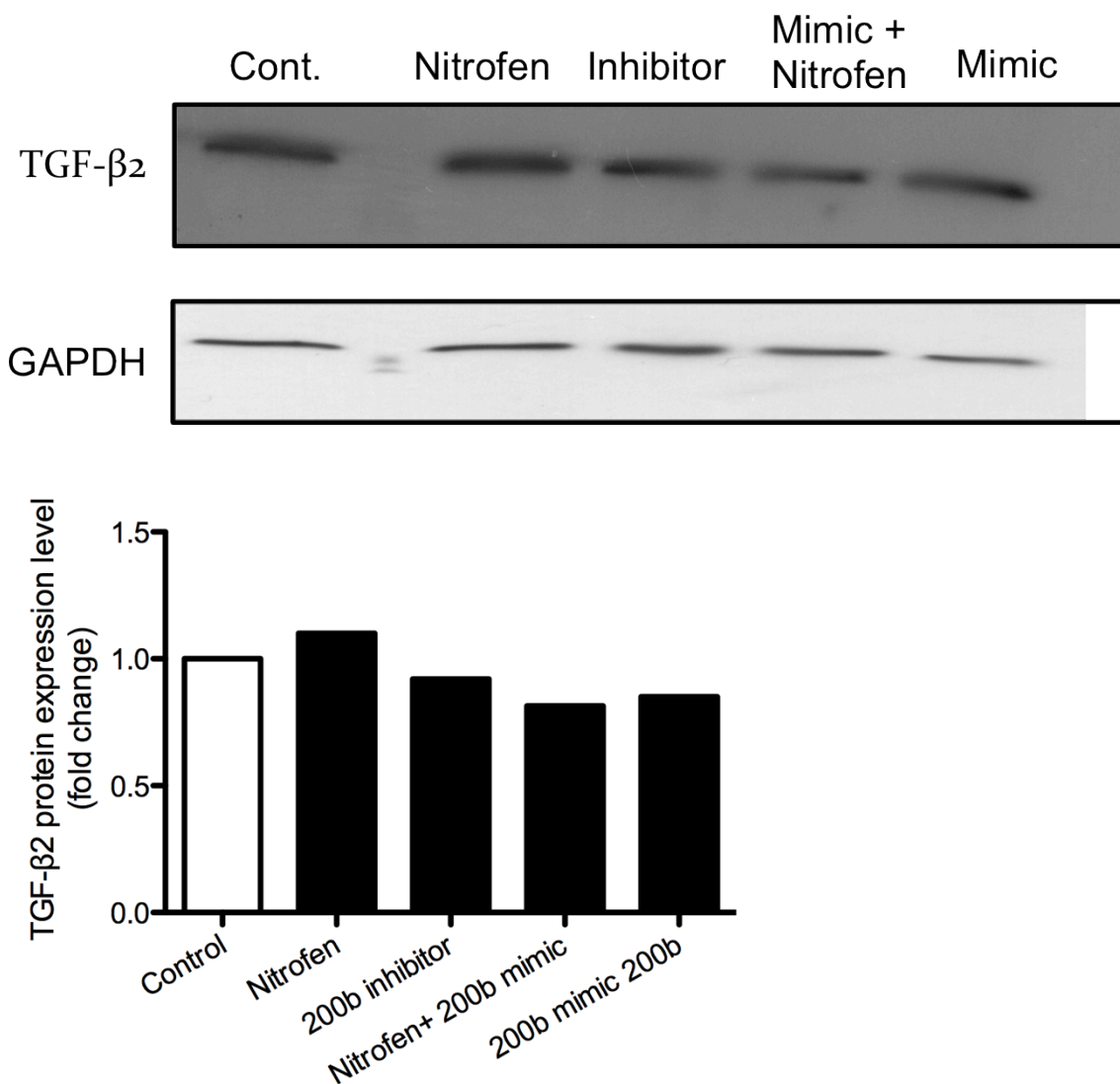
Statistical analysis

All data are presented as mean $\pm$ standard error of mean, from a minimum of three independent experiments. Statistical significance was determined by one-way ANOVA, *student's t*-test or Chi square as indicated in the figure legends. A p-value ≤ 0.05 was considered significant.

5.6. Supplementary Results

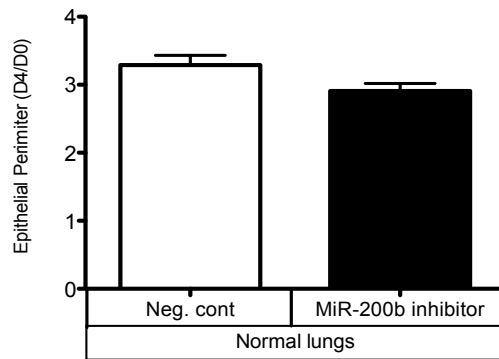


Supplementary Figure 5-8: Inhibition of miR-200b or nitrofen does not promote ZEB1 expression. Western blotting with ZEB1 antibody on BEAS-2B cells treated with nitrofen, miR-200b inhibitors, miR-200b mimics and a combination of miR-200b and nitrofen did not show any differences in ZEB1 protein.

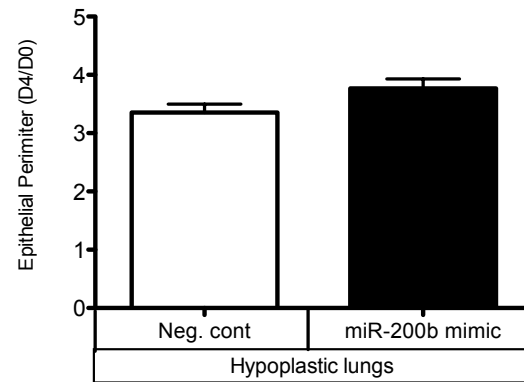


Supplementary Figure 5-9: MiR-200b does not influence TGF- β 2 expression in BEAS-2B cells. Western blotting with TGF- β 2 antibody on BEAS-2B cells treated with nitrofen, miR-200b inhibitors, miR-200b mimics and a combination of miR-200b and nitrofen did not show any differences in TGF- β 2 protein.

a

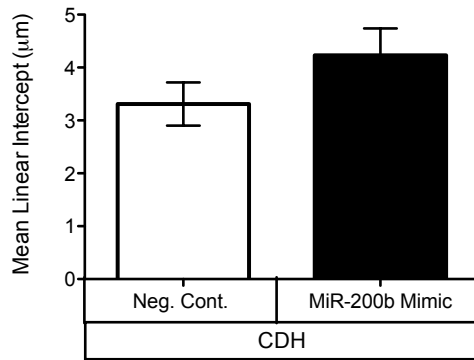


b

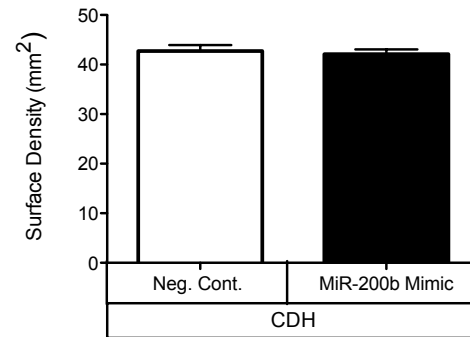


Supplementary Figure 5-10: MiR-200b had no significant effect on lung epithelial perimeter in normal or hypoplastic lung during ex-vivo culture. (a) Normal lung explants treated once with miR -200b inhibitor, after 4 days in culture, Epithelial perimeter was measured. (b) hypoplastic lung explants treated once with miR-200b mimic, after 4 days in culture, Epithelial perimeter was measured.

a



b



Supplementary Figure 5-11: The mean linear intercept of lungs with CDH in miR-200b group were higher but the differences were not significant. Pregnant rats were injected intravenously with miR-200b or oligo negative control of pregnant rat at E9 after nitrofen gavaging.

5.7. Supplementary Tables

Supplementary Table 5-1: CDH incidence after prenatal transplacental miR-200b therapy.

Treatment \ Outcome	Outcome		Total
	Pups with CDH	Pups without CDH	
miR-200b	32	89	121
Neg. Cont.	36	24	60

Number of pups with or without CDH (non-CDH) in the two treatment groups (the data is represented from at least 3 independent experiments)

Supplementary Table 5-2: *Locked nucleic acid (LNA) primer sets used in real-time quantitative PCR (qPCR)*

LNA microRNA primer set	Description
U6 snRNA (hsa, rno,mmu)	endogenous control
rno-miR-200b	tested microRNA

Supplementary Table 5-3: Double DIG labeled miRCURY LNA™ miRNA detection probes used for in situ hybridization (Exiqon).

Probe	Sequence	Concentration	Hybridization Temperature
rno-miR-200b rat	GTCATCATTACCAGGCAGTATTA	100 nM	51°C
scramble-miR negative control	GTGTAACACGTCTATACGCCCA	100 nM	57°C
U6, hasmmurno positive control	CACGAATTTGCGTGTCATCCTT	0.1 nM	54°C

5.8. Acknowledgements

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CHAPTER 6. Discussion

6.1. Overview

Every ten minutes a baby with CDH is born somewhere in the world. The survival rate is about 60% and since 2000 over 300,000 CDH babies have died. The morbidity and mortality largely depend on the degree of abnormal lung development (Colvin et al. 2005; Mah et al. 2009). Current management and treatment are very expensive. In the USA alone, the costs of caring for all CDH babies in the hospital is around \$800,000,000 each year (Lally 2016). That makes CDH a very deadly disease and expensive to manage. Therefore, a more effective treatment for abnormal lung development and CDH is much needed. Prenatal therapy of the hypoplastic lung, when the fetal lungs do not participate in gas exchange is the best time to further decrease mortality and morbidity for babies with CDH. Although fetoscopic endoluminal tracheal occlusion (FETO) demonstrated an increase in survival, this invasive intervention has several adverse effects, including premature birth due to premature rupture of the membranes (Al-Maary et al. 2016). Furthermore, there is a substantial period between the diagnosis of CDH (around 20 weeks of pregnancy) and the FETO intervention (from 28-34 weeks of gestation). Thus, it might be a situation of ‘too little, too late’. Lack of a genetic cause for more than 80% of CDH babies prompted us to investigate the role of microRNAs as an epigenetic factor. Several research groups and pharmaceutical companies across the globe are exploring microRNA-based therapy and establishing a new field of microRNA therapeutics. Few microRNAs have entered the preclinical and clinical stages, such as Phase IIa of the clinical trial for HCV using miR-122 inhibitors or Phase I of the clinical trial for liver cancer using miR-34 mimics (Christopher et al. 2016). In my Ph.D. project, I evaluated the role of miR-200b as an epigenetic factor in normal lung development and as a prenatal therapeutic agent for abnormal lung development in CDH. We hypothesized that miRNAs may be an effective, first-line treatment for CDH.

6.2. MiR-200b and CDH in human

Our microarray analysis of 319 microRNAs on human lung tissues at the end of the canalicular stage demonstrated that miR-200b and miR-10a are over-expressed in hypoplastic CDH lungs compared to lungs from age-matched controls. Higher expression of miR-200 family members in the tracheal fluid of CDH fetuses at the saccular stage is associated with a better response to FETO and sufficient lung growth, suggesting that stimulated lung development is associated with an increase in miR-200 expression. Our findings indicate that miR-200b can potentially be used as a biomarker of (catch-up) growth in hypoplastic lungs. In addition, we demonstrated that miR-200b inhibits TGF- β /SMAD signaling in human bronchial epithelial cells. Using *in situ* hybridization, we determined that miR-200b is over-expressed in the terminal sacs of postnatal CDH lungs compared to age-matched controls. TGF- β 2 expression is reduced in these hypoplastic lung tissues. These findings prompted two main questions. First, what is the role of miR-200b in normal lung development? It has been shown that miR-200 family inhibitors down-regulated pro-SP-C and SP-B expression in human explant cultures, but the comprehensive role of miR-200b in normal lung development was unknown. Second, can we use miR-200b as a therapeutic agent for abnormal lung development in CDH? This study showed the association of higher miR-200b and better lung development, which lead us to study the therapeutic potential of miR-200b in CDH and its associated abnormal lung development.

6.3. miR-200b and normal lung development

To determine the role of miR-200b in normal lung development, we generated miR-200b^{-/-} mice. We did not observe any differences in proximal branching between *miR-200b*^{-/-}, *miR-200b*^{+/+} or

miR-200b^{+/-} fetal lungs. *MiR-200b*^{-/-} mice did not show any obvious breathing problems after birth and did not have CDH. However, 8-weeks *old miR-200b*^{-/-} mice showed severe peripheral airway obstruction and lung tissue stiffness in lung function studies. *MiR-200b*^{-/-} mice had lung surfactant biophysical dysfunction with compromised pro-SP-C and SP-B expression. They had decreased distal airway branching, a denser lung tissue parenchyma with thicker alveolar walls, a higher number of fibroblast-like cells and over-expression of a marker for EMT: Twist. This abnormal lung phenotype recapitulated the hypoplastic lung observed in CDH babies. Also, we identified changes in the transcriptome of *miR-200b*^{-/-} lungs using next generation sequencing. Gene ontology analysis showed that Notch and Wnt signalling are among the most affected biological pathways affected by miR-200b absence at transcriptional level. By using qPCR, we confirmed down-regulation of *Cyp2a5*, *Plunc*, and *Cadherin-26* which explains the fibroblast-like phenotype of the lungs, airway and tissue resistance in our knockout mice and the role of miR-200b in maintaining an epithelial cell phenotype. These data encouraged us to examine the potential role of miR-200b as a prenatal therapy for hypoplastic lungs and CDH.

MiR-200b^{-/-} mice had lower expression of mature miR-200a and miR-429 as well, the two microRNAs that express as one pri-miRNA along with miR-200b. This downregulation was more noticeable in the lung than the kidney which indicates the tissue-specific regulation of the miR-200 family. Downregulation of these two miRNAs in *miR-200b*^{-/-} mice can result from lower levels of pri-miR-200 expression due to conformational change in the 3D structure of the region or higher ZEB1/2 protein levels due to absence of miR-200b (negative feedback loop). Another possible explanation could be posttranscriptional regulation of miR-200a and miR-429 by miR-200b. Assessing the levels of pri-miR-200, pre-miR-200a and pre-miR-429 could be helpful to answer some of these questions.

6.4. MiR-200b and abnormal (hypoplastic) lung in nitrofen rat model of CDH

By evaluating the temporal and spatial expression of miR-200b in normal and nitrofen-induced hypoplastic rat lungs, we demonstrated that the overall expression of miR-200b was not significantly different between rat hypoplastic lungs at the early stages of lung development compared to control. However, there was a substantial difference in spatial expression between these two groups, especially in the distal parabronchial mesenchymal cells. We demonstrated that the distal parabronchial mesenchymal cells also had disturbed ADRP expression, a marker for lipofibroblasts. We used *in vitro*, *ex vivo* and *in vivo* experiments to evaluate the effect of miR-200b on nitrofen-induced lung hypoplasia. At the cellular level, we demonstrated that miR-200b inhibits SMAD2/3 signaling, nitrofen increases SMAD2/3 signaling by decreasing miR-200b expression and reintroduction of miR-200b normalizes SMAD2/3 signaling. In *ex vivo* lung explant experiments, we showed that miR-200b inhibitors or nitrofen treatment in fetal lung explants resulted in reduced branching morphogenesis. Increasing miR-200b abundance with mimics rescued this nitrofen-induced abnormal branching morphogenesis. Finally, we showed that *in vivo* prenatal transplacental miR-200b therapy reduces the incidence of diaphragmatic defects in rat model of CDH, but also significantly improves lung hypoplasia. This finding is a critical step toward exploring if miR-200b can be used as a prenatal therapy for hypoplastic lung development in fetuses with CDH.

6.5. Summary of miR-200b expression during lung development in human and rat hypoplastic lung

According to our human study (manuscript I), the tracheal fluid of CDH fetuses undergoing FETO contains more miR-200b in CDH patients who survive and have less severe lung hypoplasia. What

we do not know and probably will never know is the expression of miR-200b in tracheal fluid of healthy fetuses undergoing FETO. In human lung tissues at the early saccular and early alveolar stages, miR-200b expression was higher in deceased CDH patients than controls, but what we do not know and will probably never know is the expression of miR-200b in healthy control lung tissues. Also, we will probably never discover the miR-200b expression at the early stages of lung development before the diagnosis of CDH. According to our studies in the nitrofen rat model (manuscript III), at late stages of lung development, miR-200b expression followed the same pattern we observed in human CDH - it was higher in the less hypoplastic lungs. At early stages of lung development, miR-200b expression was lower in hypoplastic lungs compared to controls (Figure 6-1).

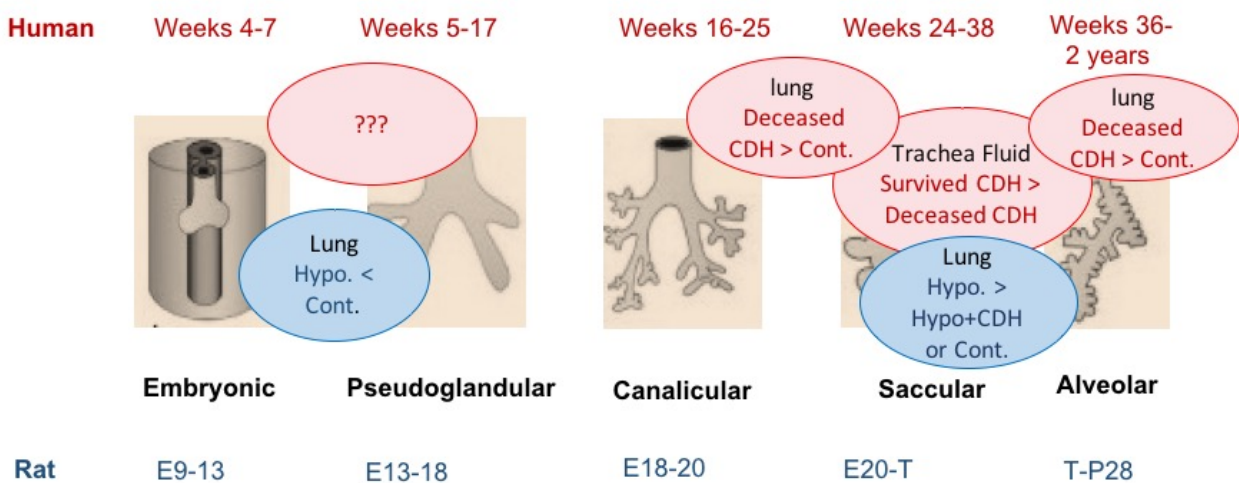


Figure 6-1: Summary of miR-200b expression in human and rat CDH.

(Stages of lung development in the figure are adopted from (Seaborn et al. 2010) used with the journal permission).

Our combined findings suggest that at the later stages of lung development, miR-200b is higher in the moderate hypoplastic lungs than the severe ones and these severe hypoplastic lungs have more expression than the healthy control lungs. At the early stages, hypoplastic lungs (severe or moderate) have lower expression of miR-200b than control lungs. These data suggest that an increase in miR-200b expression in the severe hypoplastic lung is too little and too late compared to expression in the moderate hypoplastic lung. An increase in miR-200b expression to the appropriate level at the right time is associated with survival in CDH (Figure 6-2)

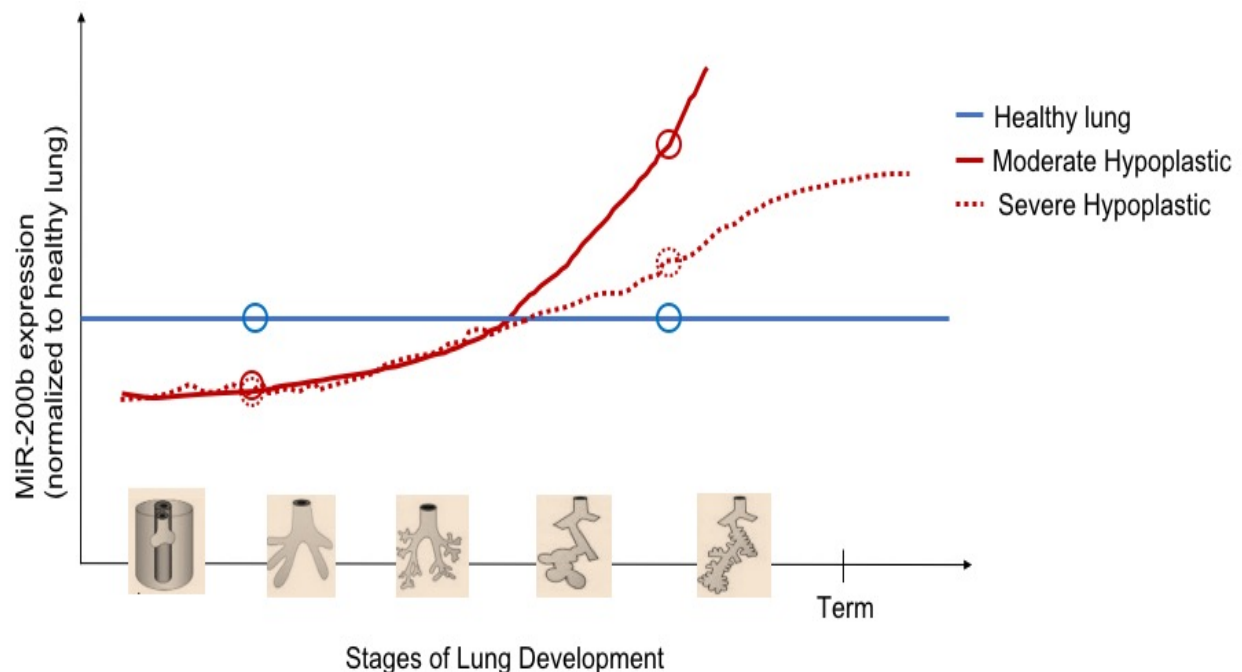


Figure 6-2: Increase in miR-200b in severe hypoplastic lung is too little and too late.

Although miR-200b^{-/-} mice do not represent the perfect model for abnormal lung development and CDH, the observed disturbed lung distal airway branching, thicker alveolar walls and downregulation of epithelial cell differentiation in these mice recapitulates some of the

characteristics of lung hypoplasia in CDH. Moreover, these characteristics indicate that the abnormal lung development probably occurs at the late stages of lung development i.e., the stages we found to have the differences in expression of miR-200b between moderate and severe hypoplastic lungs.

6.6. Conclusions

This project demonstrated that miR-200b is required for normal distal lung branching and maintaining a lung epithelial phenotype and function. The miR-200b knockout lung recapitulates lung hypoplasia observed in CDH patients. Prenatal transplacental miR-200b therapy decreases the incidence of CDH and the associated lung hypoplasia in the nitrofen rat model of CDH. Prenatal miR-200b therapy may, in fact, be able to prevent pulmonary hypoplasia from even occurring.

6.7. Limitations of the studies

6.7.1. Human samples

We only had a small sample size of human lung tissues available for microarray, PCR and ISH studies with no detailed phenotypic information on the termination of pregnancy patients. We had a relatively small sample size of amniotic and tracheal fluid samples from CDH patients undergoing FETO, but we had no control amniotic or tracheal fluids available. For obvious reasons, it would be unethical to try and obtain tracheal fluid samples from control patients.

6.7.2. Vasculature Development

The lung vasculature develops in close relation to the development of the airways and alveoli and contributes to the function and phenotype of the lungs. Although I did not assess the role of miR-200b in vasculature development in my Ph.D. project, we cannot exclude the possible involvement of abnormal vascular development in the phenotype we observed in the miR-200b knockout mice. MiR-200b prenatal therapy could have a positive influence on the vasculature in the lungs in the nitrofen model and be (partly) responsible for the observed positive outcomes.

6.7.3. Nitrofen rat model

Although lung hypoplasia, herniation and the diaphragm defect in the nitrofen model of CDH have remarkable similarities to human CDH, a significant disadvantage is that the teratogenic effects of nitrofen in rodents have never been demonstrated in humans. Therefore, conclusions drawn from the nitrofen model must be considered within the context that the pathophysiology of CDH and pulmonary hypoplasia in this model may not be 100% similar to that observed in humans. Assessing the effectiveness of miR-200b as a therapy should be evaluated in other models of CDH and lung hypoplasia as well.

6.7.4. MiR-200b based therapy

There are two significant limitations in the application of all microRNAs including miR-200b as a prenatal treatment. First, each microRNA has many targets and some of these targets can be tissue-specific. As a result, changing the abundance of a microRNA could lead to a change in a variety of undesirable pathways and biological process. Secondly, during fetal development, any subtle biological change can result in long-term effects. Therefore, we need more research to fully delineate the role of 200b in lung development and determine the short and long-term safety.

Furthermore, a lung tissue specific delivery system is required to prevent the potential side effects to the development of other organs and increase the efficacy of the therapy.

6.8. Future directions

The work outlined in the thesis is the first systematic report of the role of miR-200b in normal and abnormal lung development associated with Congenital Diaphragmatic Hernia. Despite the promising findings, to fully determine the role of miR-200b in normal and abnormal lung development in CDH, additional studies will be required as follow:

6.8.1. Lung vasculature and miR-200b

Others have shown that miR-200b has anti-angiogenic effects by directly targeting vascular endothelial growth factor (VEGF), its receptors (VEGFR-1 and VEGFR-2) (Choi et al. 2011) and Ets-1 (Chan et al. 2011). Also, injury-induced repression of miR-200b induced wound healing angiogenesis (Chan et al. 2012). A recent study showed that glucose-induced endothelial-mesenchymal transition was prevented in cardiac endothelial cells isolated from transgenic mice with endothelial-specific miR-200b overexpression (Feng et al. 2016). However, the role of miR-200b in pulmonary vasculature development has not been investigated. Here, we demonstrated that miR-200b^{-/-} mice have higher lung tissue stiffness resulting from a lower number of distal airways and more fibroblast-like cells. Future research investigating the pulmonary vascular phenotype and function in *miR-200b*^{-/-} mice and the possible involvement in the observed abnormal lung phenotype is needed. The elucidation of the pulmonary vasculature pressure and resistance along with the assessment of the right heart function in *miR-200b*^{-/-} mice will provide initial results on the contribution of a cardiovascular defect in the phenotype. Furthermore, *in vivo*

micro-CT scanning to evaluate the pulmonary vasculature using vascular contrast agents will provide greater insight into the involvement of miR-200b in pulmonary development. Also, by using primary endothelial cells isolated from *miR-200b*^{-/-} lungs, the involvement of miR-200b in important signaling pathways in pulmonary vasculature development such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) can be assessed. By using different markers of endothelial cells (such as CD31, VE-Cadherin and VEGF-R1,2) during fetal and postnatal lung development, the effect of miR-200b in the pulmonary endothelial phenotype, proliferation and differentiation, can be evaluated. We are currently evaluating the structure and function of the left and right lungs in *miR-200b*^{-/-} mice using echocardiography.

6.8.2. Regulation of miR-200b expression

Our study indicated that miR-200b expression is disturbed during abnormal lung development in the hypoplastic lung and tracheal fluid. However, the cause and mechanism of these changes were not evaluated. Recently, numerous studies have shown that miRNAs and other epigenetic factors can regulate each other (Z. Wang et al. 2013). For instance, in immortalized human mammary epithelial cells, the miR-200b/-200a/-429 cluster was silenced primarily through histone modifications whereas the miR-200c/-141 cluster was repressed by DNA methylation (Lim et al. 2013). Using immunoprecipitation coupled to quantitative PCR analysis (ChIP-qPCR) can help us to evaluate if histone modifications are the primary cause of miR-200b down-regulation during CDH pathogenesis. Also, by quantifying the abundance of Pri-miR-200b and Pre-miR-200b, we can determine if the observed changes in expression are due to post-transcriptional regulations.

6.8.3. Prenatal miR-200b therapy following the diaphragmatic defect

This project demonstrated that reintroducing miR-200b to the hypoplastic lungs during early stages of lung development - when lungs have lower miR-200b abundance - reduces the incidence of CDH and improves lung hypoplasia. The next logical step is to use miR-200b as a therapeutic agent during the later stages of abnormal lung development when the diaphragmatic defect already exists. We showed that higher miR-200b abundance is associated with better lung development (less hypoplastic lungs) in both human CDH and the nitrofen rat model. By using miR-200b at the later stages of abnormal lung development, we can evaluate if increasing miR-200b exogenously can promote growth in hypoplastic lungs after the diaphragmatic defect occurs.

6.8.4. Prenatal miR-200b therapy in a surgical model of CDH

In this project, we have demonstrated that human fetal tracheal fluid collected during FETO intervention has higher miR-200b abundance when lungs are less hypoplastic and CDH babies have better outcomes. As mentioned in the introduction, the rabbit model of CDH (surgical model) is an established model for FETO. By using this model, we can evaluate if direct miR-200b administration to the lungs during FETO can improve the lung hypoplasia and the outcomes of the fetus with CDH.

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