ROLES OF MICRORNAS IN FETAL LUNG DEVELOPMENT

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Lung alveolar type II cells uniquely synthesize surfactant, a developmentally-regulated lipoprotein that is essential for breathing. Expression of the major surfactant protein, SP-A, in midgestation human fetal lung (HFL) is dramatically induced by cAMP. cAMP induction of SP-A expression is repressed by TGF-β and by hypoxia. In this study, we found that expression of the miR-29 family was significantly upregulated in epithelial cells isolated from mouse fetal lung during late gestation and in epithelial cells isolated from HFL explants during type II cell differentiation in culture. MiR-29 expression in cultured HFL epithelial cells was increased by cAMP and inhibited by hypoxia, whereas the miR-29 target, TGF-β2, was coordinately oppositely regulated. Knockdown of the miR-29 family in cultured HFL type II cells blocked cAMP-induced SP-A expression and accumulation of surfactant-

containing lamellar bodies, suggesting its physiological relevance. This occurred through derepression of TGF-β signaling. Notably, cAMP increased binding of endogenous thyroid transcription factor-1 (TTF-1/Nkx2.1) to the *miR-29ab1* promoter in HFL type II cells and TTF-1 increased *miR-29ab1* promoter-driven luciferase activity in co-transfection assays. Together, these findings identify miR-29 family members as TTF-1-driven mediators of SP-A expression and type II cell differentiation through repression of TGF-β signaling.

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PRIOR PUBLICATIONS

RESEARCH ARTICLES

1. Dang Y, Li L, **Guo W**, Xue Z, Liu Y (2013) Convergent Transcription Induces Dynamic DNA Methylation at *disiRNA* Loci. PLoS Genet 9(9): e1003761.
2. Benlhabib H, **Guo W,** Pierce B, and Mendelson CR (2015) The miR-200 family and its targets regulate type II cell differentiation in human fetal lung, J Biol Chem. 2015 Sep 11;290(37):22409-22.
3. **Guo W**, Benlhabib H, and Mendelson CR (2016) The miR-29 family promotes type II cell differentiation in human fetal lung through inhibiting TGF-β pathway. Mol Cell Biol, in Press.

PUBLISHED ABSTRACTS

1. Benlhabib H, Pierce B, **Guo W** and Mendelson CR (2013) Surfactant protein A (SP-A) expression is regulated by microRNA-199a 5p and 3p via COX-2 and NF-κB. *Endo Rev* 33
2. **Guo W**, Benlhabib H, and Mendelson CR, The microRNA (miR)-29 family serves a kKey role in type II cell differentiation in developing fetal lung, *Endo Rev OR44-5*
3. **Guo W**, Benlhabib H, and Mendelson CR, The microRNA (miR)-29 family serves a key role in type II cell differentiation in developing fetal lung, *FASEB SRC: Lung Epithelium in Health & Disease, Meeting Abstracts, August, 2014.*
4. **Guo W**, and Mendelson CR, Transcriptional regulation of the miR-29 family, which serves a key role in type II cell differentiation in developing fetal lung, *Keystone*

Symposium on Molecular and Cellular Biology: Metabolism, Transcription and Disease Abxtracts, January, 2016.

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LIST OF DEFINITIONS

Abbreviation	Definition
cAMP	3',5'-Cyclic adenosine monophosphate
Bt_2cAMP	Dibutyryl cAMP
CBP	CREB binding protein
CCSP	Clara cell secretory protein
C/EBP	CCAAT-enhancer-binding protein
ChIP	Chromatin immunoprecipitation
CtBP	C-terminal-binding protein
DGCR8	DiGeorge syndrome critical region gene 8 protein
dpc	Days post-coitum
ERRα	Estrogen-related receptor α
EMT	Epithelial-mesenchymal transition
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal bovine serum
FGF10	Fibroblast growth factor 10
FGFR2	FGF type 2 receptor
HFL	Human fetal lung
IL-1	Interleukin-1
LPCAT1	Lysophosphatidylcholine acyltransferase 1
LTBP	Latent TGF-β-binding protein
MFL	Mouse fetal lung

miRNA/miR	microRNA
mRNA	messenger RNA
NF-κB	Nuclear factor kappa B
RISC	RNA-induced silencing complex
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
PC	Phosphatidylcholine
PE	Preeclampsia
Pri-miRNA	Primary miRNA
Pre-miRNA	Precursor miRNA
SIP1	Smad-interacting protein 1
SP	Surfactant Protein
SRC-1	Steroid receptor coactivator 1
TCF/LEF	Lymphoid enhancer-binding factor
TGF-β2	Transforming growth factor β2
TTF-1/NKX2.1	Thyroid transcription factor 1
UTR	Untranslated region
USF1	Upstream stimulatory factor 1
YY1	Yin Yang 1
ZEB1/2	E-box-binding homeobox 1 and 2

CHAPTER ONE
Introduction

Overview

The human respiratory system, which is a series of organs responsible for taking in oxygen and expelling carbon dioxide, begins at the nose and ends in the most distal alveolus. This includes the nasal cavity, the posterior pharynx, the glottis and vocal cords, the trachea, and all the divisions of the tracheobronchial tree. The upper airway consists of all structures from the nose to the vocal cords, delivers air to the respiratory tissues and is characterized by rigid walls that keep the airways open. The lower airway consists of trachea, airways, and alveoli. The trachea branches into two mainstem bronchi, which further undergo dichotomous branching pattern, form bronchioles, and end in extensive spaces surrounded by clusters of alveolar sacs(Berne, 2004). This organization creates a remarkable, highly branched, tubular structure that leads to a huge number of alveolar sacs, creating an extensive surface area through which oxygen and carbon dioxide are exchanged in blood within alveolar capillaries (Whitsett et al., 2010).

The lungs do more than gas exchange. They have essential functions in immune and microbial defense (Parker and Prince, 2011) and present a selectively permeable barrier against inhaled pathogens and toxicants (Humlicek et al., 2007). The mucus, glycoproteins, and water secreted by the epithelial cells create a thin surface layer within the airways that traps particles and is continuously swept out of the lungs by the action of the multiciliated cells (Wansleeben et al., 2013).

Alveoli are thin-walled, polygonal in shapes that are open at one side to allow air into their cavities. The alveolar surface is lined by type I and type II epithelial cells that are in direct contact with respiratory gases. Type I cells are flat with broad, attenuated cellular extensions. They comprise ~95% of the surface area of the peripheral lung and lie in close contact with capillaries to facilitate gas exchange (Williams, 2003). Type I cells have been described to be terminally differentiated, indicating that they are incapable of cell division and cannot change their phenotype. Recent data suggest that $Hopx^{+}$ Type I cells proliferate and generate type II cells during adult alveolar regrowth following partial pneumonectomy, indicating unanticipated plasticity of type I cells (Jain et al., 2015). Type II cells are cuboidal and often found in the "corners" of alveoli. They contain abundant specialized secretory vesicles called lamellar bodies. These are the primary source of pulmonary surfactant, a complex mixture of lipids and proteins, that decreases alveolar surface tension and contributes to host defense (Whitsett et al., 2010). It has long been believed that type II cells function as progenitors for other alveolar epithelial cells (Evans et al., 1975). Recent lineage tracing experiments extended these findings and showed that type II cells function as progenitors in the adult lung during homeostatic conditions and upon type II cell ablation (Barkauskas et al., 2013; Desai et al., 2014). Lineage-labeled type II cells both self-renew and generate type I cells *in vivo* and in clonal three-dimensional (3D) organoid cultures ex vivo (Barkauskas et al., 2013; Desai et al., 2014). Many serious diseases including respiratory distress syndrome and idiopathic pulmonary fibrosis involve a failure to establish or maintain type I and type II cells. Additionally, alveoli are a major site of lung cancer, the leading cause of cancer death (Siegel et al., 2013).

Lung Development

Mouse lung development involves four stages (Cardoso and Lu, 2006). First, lung development is initiated in the embryo as a ventral outpouching of endodermal cells from the anterior foregut into the surrounding mesenchyme at 9.5 days postconception (9.5 dpc) in the mouse and ~3 weeks gestation in the human (Snyder JM et al., 1985). Second, This ventral diverticulum grows caudally to form the primitive trachea and subsequently divides to form the two lung buds. The development of the buds is entirely dependent on the localized expression of Fibroblast growth factor 10 (FGF10) in the mesoderm overlying the buds and FGF type 2 receptor (FGFR2) in the endoderm (Min et al., 1998; Sekine et al., 1999). Third, Division of the foregut by a longitudinal septum separates the ventral trachea from the dorsal esophagus. Between the 7th and 16th weeks of human gestation (pseudoglandular phase; E9.5–E16 in the mouse), the lung buds undergo a program of iterative and stereotypic branching to elaborate a tree-like tubular network of airways consisting of the bronchioles, respiratory bronchioles, and alveolar ducts. Fourth, The 16^{th} through 24^{th} weeks of human gestation (E16–E17 in the mouse) make up the canalicular phase of lung development. During this period, the rapid growth rate diminishes, dichotomous branching has been completed, and differentiation of the epithelial cells lining the ducts begins to take place. This phase is characterized by the initiation of capillary growth within the developing lung and the first appearance of type II cells containing lamellar bodies, the cellular organelles that contain lung surfactant. During the terminal saccular phase of lung development (25 weeks to term in the human fetus; E17 to term in the mouse), there is continued growth of capillaries and remodeling of the distal lung to resemble adult lung parenchyma. This

remodeling involves continued development of capillary networks, cellular differentiation, thinning of mesenchyme-derived stroma, and expansion of presumptive alveoli. Augmented (Increased?) surfactant synthesis and secretion occur after ~30 weeks gestation in preparation for the transition to air breathing (Mendelson, 2000) (move this sentence to section below? You have not introduce surfactant yet).

Pulmonary Surfactant and Surfactant Proteins

Surfactant, a mixture of lipid, cholesterol (isn't cholesterol a lipd?) and four associated surfactant proteins, is required for reducing surface tension at the alveolar air-liquid interface and maintaining alveolar stability during air breathing. Surfactant is synthesized by alveolar type II epithelial cells and stored as lamellar bodies until its secretion into the lumens of the lung alveoli, where they unwind and are transformed into the quadratic lattice structure of tubular myelin, which, in turn, gives rise to a monolayer surface film of surfactant lipids and proteins, which act to reduce surface tension, increase compliance, and prevent alveolar collapse (it is better to break this long sentences into two or three simple sentences) (Haagsman and Van Golde, 1991; Mendelson, 2000). Surfactant production are developmentally and hormonally regulated (however, you did not describe hormonal regulation here). The fetal lung acquires the capacity for surfactant synthesis relatively late in gestation: In the human fetus, type II cells are first identifiable in the terminal sacs at 20-22 weeks of gestation (E16–E17 in the mouse) (Mendelson, 2000).

In addition to the function of surfactant in lowering alveolar surface tension and preventing alveolar collapse, reduced surface tension promotes smaller airway patency,

counteracts fluid accumulation, and prevents edema formation (Enhorning et al., 1995; Liu et al., 1991; Macklem, 1970). Surfactant forms a nonspecific barrier against the adhesion and invasion of microorganisms into the lung (Hills and Chen, 2000). Alterations in lung surfactant have been described in the pathophysiology of a number of respiratory diseases. Prematurely born infants with surfactant deficiency are at risk of developing respiratory distress syndrome (Avery and Mead, 1959), a leading cause of neonatal morbidity and mortality in developed countries. Surfactant dysfunction has also been implicated in the pathophysiology of acute respiratory distress syndrome and asthma (Christmann et al., 2009).

The surfactant proteins (SP) include four largely lung-specific proteins, named SP-A, SP-B, SP-C and SP-D. SP-A and SP-D are members of the collectin sub-group of the C-type lectin family of proteins (McCormack, 1998) and contain N-terminal collagen-like and C-terminal lectin-like domains. In humans, SP-A protein is encoded by two highly similar genes, *SFTPA1* and *SFTPA2*, while SP-D is encoded by *SFTPD* (Floros et al., 1996). All three genes are located in the same region on human chromosome 10 (Hoover and Floros, 1998). SP-B, encoded by *SFTPB*, belongs to the saposin-like proteins (Patthy, 1991). Human SP-C is encoded by a single gene (*SFTPC*) on chromosome 8 whose transcript directs the synthesis of a 197-amino-acid precursor protein. Both SP-B (79 amino acids (aa)) and SP-C (35 aa) are small hydrophobic proteins derived from larger precursors by proteolytic processing (Weaver, 1998).

Cyclic AMP Regulation of SP-A Gene Expression

SP-A expression is lung specific (Boggaram et al., 1988) and occurs primarily in alveolar type II cells, and to a lesser extent in bronchioalveolar epithelial (Clara) cells of the proximal and distal airways (Auten et al., 1990; Phelps and Floros, 1988; Wohlford-Lenane and Snyder, 1992). In the second trimester HFL, both SP-A mRNA and protein have also been detected in nonmucous tracheal and bronchial glands, and in isolated cells of conducting airway epithelium (Khoor et al., 1993). The transcription of *SP-A* gene is developmentally regulated in the fetal lung. It remains silenced until ~85% of gestation is completed, and then increases in concert with enhanced surfactant phospholipid synthesis,reaching the peak level just prior to term (Mendelson and Boggaram, 1991).

In cultured HFL type II cells, *SP-A* expression was found to be induced by cyclic AMP (cAMP) (Odom et al., 1987). Using transgenic mice and transfected type II cells, recent studies have identified a ~300-bp regulatory region upstream of the rabbit and human *SP-A* genes that is responsible for lung-specific and developmental regulation of *SP-A* expression (Alcorn et al., 1993; Gao et al., 1993; Gao et al., 2003; Michael et al., 1996). This region contains a number of highly conserved response elements, such as an element that binds orphan nuclear receptor estrogen-related receptor (ERR)α at −240 bp (ERRE) (Liu et al., 2006), a thyroid transcription factor 1 (TTF-1/NKX2.1) binding element (TBE) at −170 bp (Islam and Mendelson, 2002), an E-box at −87 bp that binds the basic helix-loop-helix-leucine zipper transcription factors, upstream stimulatory factor 1 (USF1) (Gao et al., 1997) and upstream stimulatory factor 2 (USF2) (Gao et al., 2003), and a GT box at −60 bp, which binds Sp1 (Young and Mendelson, 1997).

Cyclic AMP (cAMP) induces *SP-A* expression by enhancing the binding of transcription activators, e.g. TTF-1, NF-κB, and coactivators, e.g. CREB-binding protein (CBP) and steroid receptor coactivator 1 (SRC-1) (Yi et al., 2002), to the TBE site, and by regulating TTF-1 phosphorylation, acetylation, and transcriptional activity (Li et al., 1998). In addition, cAMP enhances ERRα activation of *hSP-A2* expression in lung type II cells by facilitating ERRα nuclear localization and DNA-binding (Liu et al., 2009). cAMP-mediated SP-A induction was impaired when HFL type II cells were cultured in hypoxic environment (1% O_2) (Acarregui et al., 1993). This blockage was associated with decreased acetylation and increased di- and tri-methylation of histone H3K9 in the TBE region (Benlhabib and Mendelson, 2011). These observations suggest that in late gestation, increased O_2 availability to type II cells results in changes in the chromatin structure to facilitate the access of transcription factors like TTF-1 and NF-κB to the *SP-A* promoter.

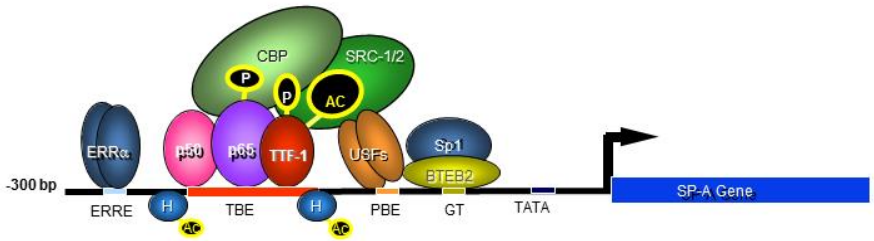

Figure 1-1. A 300-bp Region Upstream of the Human *(h)SP-A* Gene Contains Critical Response Elements for Lung Cell-specific, Developmental and Hormonal Regulation of Expression. In studies using transgenic mice and transfected type II cells, a 300-bp region upstream of the *hSP-A* gene was characterized that mediates its lung cell-specific, developmental and hormonal regulation of expression. Within this region, a number of conserved response elements for transcription factors were identified that each are essential for cAMP induction of *hSP-A* promoter activity. Of particular importance is an element, termed the TBE, which binds the homeodomain transcription factor TTF-1 and NF-κB. It was found that increased oxygen tension is essential for cAMP-induced binding of endogenous TTF-1 and NF-κB proteins p50 and p65 to the TBE, as well as recruitment of coactivators and permissive changes in histone modification.

MicroRNAs in Lung Development

Lung development involves many complex signaling pathways. The mechanisms behind these molecular pathways are not completely understood. It has recently been shown that microRNAs (miRNAs) play crucial roles in many biological processes, including lung organogenesis (Lu et al., 2007; Tian et al., 2011; Ventura et al., 2008), wherein they have been implicated in epithelial cell proliferation and differentiation.

MiRNAs are a class of small noncoding RNAs (~21–24 nt) that post-transcriptionally regulate the expression of target genes. Based on their genomic location, miRNAs are

classified as either intergenic or intragenic. Intragenic miRNAs include intronic miRNAs and exonic miRNAs that are located within the exons or introns of protein-coding genes, respectively (Berezikov, 2011). Recent studies showed that the transcription of most intronic/exonic miRNAs and their host genes are coregulated (BASKERVILLE and BARTEL, 2005; Rodriguez et al., 2004). Many miRNAs are encoded by polycistronic or bicistronic transcriptional units that generate multiple miRNAs (Bartel, 2004). MiRNA biogenesis starts with primary miRNA (pri-miRNA) transcription. The pri-miRNA then undergoes processing by a nuclear core microprocessor complex that consists of the RNase III enzyme, Drosha, and the DiGeorge syndrome critical region gene 8 protein (DGCR8) in order to generate the precursor miRNA (pre-miRNA) (Han et al., 2004; Lee et al., 2003, more references are needed). The pre-miRNA is then exported to the cytoplasm by Exportin-5 to be further processed by the RNase III enzyme, Dicer, into mature miRNAs (Kim, 2005, you need to reference the original papers that discover Dicer). The mature miRNA is then loaded onto the RNA-induced silencing complex (RISC) to inhibit expression of specific mRNA targets. Mature miRNAs repress target gene expression by binding to the complementary sequences in the 3'-untranslated region (UTR) of their specific target mRNA, resulting in inhibition of mRNA translation and/or increased mRNA degradation (Bartel, 2009). MiRNAs can also be secreted as free miRNAs or in exosomes, circulated, and transported to different cell types providing an interesting mechanism for intercellular regulation and cross-talk between cells (Chen et al., 2012; Hergenreider et al., 2012; Kosaka et al., 2013).

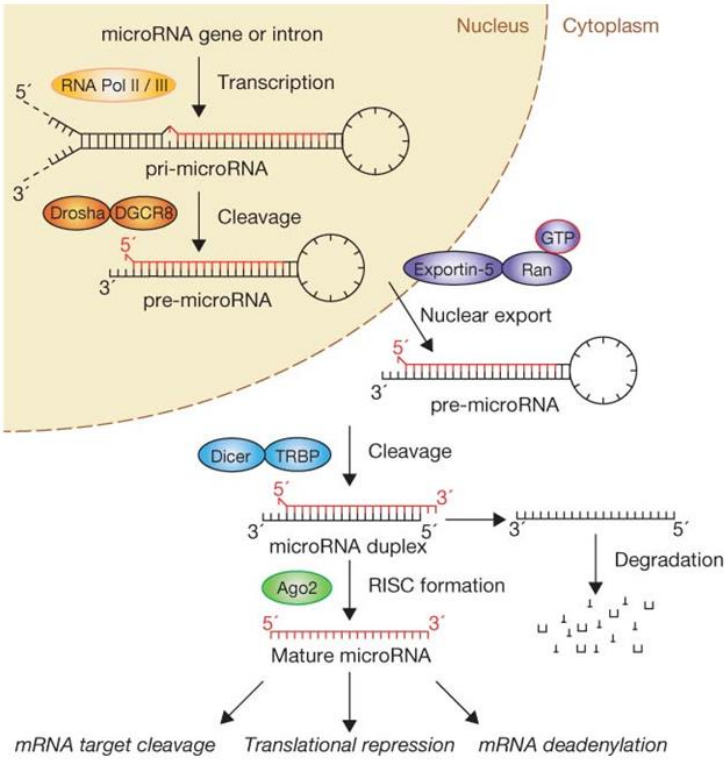

Figure 1-2 The miRNA Processing Pathway. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus. In the cytoplasm, the RNase Dicer cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression or deadenylation, whereas the passenger strand (black) is degraded.

Figure from Winter et al., 2009.

MiRNAs have been implicated in many biological processes associated with development and cell differentiation (Benlhabib et al., 2015; Boehm and Slack, 2005; Chen et al., 2012; Costinean et al., 2006; Xu et al., 2004). Accumulating studies indicate that miRNAs are involved in a diverse range of processes, including development, homeostasis, and inflammatory diseases in lung tissues and are capable of inducing differentiation, morphogenesis, and apoptosis (Johar et al., 2014). Interestingly, several components of the miRNA processing pathway have been shown to be involved in fetal lung development. A lung epithelium-specific deletion of the miRNA processing enzyme, Dicer, resulted in arrested branching morphogenesis with abnormal growth of the epithelial tubules, suggesting that downstream targets of the miRNA pathway in lung epithelium play critical roles in lung morphogenesis (Harris et al., 2006). Moreover, Argonaute1 and Argonaute2, the catalytic components of the RISC that promote miRNA-mediated mRNA silencing, are highly expressed in epithelial and mesenchymal cells within the branching embryonic lung at E11.5, suggesting a pivotal role of miRNAs in lung remodeling (Lü et al., 2005). These findings highlight the importance of miRNA machinery in fetal lung development. Furthermore, several miRNAs have been shown to be essential for lung development. The expression profiles of microRNAs between E11.5 (pseudoglandular stage) and E17.5 (late canalicular stage) in mice were analyzed and compared. miRNAs of the *miR-17~92* cluster were found to be highly expressed during early stages of lung development and to decrease in late stages and in adult lungs (Lu et al., 2007). Mice with a deletion of the *miR-17~92* cluster died shortly after birth as a result of lung hypoplasia (Ventura et al., 2008). Additionally, overexpression of the *miR-17~92* cluster in embryonic lung epithelium resulted a very

abnormal lethal phenotype through promoting lung epithelial progenitor cell proliferation and inhibition of differentiation (Lu et al., 2007). Similar to the *miR-17~92* cluster, levels of miR-302/367 are highly expressed at early stages of lung development, but decline rapidly as development proceeds (Tian et al., 2011). Overexpression of miR-302/367 leads to a thickening of distal epithelium and reduction in sacculation (Tian et al., 2011). These studies highlight the importance of specific miRNAs in embryonic lung development.

The roles of miRNAs in type II cell differentiation and surfactant lipoprotein production has been relatively unexplored until recently. To identify and characterize differentially expressed miRNAs during cAMP stimulation of type II cell differentiation and the associated induction of SP-A expression, miRNA microarray analysis of RNA from epithelial cells isolated from mid-gestation HFL explants before and after culture with Bt_2cAMP has been conducted (Benlhabib et al., 2015). In these studies, we obtained evidence to suggest that members of the miR-200 family, which are upregulated during type II cell differentiation, and their targets, zinc finger E-box-binding homeobox 1 and 2 (ZEB1/2) and transforming growth factor beta 2 (TGF-β2), which are downregulated, serve important roles in the developmental regulation of type II cell differentiation and function in HFL (Benlhabib et al., 2015). We found that miR-200 antagonists inhibited TTF-1 and surfactant proteins and up-regulated TGF-β2 and ZEB1 expression in type II cells. Overexpression of ZEB1 in type II cells decreased DNA binding of endogenous TTF-1, blocked cAMP stimulation of surfactant proteins, and inhibited miR-200 expression, whereas cAMP markedly inhibited ZEB1/2 and TGF-β. Importantly, overexpression of ZEB1 or miR-200 antagonists in HFL type II cells also inhibited LPCAT1 and ABCA3, enzymes involved

in surfactant phospholipid synthesis and trafficking, and blocked lamellar body biogenesis. Our findings suggest that the miR-200 family and ZEB1, which exist in a double-negative feedback loop regulated by TGF-β, serve important roles in the developmental regulation of type II cell differentiation and function in HFL (Benlhabib et al., 2015). In my thesis research, I have focused on the roles of miR-29 family in the regulation of type II cell differentiation and surfactant synthesis.

The miRNA-29 Family

The miR-29 family, which includes three members, miR-29a/b/c, are generated from two alleles containing a bicistronic transcriptional unit, *miR-29ab1* and *miR-29b2c*, in human, rat and mouse genomes (Figure 1-3). RACE and RT-PCR analyses have confirmed that miR-29b-1 and miR-29a are transcribed together as a polycistronic primary transcript (Chang et al., 2008; Mott et al., 2010). Likewise, miR-29b-2 and miR-29c are transcribed together. Human miR-29b-2 and miR-29c are encoded by the last exon of the miR-29b-2/c primary transcript (GenBank accession numbers: EU154351 and EU154352) (Chang et al., 2008). On the other hand, the precursors of miR-29b-1 and miR-29a are processed from the last intron of the primary transcript EU154353 (Chang et al., 2008). The *miR-29ab1* cluster, which encodes the precursors of miR-29b-1 and miR-29a, is located in chromosome 7q32.3 in human, while the *miR-29b2c* cluster encoding miR-29b-2 and miR-29c is on chromosome 1q32.2. In rats, the same two clusters, 29a with 29b1 and 29b2 with 29c, map to chromosomes 4q22 and 13q27, respectively, whereas, in mice, the miR-29 miRNAs cluster on chromosomes 6qA3.3 and 1qH6, respectively (Slusarz and Pulakat, 2015). Mature

miRNA sequences of miR-29a/b/c are well conserved in human, mouse, and rat. Analysis of the individual miR-29 family members revealed that they contain identical seed sequences at nucleotide positions 2–7, and therefore are likely to target the same transcripts. However, differences in processing, stability, and cellular distribution are reported for the three family members indicating their distinct expression, regulation, and function (Kapinas et al., 2010; Mott et al., 2010; Wang et al., 2008; Xiao et al., 2012a). The expression levels of all members of miR-29 family were found to be more than 50-fold higher in adult, compared to neonatal mouse lungs (Dong et al., 2010), suggesting a role of miR-29 family in promoting pulmonary epithelial cell differentiation.

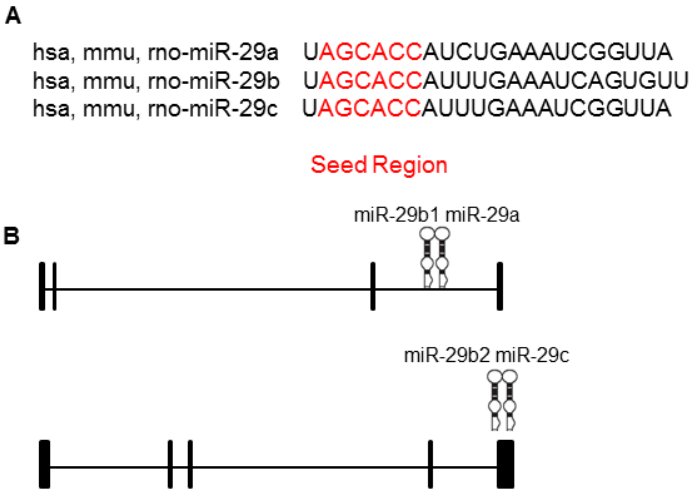

Figure 1-3. Mature Sequence and Graphical Summary of Primary Transcripts of the miR-29 Family. (A) mature sequences of the miR-29 family members are conserved in human (hsa-), mouse (mmu-), and rat (rno-), and share identical seed regions (shown in red). (B) primary transcripts of the miR-29 gene clusters in human genome. Exons and introns are shown as vertical boxes and horizontal lines, respectively. The hairpins indicate the locations of the sequences encoding precursors of miR-29s.

A number of studies show that miR-29 family contributes to normal tissue differentiation: upregulation of miR-29 family promotes myogenesis and attenuates the inhibitory action of TGF-β on myogenesis through targeting HDAC4, a key inhibitor of muscle differentiation (Winbanks et al., 2011). De-repression of miR-29 resulted in an increase of skeletal myogenesis by targeting its repressor YY1 (Wang et al., 2008). MiR-29b was shown to serve as a key regulator of osteoblast differentiation through targeting anti-osteogenic factors and modulating extracellular matrix proteins (Li et al., 2009b). It was postulated is that specific microRNA may have significant impact on a biological process by regulating a cohort of genes that are involved in the same or related pathways (Chou et al., 2013; Liang, 2009). The regulation of extracellular matrix (ECM) components, including collagen isoforms, laminin γ1, fibrillin 1, elastin, matrix metalloproteinase 2, and integrin β1 (Li et al., 2009b; Liu et al., 2010; van Rooij et al., 2008) and pro-metastatic genes involved in differentiation and modification of the tumour microenvironment (Chou et al., 2013; Sengupta et al., 2008) by the miR-29 family are examples of a single miRNA family regulating a large set of functionally related genes.

It was recently suggested that the miR-29 family is involved in many diseases and pathophysiological processes. For instance, miR-29-mediated downregulation of ECM proteins may sensitize the aorta to the formation of aneurysms in aged mice, while inhibition of miR-29 in vivo abrogates aortic dilation in mice (Boon et al., 2011). Moreover, miR-29a was strongly down-regulated in systemic sclerosis involving fibroblasts and skin sections and regulates type I and III collagen expression (Maurer et al., 2010). Up-regulation of miR-29b

expression may contribute to the onset of preeclampsia (PE) through repressing of trophoblast cell invasion, angiogenesis and increasing of trophoblast cell apoptosis (Li et al., 2013). Furthermore, miR-29 was also found to have critical impact on pathophysiological processes of liver fibrosis (Roderburg et al., 2011), cardiac fibrosis (van Rooij et al., 2008), renal fibrosis (Wang et al., 2012) and myocardial ischemia-reperfusion injury (Ye et al., 2010). Other studies indicate that miR-29 participates in the processes of apoptosis, proliferation and epithelial-mesenchymal transition (EMT) in cancer (Jiang et al., 2014).

Like most miRNAs and mRNAs, miR-29 family members are transcribed by RNA polymerase II. A number of critical *cis* elements have been identified in the proximal region of the *miR-29* gene promoters. Chromatin immunoprecipitation (ChIP) analysis has identified significant c-Myc binding in the vicinity of the transcription start site of both *miR-29ab1* and *miR-29b2c* clusters (Chang et al., 2008; Mott et al., 2010). Other transcription factors, including GATA3, NF-κB, CCAAT-enhancer-binding proteins (C/EBP), GLI-Kruppel family member (GLI), Yin Yang 1 (YY1), SMAD3 and lymphoid enhancer-binding factor (TCF/LEF) have been found to bind the promoter regions of *miR-29* genes (Chang et al., 2008; Chou et al., 2013; Cushing et al., 2014; Mott et al., 2010; Wang et al., 2008; Xiao et al., 2012a; Zhou et al., 2012).

Transforming Growth Factor β2 (TGF-β2)

TGF-β, which includes three different isoforms (TGF-β 1-3), is a pleiotropic cytokine belonging to the TGF-β superfamily. The TGF-β pathway occupies a central position in the signaling networks that control cell proliferation, differentiation, apoptosis, ECM remodeling

and final fate of metazoan cells (Massague et al., 2000). The active 25-kD TGF-β molecule consists of two identical disulfide-linked 12.5-kD polypeptide chains (Assoian et al., 1983). Each monomer is synthesized as a homodimeric proprotein containing the mature form of TGF-β at the carboxyterminal region. The dimeric propeptide is cleaved intracellularly from the growth factor (Taipale et al., 1998) and secreted as an inactive homodimer (latent TGF-β). Two copies of the propeptide remain in a complex with the TGF-β and maintain it in an inactive complex known as latent TGF-β-binding protein (LTBP). An LTBP is often linked to the prosegment and plays a critical role in targeting the complex to the extracellular matrix, where TGF-β is activated and released by proteolytic cleavage of the prosegment (Feng and Derynck, 2005). The TGF-β receptor complex consists of two type II (Tβ-RII) and two type I (Tβ-RI) transmembrane serine/threonine kinases (Feng and Derynck, 2005). TβR-I is phosphorylated by TβR-II upon ligand binding and mediates specific intracellular signaling through phosphorylation of Smad2 and Smad3. Upon phosphorylation by the receptors, Smad complexes translocate into the nucleus from cytosol. Nuclear Smad complexes cooperate with sequence-specific transcription factors and coactivators, to regulate gene transcription (Figure 1-4) (Feng and Derynck, 2005; Massagué, 1998).

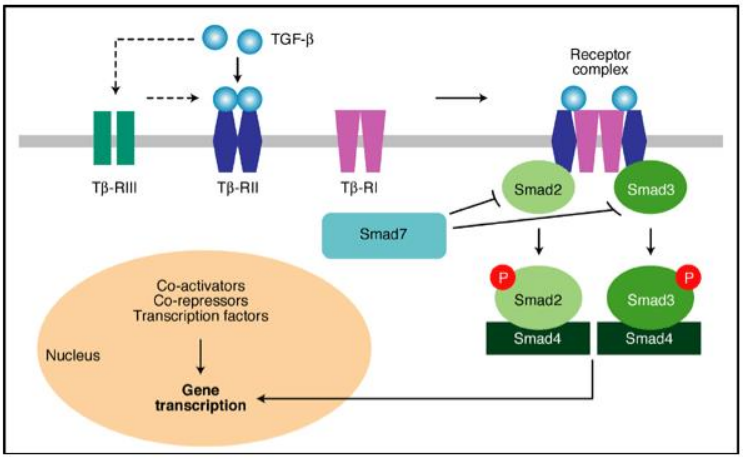

Figure 1-4. The TGF-β signaling pathway. TGF-β binds to the receptor Tβ-RII, recruits and phosphorylates Tβ-RI, leading to activation of Smad2 and Smad3 by phosphorylation (P). This is inhibited by Smad7. Activated Smad2 and Smad3 form heterodimers with Smad4 and translocate to the nucleus. Together with co-activators, co-repressors and other transcription factors, the Smad complex regulates gene expression.

Figure from Hui and Friedman, 2003.

TGF-β has received much attention as a major inducer of EMT during embryogenesis, cancer progression and fibrosis (Moustakas and Heldin, 2007). EMT is a process that reorganizes epithelial cells to become migratory mesenchymal cells. Consistent with the critical roles of Smads, the loss of epithelial marker expression and acquisition of mesenchymal features are achieved through a well-orchestrated transcription program that involves three families of transcription factors, the Snail, ZEB and basic helix-loop-helix (bHLH) families (Peinado et al., 2007). Their expression is induced by TGF-β, either in a Smad-dependent manner or activation through other transcription factors. Upon activation,

these transcription factors in turn repress epithelial marker gene expression and concomitantly activate mesenchymal gene expression (Peinado et al., 2007).

TGF-β signaling induces the expression of ZEB proteins during EMT through an indirect mechanism mediated in part by Ets-1 (Shirakihara et al., 2007). ZEB proteins then interact with Smad3 and directly repress the expression of epithelial marker genes, by recruiting the co-repressor C-terminal-binding protein (CtBP) (Postigo, 2003; Postigo et al., 2003). Two ZEB family transcription factors are known in vertebrates: ZEB1, also known as δEF1 or AREB6, and ZEB2, also known as Smad-interacting protein 1 (SIP1). The ZEB family members are structurally similar, containing two zinc-finger domains, whose simultaneous binding to bipartite E-boxes mediates their interaction with DNA sequences. The central repression region contains a Smad-interaction domain, a homeodomain and a CtBP binding domain (van Grunsven et al., 2001; Verschueren et al., 1999). Both ZEB1 and ZEB2 can repress transcription by directly binding to E-box-binding sequences in gene promoters (Remacle et al., 1999; Sekido et al., 1994), including genes encoding several cell junctional proteins, desmosomes and gap junctions (Vandewalle et al., 2005).

TGF-β plays important regulatory roles in lung branching morphogenesis and epithelial cell differentiation. Mice homozygous for a deletion of TGF-β3 gene manifest delayed pulmonary development and decreased SP-C expression (Kaartinen et al., 1995), suggesting a critical role of TGF-β3 in lung morphogenesis. Similarly, global loss of TGF-β2 led to perinatal mortality and a wide range of developmental defects, including defects in lung development (Sanford et al., 1997). By contrast, TGF-β1 decreases branching morphogenesis and formation of saccular buds in cultured embryonic lung buds (Serra et al.,

1994); transgenic mice overexpressing a constitutively active form of TGF-β in lung epithelium showed decreased epithelial cell differentiation, which was manifested by decreased levels of SP-C and Clara cell secretory protein (CCSP) gene expression (Zhou et al., 1996), while abrogation of TGF-β type II receptor signaling results in increased embryonic lung branching morphogenesis in culture (Zhao et al., 1996). Moreover, TGF-β1, -β2, and -β3 were found to be equally effective in inhibiting *SP-A* expression in NCI-H441 lung adenocarcinoma cells (Whitsett et al., 1992), and exogenous TGF-β1 disrupted culture-induced maturation of fetal lung epithelial cells and inhibited expression of surfactant components including SP-A, SP-B, and SP-C through effects on gene transcription (Beers et al., 1998).

Previous work demonstrated that miR-29b targets TGF-β2 mRNA (Chou et al., 2013) and is inhibited by TGF-β-Smad3 signaling (Zhou et al., 2012), indicating the existence of a double-negative feedback loop. Notably, TGF-β2 mRNA transcripts were localized exclusively in mouse bronchiolar epithelium at 12.5 dpc; the signal was increased at 14.5 dpc (Schmid et al., 1991), while relatively low levels of TGF-β mRNA and protein as well as TGF-β type I and II receptors were found to be present in postnatal lung after completion of development (Bartram and Speer, 2004).

In the present study, we have elucidated a role for the miR-29 family and its target, TGF-β2, in the regulation of alveolar type II cell differentiation and surfactant synthesis during fetal lung development. Our investigations have revealed that the levels of miR-29 family in both human and mouse fetal lung were significantly increased during type II cell differentiation, whereas the miR-29 target, TGF-β2, was coordinately decreased. Knockdown

of the miR-29 family in cultured HFL epithelial cells blocked cAMP-induced surfactant protein expression and lamellar body accumulation through increasing TGF-β2 expression and de-repressing TGF-β signaling, indicating its physiological relevance. Furthermore, we found that miR-29 family expression was increased by cAMP, via transcriptional activation of TTF-1. Our collective findings indicate that the developmental increase in TTF-1 transcriptional activity in fetal lung during late gestation promotes upregulation of miR-29 family members, which suppress TGF-β2 signaling to promote and maintain the type II epithelial cell differentiation and surfactant protein gene expression.

CHAPTER TWO

Section 1
Identifying miRNAs that are Upregulated during HFL Type II Cell Differentiation

Introduction and Experimental Design

Previously, we observed that upon culture of HFL explants in serum-free medium, type II cells differentiate spontaneously within the prealveolar ducts and develop the capacity to produce surfactant (Snyder et al., 1981). Moreover, cAMP enhances the rate of type II cell differentiation and induction of SP-A gene expression (Odom et al., 1987). To identify and characterize differentially regulated miRNAs during differentiation of type II cells isolated from mid-gestation HFL explants in culture, we previously performed miRNA microarray analysis of RNA from epithelial cells isolated from the HFL explants before culture and after 48 and 96 h of culture in the presence of Bt_2cAMP (Benlhabib et al., 2015). In these studies, we observed that 5 miRNAs were upregulated ~2-fold at both 48 and 96 h time points, as compared to the HFL epithelial cells before culture, including miR-200b and miR-200c, members of the miR-200 family, and miR-29a, member of the miR-29 family (Figure 2-1.2).

In my thesis research, I have focused on the miR-29 family, which includes miR-29a/b/c. The three family members are generated from two alleles containing a bicistronic transcriptional unit, *miR-29ab1* and miR-*29b2c*, in both human and mouse genomes (Chang et al., 2008). Mature miRNA sequences of miR-29a/b/c are well conserved, contain identical seed sequences, and are likely to target the same transcripts. MiR-29 family members are

known to target collagens, integrins, basement membrane and extracellular matrix remodeling proteins, as well as stem cell factor, KLF4 (Chou et al., 2013; Cittelly et al., 2013; Cushing et al., 2014), suggesting that miR-29 may mediate reprogramming of cells toward a differentiated epithelial phenotype.

In an effort to confirm and extend findings from the microarray that miR-29 family expression increases in epithelial cells from HFL explants during differentiation in culture, epithelial cells isolated from mid-gestation HFL were analyzed for SP-A and miR-29a, b and c before and after 24, 48 and 72 h of culture on extracellular matrix-coated dishes in medium containing Bt_2cAMP using RT-qPCR. In order to determine if the putative targets of miR-29 family members are regulated by these miRNAs in HFL epithelial cells, we conducted RT-qPCR and immunoblotting analysis on HFL epithelial cells during differentiation in culture. We also conducted parallel studies in mouse fetal lung during late gestation from 15.5 dpc to 18.5 dpc to confirm that the expression pattern of the miR-29 family and their respective targets is conserved from mouse to human.

Results: The miR-29 Family is Developmentally Upregulated during HFL Epithelial Cell Differentiation

As mentioned, miR-29a was identified by miRNA microarray to be significantly upregulated during in epithelial cells isolated from HFL explants during differentiation in culture in the presence of Bt_2cAMP (Figure 2-1.1 and Figure 2-1.2). To further confirm this result, RT-qPCR analysis of RNA isolated from mid-gestation HFL epithelial cells before and after 24, 48 and 72 h of culture on extracellular matrix-coated dishes in medium containing Bt_2cAMP was conducted. Since miR-29a belongs to a conserved miRNA family that includes miR-29a/b/c, we extended our study to all the family members. Interestingly, RT-qPCR analysis revealed that cAMP induction of hSP-A mRNA (Figure 2-1.3A) was associated with a marked increase in expression of miR-29a/b and a modest albeit significant increase in miR-29c (Figure 2-1.3B) compared with samples from before culture (BC). Because all miR-29 family members showed significant increases in expression in HFL epithelial cells between BC and 24, 48, 72 h after culture, we chose all of the 3 members to further investigate throughout the course of our investigations.

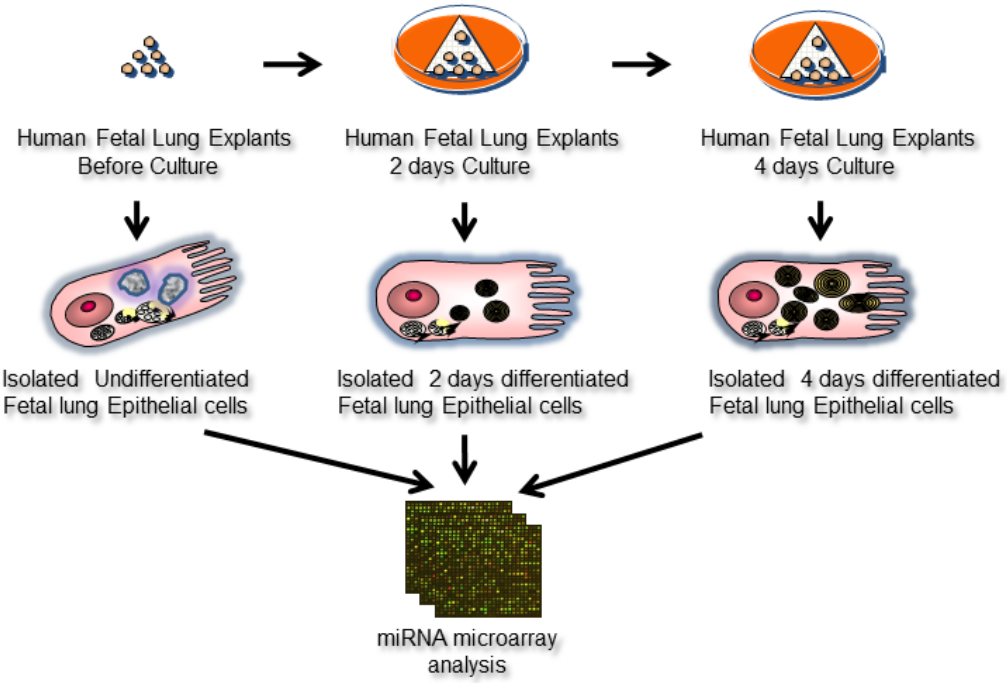

Figure 2-1.1. miRNA Microarray Analysis of Epithelial Cell RNA from HFL Explants Before and After Differentiation in Culture. Mid-gestation HFL explants were harvested before and after 48 and 96 h of culture ± Bt_2cAMP in serum-free medium. At each time point, epithelial cells were isolated, RNA was processed and subjected to miRNA microarray analysis using a commercial vendor (LC Systems, Houston, TX).

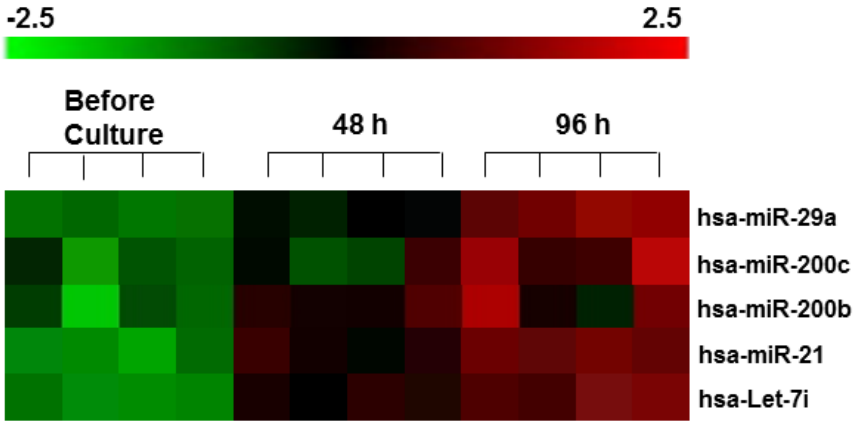

Figure 2-1.2. Heat Map of miRNAs Upregulated >2-Fold in Epithelial Cells from HFL Explants During Differentiation in Culture. Heat map of five miRNAs that were up-regulated ≥2-fold at both 48 and 96 h, as compared with the tissue before culture. miR-29a, together with members of the miR-200 family, miR-21 and Let-7i were found to be significantly upregulated in concert with type II cell differentiation.

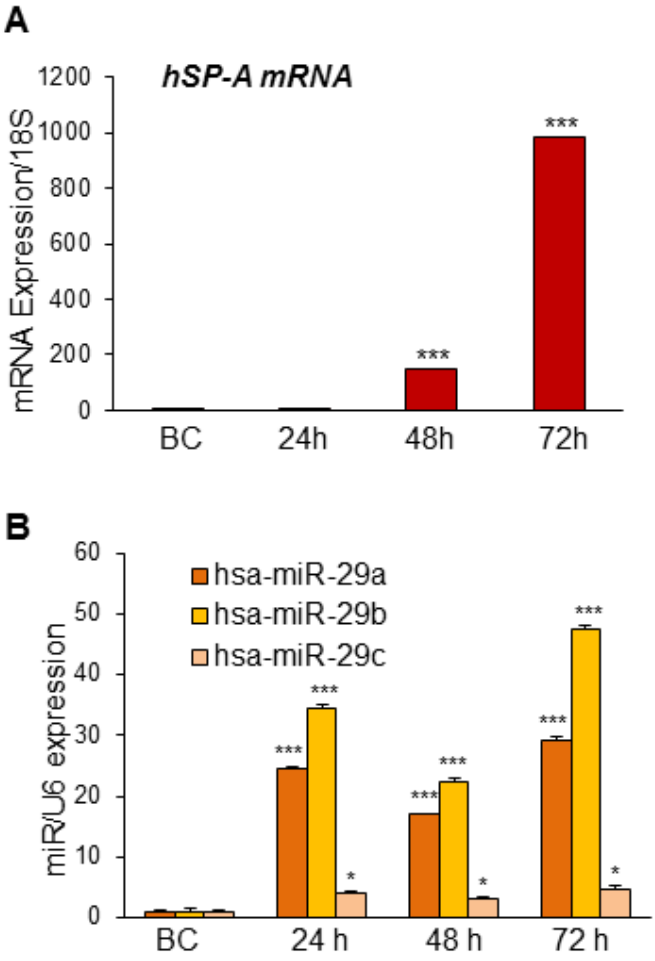

Figure 2-1.3. miR-29 Family Members are Significantly Upregulated in Concert with SP-A mRNA During Differentiation of HFL Epithelial Cells Cultured with cAMP. (A) Epithelial cells isolated from mid-gestation HFL were analyzed for SP-A mRNA before (BC) and after 24 – 72 h of culture in the presence of Bt_2cAMP (1 mM). (B) Expression of mature miR-29 family members was analyzed in the same sets of samples (hsa, *homo sapiens*). Expression of mRNA and miRNAs was determined by RT-qPCR, normalized to h18S and U6, respectively, and expressed as the fold increase over BC samples. Significantly different from values BC (*P < 0.05; ***P < 0.001)

Discussion

In the present study, we have obtained compelling evidence that miR-29 family members are developmentally upregulated in concert with fetal lung epithelial cell differentiation during late gestation. Interestingly, all members of the miR-29 family appear to be similarly upregulated. Members of the miR-29 family contain identical seed sequences (Figure 1-1.3), and thus target the same pool of mRNAs. Since known targets of miR-29 family members include components of the TGF-β signaling cascade, which is known to inhibit type II cell differentiation and function, we hypothesize that collectively this miRNA family serves an important role in regulating type II epithelial cell differentiation and surfactant synthesis during fetal lung development

Having established the temporal expression pattern of miR-29 family in HFL epithelial cells during late gestation, we next sought to explore the functional relevance of the miR-29 family in fetal lung, further identify specific mRNA targets and examine how miR-29 family members influence surfactant protein synthesis and HFL type II cell differentiation.

CHAPTER TWO

SECTION 2
miR-29 Regulates Surfactant Protein Expression and HFL Type II Cell Differentiation

Experimental Design

In light of our findings regarding developmental upregulation of expression of miR-29 family members in HFL epithelial cells during differentiation, we hypothesized that the miR-29 family might play a critical role in regulating type II cell differentiation and sought to further assess the functional roles of miR-29 family in type II cell differentiation.

To determine whether endogenous miR-29 family members are involved in the regulation of expression of the surfactant proteins, HFL epithelial cells were transfected with miR-29 family LNA inhibitors (anti-miR-29); surfactant protein expression was analyzed by RT-qPCR and immunoblotting. To assess whether transfection with anti-miR-29 influences HFL type II cell differentiation, osmium tetroxide (OsO_4) staining was conducted to visualize accumulation of lamellar bodies in HFL type II cells.

Results: Knockdown miR-29 Impairs cAMP-induced Surfactant Protein Expression and Lamellar Body Accumulation

To access the functional roles of miR-29 in type II cell differentiation, we knocked down miR-29 family using anti-miR-29 in HFL epithelial cells cultured in the absence or presence of Bt_2cAMP. RT-qPCR was performed to confirm effects of anti-miR-29 on expression levels of all miR-29 family members (miR-29a/b/c) (Figure 2-2.1A). In cultured HFL epithelial cells transfected with the non-targeting (NT) LNA control, Bt_2cAMP significantly induced mRNA encoding surfactant proteins, SP-A and SP-C, and expression of lysophosphatidylcholine acyltransferase 1 (LPCAT1), a key enzyme surfactant phospholipid synthesis (Bridges et al.; Chen et al., 2006; Gao et al.). By contrast, in cells transfected with anti-miR-29, the inductive effect of Bt_2cAMP on SP-A, SP-C and LPCAT1 was blocked (Figure 2-2.1B, C), suggesting that miR-29 is required for AMP-induced surfactant protein expression.

To determine whether the morphology and function of type II cells was altered by anti-miR-29, we knocked down miR-29 family in HFL type II cells. Previously, it was shown that HFL type II cells cultured at an air/liquid interface in the presence of cAMP maintain their differentiated state for extended periods, as demonstrated by increased SP-A expression and accumulation of osmiophilic lamellar bodies, the storage form of surfactant (Alcorn et al., 1997). Notably, in type II cells transfected with anti-miR-29, a pronounced diminution of lamellar body accumulation was evident by the absence of OsO_4 staining, whereas cells transfected with NT controls showed numerous osmiophilic granules (Figure 2-2.1D). These findings suggest that transfection of anti-miR-29 caused a marked reduction in enzymes

required for surfactant lipoprotein synthesis. This is indicated by the loss of cAMP induction of LPCAT1 expression in anti-miR-29 transfected cells. Altogether, these results demonstrate that miR-29 serves a critical role in HFL type II cell differentiation.

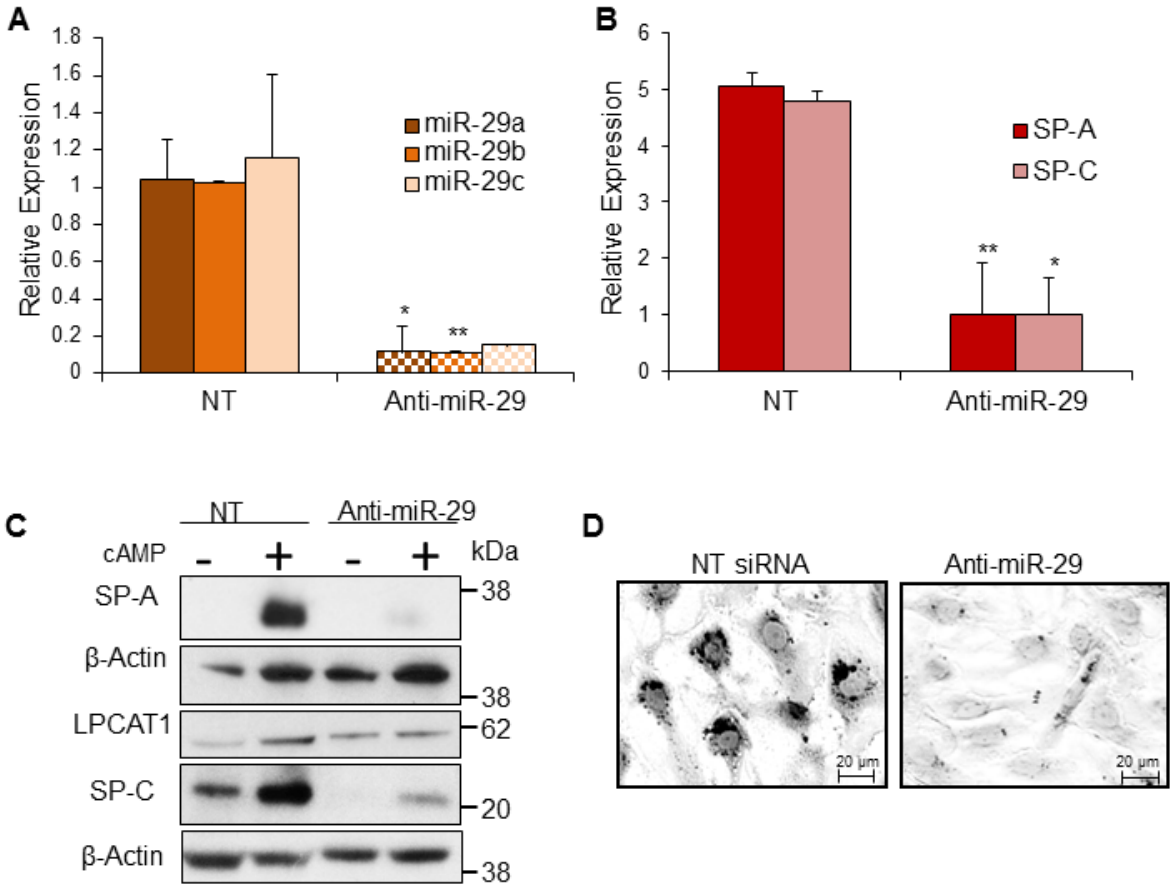

Figure 2-2.1. Knockdown miR-29 abolishes SP-A and SP-C induction by cAMP and reduces lamellar body accumulation in cultured HFL type II cells. (A–D) primary HFL epithelial cells were transfected with LNA-anti-miR-29 (Anti-miR-29) or LNA non-targeting (NT) control. (A) Expression levels of miR-29 family members were analyzed in HFL epithelial cells. The levels of each miRNA were normalized to U6 and expressed as relative fold change. Data shown are the mean ± SEM of triplicate dishes of cells. (B) SP-A and SP-C mRNA were analyzed in HFL epithelial cells after treatment for 48 h. The levels of each mRNA were normalized to h18S and expressed as relative fold change. Data shown are the mean ± SEM of triplicate dishes of cells. Significantly different from values at NT group (*P < 0.05, **P < 0.01). (C) SP-A, SP-C and LPCAT1 protein levels were analyzed by immunoblotting. The immunoblots shown are representative of three replicate experiments. (D) HFL type II cells were transfected with NT or anti-miR-29 and cultured ± Bt_2cAMP for 48 h. Lamellar body accumulation was accessed by osmium tetroxide staining. A representative image is shown for each treatment group.

Discussion

Collectively, these findings provide strong evidence for the functional role of the miR-29 family in promoting HFL epithelial cell differentiation and surfactant protein expression during fetal lung development.

In previous studies, we observed that HFL type II cells cultured at an air/liquid interface at the presence of cAMP maintain their differentiated state for extended periods, as demonstrated by increased SP-A expression and accumulation of osmiophilic lamellar inclusions, the storage form of surfactant lipoprotein (Alcorn et al., 1997). Although the involvement of different clusters and families of miRNAs has been evaluated in lung development (Tian et al., 2011; Ventura et al., 2008), the roles of miRNAs in alveolar type II cell differentiation have not been explored until recently (Benlhabib et al., 2015). Using a microarray based approach and a number of cell culture models, we recently uncovered a role for the miR-200 family and its targets, ZEB1/2, TGF-β2, TGF-βR1 and SMAD2, which serve important roles in the developmental regulation of type II cell differentiation and function (Benlhabib et al., 2015). ZEB1/2 and TGF-β exist in a positive feedforward loop (Gregory et al., 2011) and in a double-negative feedback loop with miR-200s (Benlhabib et al., 2015; Bracken et al., 2008; Burk et al., 2008). In the present study, we found that type II cells transfected with anti-miR-29 manifested a marked decline in cAMP induced SP-A, SP-C and LPCAT1 expression, and a pronounced decrease in OsO_4-stained inclusion bodies within 48 h, indicating a reduction in surfactant lipoprotein synthesis.

Previous studies in gene targeted mice identified roles of miR-29 in distal lung vascular smooth muscle cell differentiation: deficiency of miR-29s *in vivo* invoked an immature lung vascular smooth muscle cell phenotype, specifically associated with distal lung, by regulating FBXO32 and SMC protein degradation (Cushing et al., 2015). Our investigations demonstrated for the first time that the miR-29 family serves a key role in lung epithelial cells by promoting type II cell differentiation. This was indicated by the marked loss of cAMP induced expression of SP-A, SP-C, and LPCAT1, an enzyme required for surfactant lipoprotein synthesis, as well as a profound reduction of lamellar body accumulation in HFL type II cells after miR-29 knockdown.

With these major findings in mind, our next aim was to determine the specific targets of the miR-29 family during HFL type II cell differentiation and to examine the role of these targets in regulating surfactant synthesis and cell differentiation.

CHAPTER TWO

SECTION 3
miR-29 Family Represses TGF-β2 Signaling in HFL Epithelial Cells

Experimental Design

Using computational analysis (Target Scan; http://www.targetscan.org/), we found that miR-29 family is predicted to target over 1000 mRNAs. In order to refine the list of potential targets, we first excluded those targets that were not conserved in both mice and humans. Next, we performed a literature review to search for those remaining putative and known targets that function in processes associated with type II cell differentiation, such as cAMP signaling.

After accessing the relevance of these putative and known targets to surfactant protein production and type II cell differentiation, we selected several for further study (Table). The miR-29 family is known to target collagens, integrins, basement membrane and extracellular matrix remodeling proteins, as well as stem cell factor, KLF4 (Chou et al., 2013; Cittelly et al., 2013; Cushing et al., 2014), suggesting that miR-29 may mediate reprogramming of cells toward a differentiated epithelial phenotype. Interestingly, TGF-β2 is a known target of the miR-29 family in mouse and human (Chou et al., 2013) that is known to transcriptionally repress SP-A expression in NCI-H441 lung adenocarcinoma cell line (Whitsett et al., 1992).

Notably, our previous work showed that TGF-β1, -2, and -3 mRNA declined significantly in mid-gestation HFL epithelial cells cultured in medium containing cAMP for

24-72 h. This was associated with pronounced induction of SP-A mRNA (Benlhabib et al., 2015). ZEB1/2 was correspondingly downregulated, as was the EMT marker vimentin, whereas E-cadherin, an index of epithelial cell differentiation, increased with the temporal induction of SP-A (Benlhabib et al., 2015). Notably, cAMP treatment significantly decreased mRNA levels of TGF-β1, TGF-β2, and TGF-β3. Nuclear levels of phospho-Smad2, a downstream transcriptional mediator of the TGF-β signaling pathway, and CtBP1, known to interact with ZEB1 at target gene promoters and to enhance its repressive activity, were also reduced by cAMP treatment. ZEB1/2 mRNA and protein expression also were profoundly decreased by cAMP treatment, suggesting that cAMP enhances type II cell differentiation, in part, by inhibiting TGF-β signaling and ZEB1/2 expression and function (Benlhabib et al., 2015).

To determine whether expression of TGF-β2 mRNA and protein was directly targeted by miR-29 family, we transfected HFL epithelial cells with LNA miR-29 family inhibitor (anti-miR-29) to repress endogenous miR-29 expression and signaling. Then, to evaluate whether miR-29 family promotes surfactant protein expression and type II cell differentiation by directly targeting the TGF-β2 signaling pathway, we transfected HFL epithelial cells with miR-29 family inhibitor in the absence or presence of LY364947, a selective, ATP-competitive inhibitor of TGF-β Type I receptor kinase (TGF-β RI).

Results: TGF-β2 is De-repressed in HFL Epithelial Cells Transfected with Anti-miR-29

As mentioned, the 3'-UTR of TGF-β2 contains one putative binding site for miR-29 family members; direct binding of miR-29 to the TGF-β2 3'-UTR and miR-29 inhibition of TGF-β2 expression have previously been demonstrated (Chou et al., 2013). Although previous studies indicated that miR-29 family members downregulate expression of TGF-β2 in a variety of cells types, the question of whether miR-29 promotes HFL type II cell differentiation through inhibition TGF-β2 expression and signaling has not previously been explored. To investigate the regulation of TGF-β2 signaling by members of the miR-29 family during HFL development, we analyzed the effects of miR-29 knockdown on TGF-β2 expression. As expected, following transfection of miR-29 family inhibitor, levels of the endogenous TGF-β2 precursor protein were increased. Furthermore, anti-miR-29 transfected cells manifested higher levels of p-Smad2 and the downstream mediator ZEB1 (Figure 2-3.1). This suggests that miR-29 knockdown enhances TGF-β signaling and thereby reduces SP-A expression.

To further determine whether the inhibitory effects of miR-29 knockdown on type II cell differentiation were mediated by an upregulation of TGF-β2 signaling, we analyzed the effects of anti-miR-29 on HFL epithelial cells in the absence or presence of the TGF-βRI signaling inhibitor LY364947. Our findings that LY364947 blocked the inhibitory effects of anti-miR-29 on SP-A mRNA (Figure 2-3.2A) and protein (Figure 2-3.2B) expression suggest that the actions of miR-29 to promote surfactant protein gene expression are mediated, at least in part, by inhibition of TGF-β2 signaling.

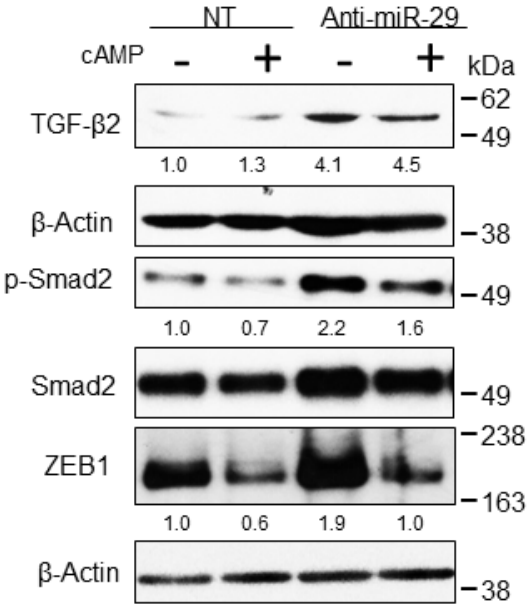

Figure 2-3.1. Knockdown miR-29 Enhances TGF-β2 Signaling in Cultured HFL Epithelial Cells. Transfection of anti-miR-29 increased protein levels of TGF-β2 precursor and downstream mediators, p-SMAD2 and ZEB1.

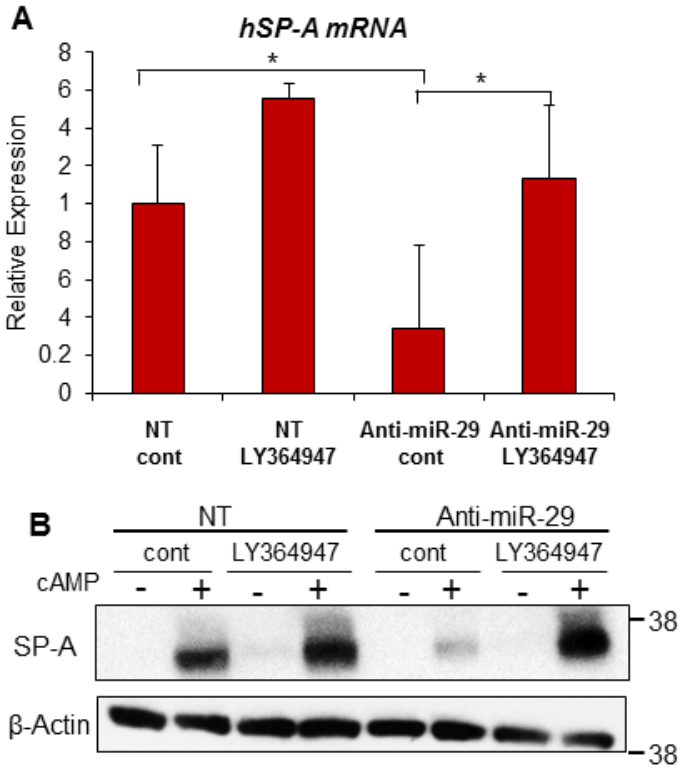

Figure 2-3.2. Anti-miR-29 Exerts Its Inhibitory Effects on SP-A Expression by Upregulating the TGF-β Signaling Pathway in HFL Epithelial Cells. HFL epithelial cells were transfected with NT control or with anti-miR-29 overnight and treated ± 10 μM LY364947 ± Bt_2cAMP for 48 h and analyzed for SP-A mRNA (A) and protein (B).The profound decrease in SP-A mRNA and protein expression caused by anti-miR-29 was blocked by co-treatment with LY364947. Data shown in B are the mean ± SEM of values from 3 replicates (Student's t test, *P < 0.05).

Discussion

These findings establish the presence of the miR-29/TGF-β2 relationship during cAMP-induced HFL type II cell differentiation. In the mouse fetal lung, TGF-β1 expression is detected in epithelial cells as early as day 11 of gestation (Heine et al., 1990). TGF-β2 has previously been observed to be expressed in HFL lung epithelial cells and downregulated by cAMP (Benlhabib et al., 2015). Notably, treatment of HFL explants with TGF-β suppresses hormone-induced (glucocorticoid + cAMP) type II cell differentiation (Beers et al., 1998). These and other observations support the concept that TGF-β is a negative regulator of lung development.

Our investigations implicate an important regulatory role of TGF-β2, which was downregulated by cAMP during HFL type II cell differentiation and targeted by the miR-29 family. Anti-miR-29 caused a de-repression of TGF-β2 protein expression and increased expression of p-SMAD2 and ZEB1. This supports our hypothesis that miR-29 enhances SP-A expression and type II cell differentiation by inhibiting TGF-β expression and signaling . To further substantiate our hypothesis, we examined the influence of inhibiting TGF-β signaling in HFL epithelial cells transfected with anti-miR-29. Consistent with our hypothesis, inhibiting TGF-β signaling using LY364947 prevented the inhibition of SP-A mRNA and protein in cells transfected with anti-miR-29.

As mentioned above, members of the miR-29 family directly bind and downregulate expression of TGF-β2 (Chou et al., 2013). Furthermore, TGF-β treatment, in turn, significantly downregulates expression of miR-29 in many cell types, including the proximal renal tubular cell line HK-2 cells (Du et al., 2010), HFL fibroblast cells (Cushing et al.,

2011), human and mouse hepatic stellate cells (Roderburg et al., 2011; Zhang et al., 2014), and renal tubular epithelial cells (Jiang et al., 2013; Qin et al., 2011; Wang et al., 2012). Thus, the miR-29 family and TGF-β interact in a double-negative feedback loop. This reciprocal repression maintains a balance of miR-29 and TGF-β within the cell that can be tipped at any given time by changes in TGF-β expression. This is exemplified in a mouse model of progressive renal fibrosis and obstructive nephropathy, in which the induction of renal fibrosis was accompanied by reduced expression of miR-29 and enhanced TGF-β signaling (Qin et al., 2011). In the current investigation, we found that cAMP treatment triggered a decline TGF-β signaling and the induction of miR-29 family expression.

TGF-β signaling is known to be a critical regulator of cell spreading, adhesion, and tissue morphogenesis (Massague, 2012). Notably, TGF-β inhibition of IL-2 gene expression in T-cells was reported to be mediated by Smad2/3 interaction with NF-κB and recruitment of Suv39H1 to the *IL-2* promoter (Wakabayashi et al., 2011). In addition, in H441 lung adenocarcinoma cells, TGF-β inhibition of SP-B expression was associated with Smad3 interaction with TTF-1, HNF-3, and FoxA1 (Li et al., 2002; Minoo et al., 2008). Thus, TGF-β inhibition of SP-A expression in HFL type II cells may be mediated, in part, by Smad2/3 interaction with both TTF-1 and NF-κB.

The current studies have elucidated a role for the miR-29 family and its reciprocal targets, TGF-β2 and TGF-βRI, in the regulation of HFL type II cell differentiation and surfactant protein expression. Our investigations have revealed that the miR-29 family is significantly upregulated upon HFL type II cell differentiation and coordinated with the downregulation in TGF-β2 signaling in these same tissues. Furthermore, knockdown of miR-

29 results in a de-repression of TGF-β2 and an enhancement of downstream TGF-β signaling mediators including p-SMAD2 and ZEB1. The inhibition of TGF-β signaling through LY364947 in miR-29 knockdown cells was found to be sufficient to reverse anti-miR-29 inhibition of differentiation as indicated by the induction of SP-A mRNA and protein. Taken together, these results indicate that miR-29 family promotes HFL type II cell differentiation, and loss of miR-29 family causes dramatic loss of differentiation markers including SP-A, SP-C, LPCAT1 and lamellar body accumulation; this is mediated, at least in part, by an increased level of TGF-β signaling.

Considering the essential role of cAMP in the induction of surfactant protein expression and type II cell differentiation, it was of importance to investigate the effects of cAMP on miR-29 family expression in HFL epithelial cells.

CHAPTER TWO

SECTION 4
The miR-29/TGF-β2 Relationship is Conserved from Human to Mice

Experimental Design

As mentioned above, the lung development extends from the embryonic period through the fetal period up to birth (Cardoso and Lu, 2006). The saccular stage of lung development, which extends from approximately embryonic day 16.5 (E16.5) to E18.5 of mouse gestation, is a pivotal step when the distal airspace saccules are generated as a first step toward alveologenesis and results in the extensive dilation and expansion of the distal lung region after initial specification and differentiation of the alveolar epithelium (Wang et al., 2016). As found previously, SP-A mRNA levels are barely detectable in mouse fetal lung at 15.5 dpc, increase modestly at 16.5 dpc, and rise dramatically thereafter toward term (19.0 dpc) (Condon et al., 2004; Korfhagen et al., 1992).

Since miR-29 family members are highly conserved in human, mouse and rat, we hypothesized that the roles and relative expression pattern of miR-29 family and its targets during pulmonary alveolar epithelial differentiation would be conserved from human to mouse. Importantly, miR-29 family members are developmentally regulated both in mouse and human fetal lung and their seed sequences are identical suggesting common targets.

To investigate whether the developmental regulation of the miR-29 family and its targets is conserved from mouse to human during fetal lung alveolar epithelial cell

differentiation, we isolated epithelial cells from mouse fetal lung from 15.5 dpc to 18.5 dpc and analyzed the expression levels of miR-29 family members and their target, TGF-β2. Downstream TGF-β signaling mediators, including p-Smad2, and Zeb1 and 2 were also analyzed in mouse fetal lung extracts at the same developmental timepoints by immunoblotting.

Results: TGF-β2 is Downregulated in MFL Epithelial Cells during Late Gestation

To validate the findings obtained using cultured HFL epithelial cells in an *in vivo* system, we isolated a highly enriched population of epithelial cells from MFL (Fig. 2-4.1A) at 15.5 to 18.5 days of gestation. Previous work showed that *SP-A* mRNA levels were virtually undetectable in MFL at 15.5 dpc, increased modestly at 16.5 dpc, and rose dramatically thereafter toward term (19.0 dpc) (Benlhabib and Mendelson, 2011; Condon et al., 2004; Korfhagen et al., 1992). We observed a similar developmental increase of SP-A mRNA in isolated MFL epithelial cells (Figure 2-4.1B). Notably, the expression level of miR-29 family members was significantly increased in MFL epithelial cells at 17.5 and 18.5 dpc, as compared with epithelial cells isolated at 15.5 dpc (Figure 2-4.1C). This suggests that the developmental pattern of miR-29 family members during epithelial cell differentiation in fetal lung is conserved in human and mouse.

Notably, previous work showed that TGF-β1, -2, and -3 mRNA declined significantly in epithelial cells isolated from mid-gestation HFL cultured in medium containing cAMP for 24-72 h (Benlhabib et al., 2015). This was correlated with an upregulation of miR-29 expression in HFL epithelial cells (Figure 2-1.3B).

Supporting our hypothesis that the inverse regulatory relationship between miR-29 and TGF-β2 may play a role in fetal lung type II cell differentiation in mouse and human, the miR-29 family was found to be significantly upregulated in close association with SP-A induction in epithelial cells isolated from MFL between 15.5 dpc to 18.5 dpc of gestation. In contrast, we observed that TGF-β2 mRNA and protein precursor significantly declined in isolated MFL epithelial cells at 17.5 and 18.5 dpc, compared to 16.5 dpc (Figure 2-4.2 A and

B). Additionally, transcription factors p-Smad2, Zeb1 and 2, which are induced by TGF-β signaling and promote EMT, were decreased significantly in MFL extracts from 15.5 dpc to 17.5 dpc and 18.5 dpc (Figure 2-4.3 A and B). Collectively, these findings suggest that the TGF-β signaling pathway is repressed during late fetal lung development in both mouse and human and that this is due, in part, to the developmental up-regulation of miR-29 family members.

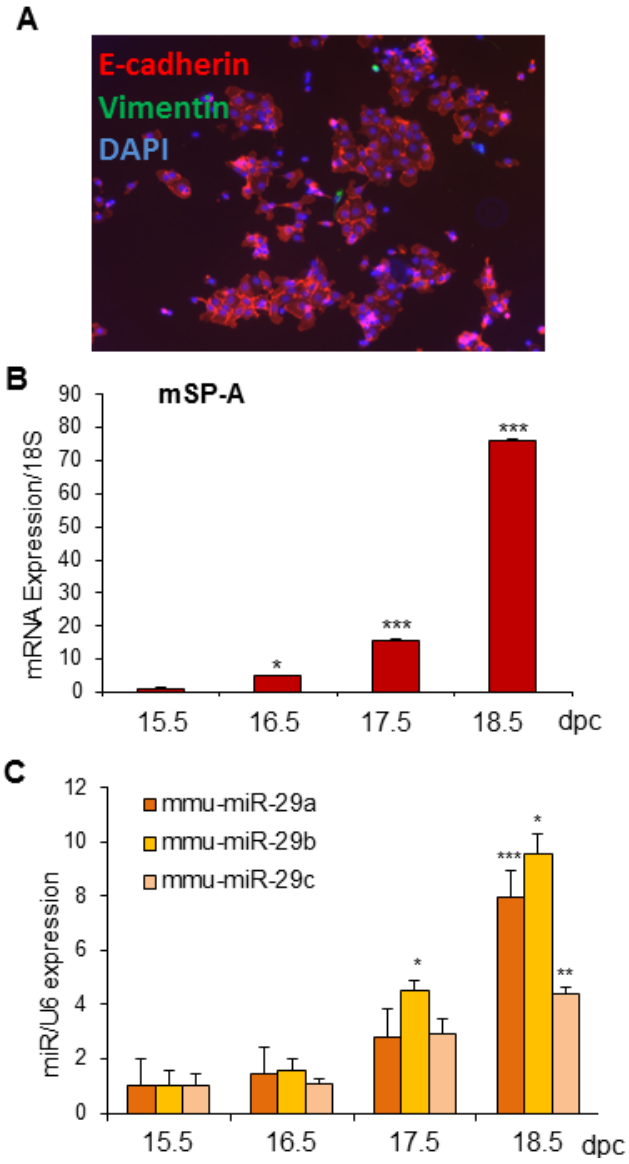

Figure 2-4.1. miR-29 Family Members are Significantly Upregulated During Differentiation of MFL Epithelial Cells from 15.5 dpc to 18.5 dpc. (A) Isolated MFL epithelial cells were double stained for epithelial cell marker, E-cadherin (red), and the mesenchymal cell marker, Vimentin (green), demonstrating the enrichment of epithelial cells. Nuclei were stained using DAPI (blue). (B) SP-A mRNA was analyzed in epithelial cells isolated from MFL at 15.5 – 18.5 dpc (N ≥ 3 mouse fetal lungs per time point). (C) miR-29 family expression was analyzed in the same sets of samples (mmu, mus musculus). Expression of each mouse mRNA/miRNA was analyzed by RT-qPCR, normalized to m36B4/U6, and expressed as the fold increase over values at 15.5 dpc. Significantly different from values at 15.5 dpc (*P < 0.05; **P < 0.01; ***P < 0.001; N ≥ 3 mouse fetal lungs per time point). Data were analyzed by the Student's t test: *P < 0.05, **P < 0.01, ***P < 0.001; Mean ± SEM values are shown.

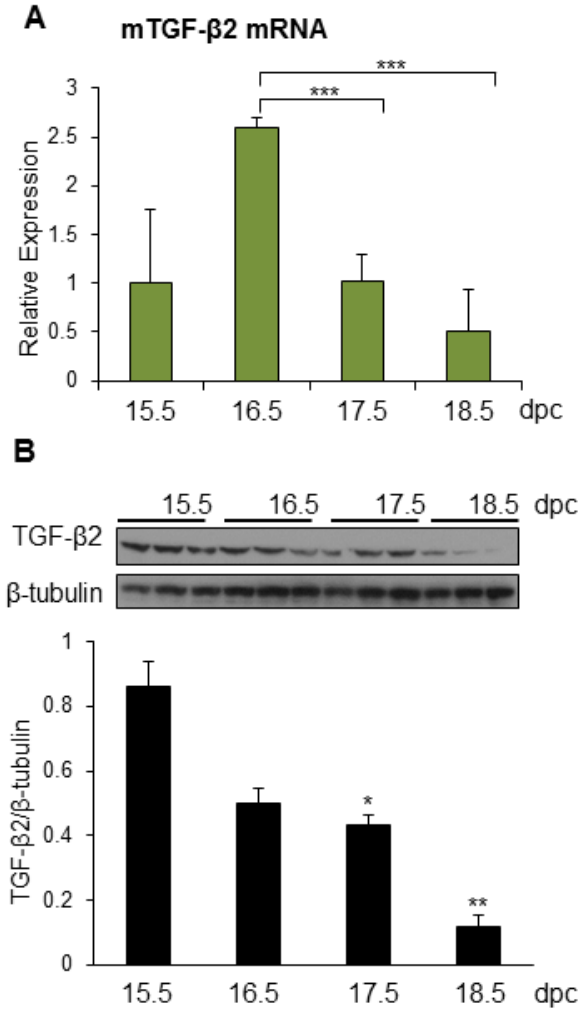

Figure 2-4.2. TGF-β2 is Downregulated in MFL during Late Gestation. (A) Expression of TGF-β2 mRNA was analyzed in epithelial cells isolated from MFL at 15.5 - 18.5 dpc by RT-qPCR, normalized to m36B4, and expressed as the fold change over values at 15.5 dpc. Mean ± SEM values are shown. Significantly different from values at 16.5 dpc (***$P < 0.001$). $N \geq 3$ mice at each gestational time point. (B) Expression of TGF-β2 protein precursor was analyzed by immuno-blotting of whole cell extracts isolated from MFL at 15.5 – 18.5 dpc. β-tubulin was used as a loading control. Densitometry analysis revealed that TGF-β2 protein was significantly decreased at 17.5 and 18.5 dpc from values at 15.5 dpc (*$P < 0.05$; **$P < 0.01$; $N = 3$ mice per group).

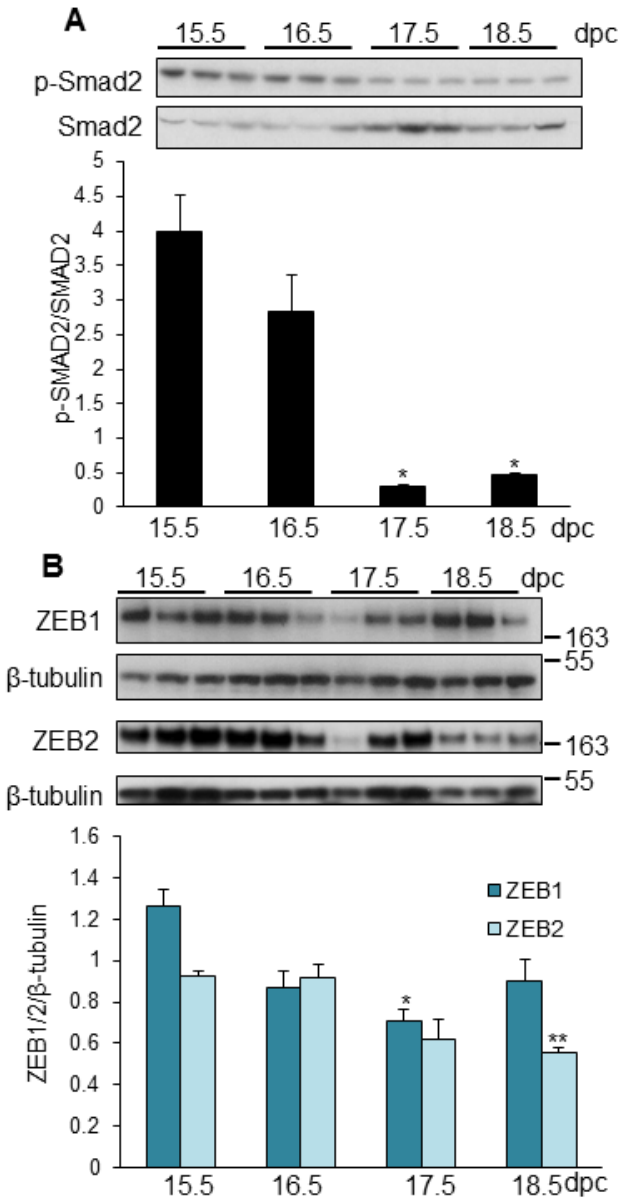

Figure 2-4.3. TGF-β2 Signaling Pathway Activity is Downregulated in MFL during Late Gestation. (A and B) Expression of TGF-β2 signaling downstream factors, p-SMAD2 and ZEB1/2, were analyzed by immunoblotting of whole cell extracts isolated from MFL at 15.5 – 18.5 dpc. Total SMad2 and β-tubulin were used as loading controls. Densitometry analysis revealed a significant decrease in p-SMAD2 (A), ZEB1 and 2 (B) proteins from values at 15.5 dpc (*P < 0.05; **P<0.01; N = 3 mice per group).

Discussion

Importantly, our findings that miR-29 family expression is upregulated in MFL epithelial cells during late gestation agrees with our findings of HFL epithelial cells during differentiation in culture. This supports our hypothesis that the miR-29/TGF-β relationship in pulmonary alveolar epithelial cells is conserved during fetal lung epithelial cell differentiation in mouse and human. These findings in fetal lung epithelial cells provide important insight into the regulation of the TGF-β signaling pathway, whose function is essential as a major inducer of EMT during embryogenesis, cancer progression and fibrosis (Moustakas and Heldin, 2007).

Previous studies identified important regulatory roles of TGF-β in lung morphogenesis. Mice homozygous for deletion of *TGF-β3* gene manifested delayed pulmonary development (Kaartinen et al., 1995), while loss of TGF-β2 led to perinatal mortality and a wide range of developmental defects, including those in lung (Sanford et al., 1997), suggesting a critical role of TGF-β in lung morphogenesis. On the other hand, TGF-β1, -β2, and -β3 were found to be equally effective in inhibiting SP-A expression in NCI-H441 lung adenocarcinoma cells (Whitsett et al., 1992). Our findings suggest that during early to mid-gestation there is increased expression of TGF-β and ZEB1/2, which maintain suppression of miR-29 family members, inhibit TTF-1 transcriptional activity, and promote increased plasticity of epithelial cells and their invasive/mesenchymal properties without entirely committing to a mesenchymal phenotype (Benlhabib et al., 2015). Near term, the decline of TGF-β2 mRNA and downstream signaling may be due at least partially to the marked associated increase in expression of the miR-29 family members. The reciprocal

regulation of TGF-β2 and miR-29 family expression during fetal lung epithelial cell differentiation in mice and human suggests that the role of these miRNAs in *TGF-β2* expression regulation is conserved between these species.

CHAPTER TWO

SECTION 5
Transcriptional Regulation of the miR-29 Expression in HFL Epithelial Cells

Experimental Design

The miR-29 family has been reported to be expressed in a variety of tissue and cell types, and a number of transcription factors, including c-Myc, GATA3, NF-κB, CCAAT-enhancer-binding proteins (C/EBP), GLI-Kruppel family member (GLI), Yin Yang 1 (YY1), SMAD3 and lymphoid enhancer-binding factor (TCF/LEF) have been found to bind the promoter regions of *miR-29* genes to regulate their expression (Chang et al., 2008; Chou et al., 2013; Cushing et al., 2014; Mott et al., 2010; Wang et al., 2008; Xiao et al., 2012a; Zhou et al., 2012). However, little is known about transcriptional regulation of the miR-29 family in developing lung epithelium.

Previously, we observed that cAMP treatment enhanced the rate of type II cell differentiation and caused induction of *hSP-A* gene expression (Odom et al., 1987). The mechanisms by which cAMP induces type II cell differentiation are notably complex, one of them is through increased activation and binding of TTF-1 to a composite response element 175-bp upstream of the *hSP-A* transcription initiation site (Islam and Mendelson, 2002; Li et al., 1998). In light of the stimulatory effect of cAMP on type II cell differentiation and *SP-A* expression, it was of interest to investigate the effects of cAMP on expression of the miR-29 family in HFL epithelial cells in culture.

To analyze the effects of cAMP on miR-29 expression, HFL epithelial cells were cultured in the absence or presence of cAMP for 24 h. Potential transcription factor binding sites within the *miR-29* promoter region were analyzed using the PROMO prediction program. *In vivo* binding of potential transcription factors to the *miR-29* promoter in HFL type II cells was analyzed by chromatin immunoprecipitation coupled to detection by quantitative real-time PCR (ChIP-qPCR). To assess the effects of potential transcription factors on *miR-29* promoter activity, A549 lung adenocarcinoma cells were cotransfected with a reporter construct containing 539 bp of the human *miR-29b-1/a* gene (*miR-29ab1*) 5'-flanking sequence subcloned upstream of a luciferase reporter and with an expression vector encoding the transcription factor. The cells were cultured for 48 h and assayed for luciferase activity.

Results: cAMP upregulation of miR-29 in HFL type II cells is mediated by TTF-1

We observed that cAMP significantly increased miR-29a, -b and -c expression in HFL type II cells compared to control after 24 h (Figure 2-5.1A). This was associated with a cAMP-mediated reduction of TGF-β2 mRNA and protein and a coordinate repression of p-SMAD2 and ZEB expression (Benlhabib et al., 2015).

To identify transcription factors that might regulate miR-29 expression in HFL type II cells, we used the PROMO prediction program (Farré et al., 2003; Messeguer et al., 2002) to search for putative transcription factor binding sites upstream of the *miR-29ab1* promoter. Notably, several potential TTF-1 binding elements were found.

In order to determine whether TTF-1 binds to the *miR-29ab1* promoter and activates its transcription, we first analyzed a published TTF-1 ChIP-seq (ChIP combined with deep sequencing) database (GES51510) obtained using NCI-H441 bronchioalveolar adenocarcinoma cells (Isogaya et al., 2014). Interestingly, we found significant TTF-1 binding peaks at the human *miR-29ab1* promoter and within the gene body (Figure 2-5.1B). To determine whether endogenous TTF-1 binds to the *miR-29ab1* promoter in HFL type II cells, we designed primers flanking putative TTF-1-binding elements (TBEs) and performed ChIP-qPCR using TTF-1 antibodies. In HFL type II cells cultured in control medium, TTF-1 binding was increased significantly over the IgG control; moreover, TTF-1 binding was markedly increased by cAMP-treatment (Figure 2-5.1C).

To determine whether TTF-1 transactivates the *miR-29ab1* promoter, we cotransfected A549 lung adenocarcinoma cells with a reporter construct containing 539 bp of the human *miR-29b-1/a* gene (*miR-29ab1*) 5'-flanking sequence subcloned upstream of a

luciferase reporter and increasing amounts of an expression vector containing either wild-type (WT) TTF1 or TTF1 lacking its homeodomain (ΔHD), which is known to be critical for transcriptional activation of its target gene promoters (De Felice et al., 1995; Runkle et al., 2012). As can be seen, increasing amounts of TTF-1 expression vector caused upregulation of *miR-29ab1* promoter activity in a dose-dependent manner (Figure 2-5.2A); however, this was largely prevented in cells co-transfected with TTF-1ΔHD (Figure 2-5.1 B and C). These findings suggest that TTF-1 enhances *miR-29ab1* expression by direct binding to the *miR-29ab1* promoter and that TTF-1 promoter binding and activity are increased by cAMP. Together, these findings suggest that *miR-29ab1* gene transcription is directly regulated by TTF-1.

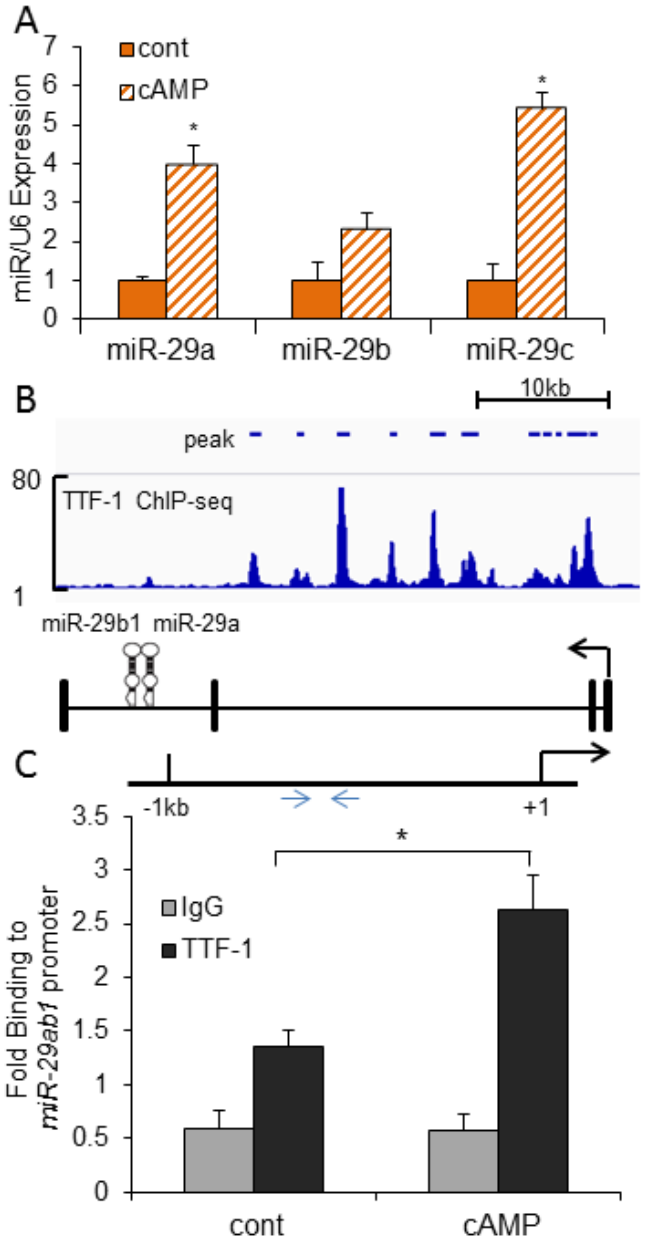
A
cont
cAMP
miR/U6 Expression
0
1
2
3
4
5
6
7
*
*
miR-29a
miR-29b
miR-29c
B
10kb
peak
80
TTF-1 ChIP-seq
1
miR-29b1 miR-29a
C
-1kb
+1
Fold Binding to miR-29ab1 promoter
0
0.5
1
1.5
2
2.5
3
3.5
*
IgG
TTF-1
cont
cAMP

Figure 2-5.1. cAMP Induction of miR-29 Expression in HFL Type II Cells is Mediated by Increased Binding of TTF-1 to the *miR-29ab1* Promoter. (A) miR-29 family expression was increased by treatment of HFL type II cells for 24 h with Bt_2cAMP. Expression of miRNAs was determined by RT-qPCR, normalized to U6, and expressed as the fold increase over control samples. Significantly different from control values (*P < 0.05). (B, upper panel) ChIP-seq profile of TTF-1 binding to the human *miR-29ab1* locus in NCI-H441 cells (GEO database, GSE51510 (36)). (B, lower panel) Validated model of miR-29ab1 transcript (EU154353(21)). (C) cAMP induction of miR-29 expression was associated with increased binding of endogenous TTF-1 to the *miR-29ab1* promoter region, analyzed by ChIP-qPCR. HFL type II cells, cultured ± Bt_2cAMP for 48 h were analyzed for binding of endogenous TTF-1 to the *miR-29ab1* upstream region using ChIP-qPCR with rabbit non-immune or anti-TTF-1 IgG. TTF-1 binding was quantified by qPCR using primers flanking -484 bp to -627 bp upstream of the miR-29 transcription unit, normalized to input and expressed as fold-increase over IgG control. Data are the mean ± SEM from four independent experiments. Significantly different from Control (*P < 0.05).

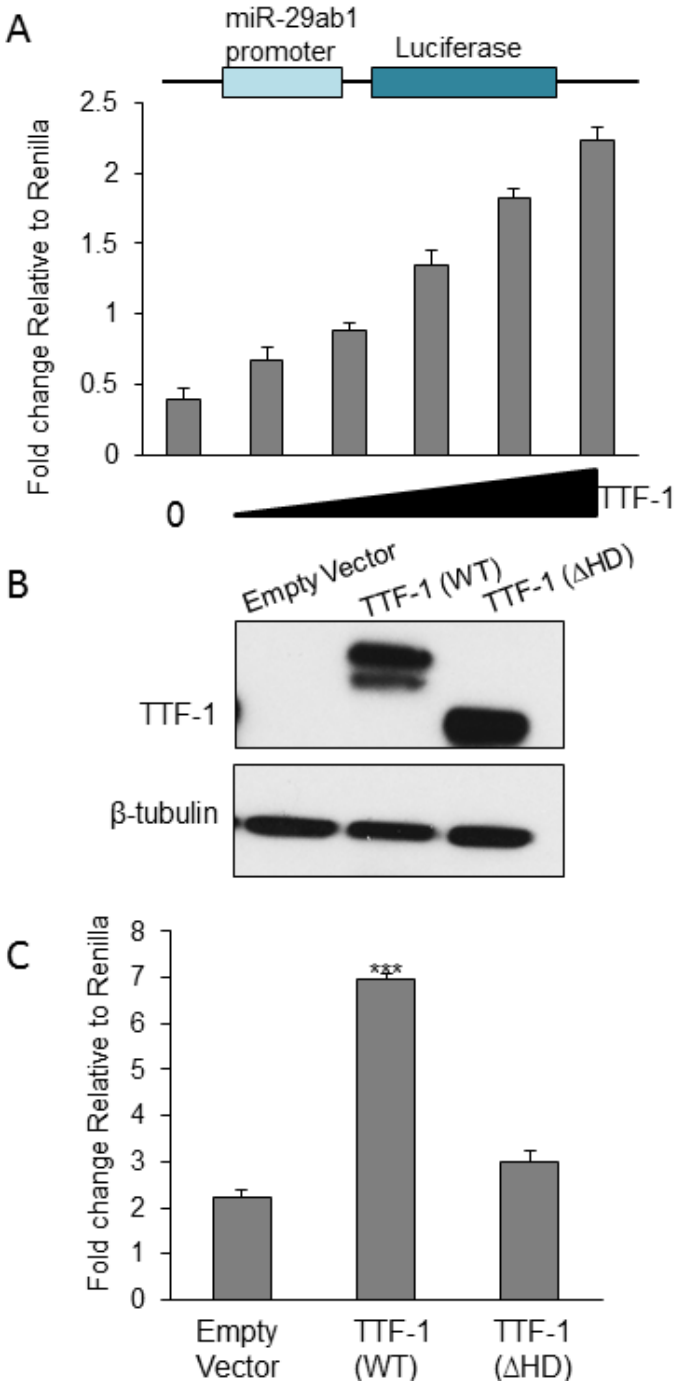
A
miR-29ab1
promoter
Luciferase
Fold change Relative to Renilla
2.5
2
1.5
1
0.5
0
0
TTF-1
B
Empty Vector
TTF-1 (WT)
TTF-1 (ΔHD)
TTF-1
β-tubulin
C
Fold change Relative to Renilla
8
7
6
5
4
3
2
1
0
***
Empty Vector
TTF-1 (WT)
TTF-1 (ΔHD)

Figure 2-5.2. cAMP Induction of miR-29 Expression in HFL Type II Cells is Mediated by TTF-1 Stimulation of *miR-29ab1* Promoter Activity. (A) A549 cells were transiently transfected with a *miR-29ab1* promoter-luciferase reporter construct containing 539 bp of 5′-flanking sequence from the hsa-*miR-29ab1* gene, Renilla luciferase and increasing amount of human TTF-1 expression plasmid (0, 10 ng, 20 ng, 40 ng, 80 ng, 160 ng). After 48 h, the cells were harvested and luciferase activity was assayed and normalized to Renilla luciferase activity. (B and C) The TTF-1 homeodomain is required for induction of *miR-29ab1* promoter activity. A549 cells were co-transfected with human *miR-29ab1* promoter-luciferase reporter construct with 100 ng of empty vector, TTF-1 (wt), or TTF-1 (ΔHD) expression vectors. Overexpression of TTF-1 and TTF-1ΔHD was confirmed by immunoblotting (E). Luciferase activities were assayed 48 h after transfection. Data in D and F are the mean ± SEM values of four replicates. Significantly different from empty vector (***P < 0. 001).

Discussion

Despite the importance of the terminal stages of pulmonary development and their relation to neonatal respiratory diseases, little is understood about the molecular events underlying alveologenesis during lung development. In this regard, the molecular mechanisms that promote type II cell differentiation and surfactant synthesis during late gestation remain incompletely understood. It is clear that cAMP analogues enhance the rate of type II cell differentiation and of enlargement of the prealveolar ducts in midgestation human fetal lung explants (Odom et al., 1987). cAMP mediates the induction of *SP-A* expression, in part, by recruitment to the *hSP-A* promoter of the critical transcription factors, TTF-1, NF-κB, and histone-modifying cofactors, which promote permissive changes in chromatin structure (Benlhabib and Mendelson, 2011; Islam and Mendelson, 2002; Li et al., 1998). Our recent studies have revealed that cAMP promotes type II cell differentiation and surfactant protein gene expression in HFL, in part, by its marked effect to increase expression of the miR-200 family and repress ZEB1 and TGF-β expression and signaling, resulting in increased TTF-1 transcriptional activity (Benlhabib et al., 2015).

Our present findings suggest that another microRNA family, the miR-29 family, serves a critical role in type II cell differentiation via its action to downregulate expression of TGF-β2 and repress the TGF-β signaling pathway. We also provide strong evidence to suggest that *miR-29ab1* is a direct TTF-1 target gene in HFL epithelial cells. We hypothesize that during early to mid-gestation when the fetal lung is relatively hypoxic, cAMP signaling is impaired (Acarregui et al., 1993; Benlhabib and Mendelson, 2011; Islam and Mendelson, 2006), allowing increased expression of TGF-β and ZEB1/2 (Benlhabib et al., 2015), which

inhibit type II cell differentiation. The increased TGF-β and ZEB1/2 levels maintain suppression of miR-29s, inhibit TTF-1 transcriptional activity, and promote increased plasticity of epithelial cells and their invasive/mesenchymal properties without entirely committing to a mesenchymal phenotype (Benlhabib et al., 2015). Suppressed TTF-1 transcriptional activity further decreases miR-29 family expression.

Near term, increased vascularization of the fetal lung and elevated local O_2 tension increases cAMP signaling and stimulates TTF-1 DNA-binding and transcriptional activity, resulting in increased miR-29 expression. Elevated expression of the miR-29 family, in turn, inhibits miR-29 targets, such as TGF-β2, resulting in a further decline of p-SMAD and ZEB levels. This relieves suppression on their downstream targets, including SP-A to further promote surfactant lipoprotein synthesis and type II cell differentiation (Figure 2-5.3).

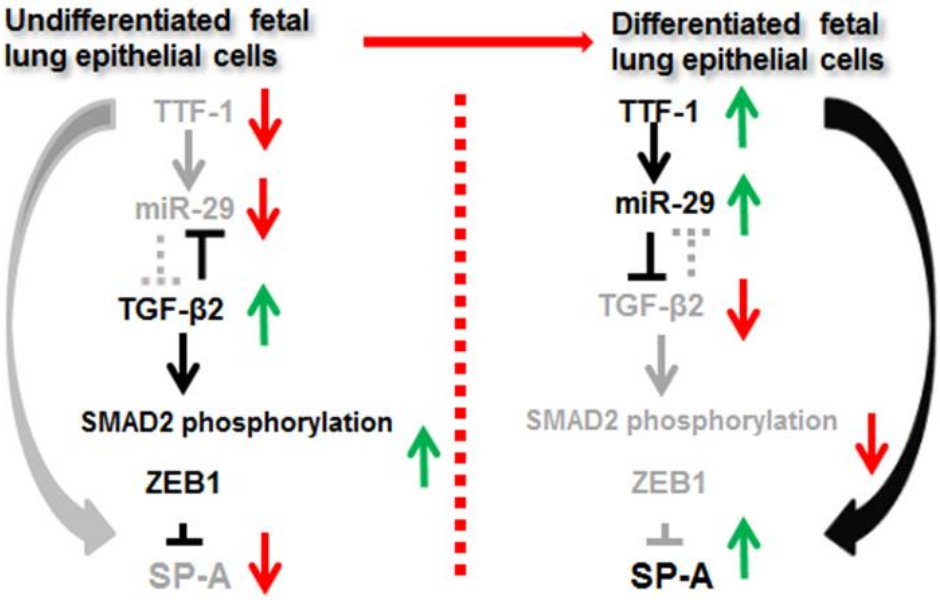

Figure 2-5.3. Increased Expression of the miR-29 Family in Fetal Lung Near Term Serves a Key Role in Promoting Type II Cell Differentiation and Function through Repression of TGF-β2 Signaling. During early to mid-gestation, the fetal lung is relatively hypoxic and cAMP signaling is decreased. This allows for increased expression of TGF-β and ZEB1/2, which inhibit type II cell differentiation. The increased TGF-β and ZEB1/2 levels inhibit TTF-1 transcriptional activity and maintain decreased miR-29ab1 expression. Near term, increased vascularization of the fetal lung and elevated local O_2 tension increases cAMP signaling and stimulates TTF-1 DNA-binding and transcriptional activity, resulting in increased miR-29 expression. This, in turn, suppresses miR-29 targets, such as TGF-β2, resulting in increased type II cell differentiation and surfactant lipoprotein synthesis.

CHAPTER TWO

SECTION 6
Generation of the miR-29 Tissue Specific Knockout Mouse

Experimental Design

The findings presented thus far strongly suggest that miR-29 family members serve a critical role in promoting differentiation of HFL type II cells. Notably, introduction of miR-29 into the lungs of bleomycin treated mice reduced the severity of fibrosis (Xiao et al., 2012a), suggesting a possible action to promote mesenchymal to epithelial transition (MET). Notably, miR-29 family members transcribed from the two genomic loci, *miR-29a/b1* and *miR-29b2/c*, are the most abundant miRNAs in adult mouse lungs (REF). To understand roles of the miR-29 family and their targets in alveolarization, type II cell differentiation and lung pathology, our next objective was to examine the impact of conditional ablation of miR-29 family members in lung epithelium of mice.

As mentioned, miR-29 family members exist in two conserved chromosomal clusters comprised of *miR-29a/b1* and *miR29b2/c* (Chang et al., 2008; Mott et al., 2010). Previous studies showed that global deletion of the *miR-29a/b1* genomic cluster resulted in unrestrained T-bet expression and IFN-γ production (Smith et al., 2012). In more recent studies, global double-knockout mice for *miR-29ab1* and *miR-29b2c* alleles were founbd to manifest significant postnatal growth retardation, and died at 4-6 weeks after birth from presumed defects in differentiation of lung vascular smooth muscle cells within distal vessels

(Cushing et al., 2015). However, the specific functions of miR-29 family members in differentiation of the mouse lung epithelium remain undefined.

To assess the lung epithelial-specific roles of the miR-29 family in branching morphogenesis and alveolarization, we generated epithelium-specific *miR-29* single and double cluster knockout mouse lines by crossing *miR-29ab1*$^{flox/flox}$ and *miR-29ab1*$^{flox/flox}$*/miR-29b2c*$^{flox/flox}$ mouse line with *shh-Cre* line. The *miR-29ab1*$^{flox/flox}$ mouse and *miR-29b2c*$^{flox/flox}$ mouse were generously provided by the Carlo M. Croce lab. This section details the evidence that *miR-29ab1* and *miR-29b2c* alleles were deleted in lung epithelial cells of knockout mice, however, defects in alveolar development in *miR-29* single and double cluster knockout mice remain to be determined.

Results:

We generated *miR-29ab1*$^{flox/flox}$*;Shh-cre* (referred to as *miR-29ab1* conditional knockout mice) and *miR-29ab1*$^{flox/flox}$*;miR-29b2c*$^{flox/flox}$*;Shh-cre* mice in which either a single miR-29 allele or both loci were efficiently excised within the of the lung epithelium. In previous studies (Ref), it was shown that *Shh* gene expression is activated at E9.5 in embryonic lung endoderm (REF), suggesting that *miR*-29 genomic clusters were excised at an early stage of lung development. We used *miR-29ab1*$^{flox/flox}$ and *miR-29ab1*$^{flox/flox}$*;miR-29b2c*$^{flox/flox}$ animals as controls. PCR analysis of genomic DNA extracted from sorted E18.5 lung epithelial cells based on the expression of EpCAM (McQualter et al., 2010) confirmed the deletion of *miR-29* alleles specifically in the epithelium of conditionally targeted mice, while *miR-29* expression in non-epithelial cells were unaffected (Figure 2-6.1). Furthermore, we collected E18.5 lung epithelial cells and non-epithelial cells from *miR-29ab1*$^{flox/flox}$*;Shh-cre* and control embryos, extracted RNA and conducted RT-qPCR analysis. Levels of endogenous miR-29a and miR-29b were significantly decreased in EpCAM^{+} cells in lungs of E18.5 *miR-29ab1*$^{flox/flox}$*;Shh-cre* mice compared to epithelial cells from *miR-29ab1*$^{flox/+}$*;Shh-cre* and *miR-29ab1*$^{flox/flox}$ mice (Figure 2-6.2B and D), whereas the expression of miR-29 family members were not affected in EpCAM^{-} cells (Figure 2-6.2A and C).

Deletion of *miR-29ab1* alleles in lung epithelium resulted in no overt developmental defects and the targeted alleles were transmitted at the expected Mendelian ratios, suggesting that there were no major structural or functional abnormalities in lung development. Next, we examined whether any lung developmental deficiencies were manifest in *miR-29* double

cluster conditional knockout mice. Initial histological examination of *miR-29ab1*$^{flox/flox}$*;miR-29b2c*$^{flox/flox}$*;Shh-cre* adults revealed no apparent differences in lung morphology in the *miR-29* conditional knockout mouse up to 20 weeks of age (Figure 2-6.3). It is possible that *miR-29* double-cluster conditional knockout mice did not manifest respiratory deficiency because of redundant function and compensatory upregulation of miR-200 family members. Since we previously observed that the miR-200 family shared several targets (*e.g.* TGF-β2) with miR-29 family and was critical for HFL type II cell differentiation, we examined expression of miR-200 family members in *miR-29* conditional double knockout mouse lung. Notably, we found that miR-200a and 200b levels were increased ~2 fold in whole lungs from *miR-29ab1*$^{flox/flox}$*;miR-29b2c*$^{flox/flox}$*;Shh-cre* embryos at E18.5 compared with *29ab1*$^{fl/fl}$*;miR-29b2c*$^{fl/fl}$ controls (Figure 2-6.4). In ongoing studies, we will analyze miR-200 expression in EpCAM^{+} and EpCAM^{-} cells from the *double-cluster* KO *vs. double-floxed* fetal lungs.

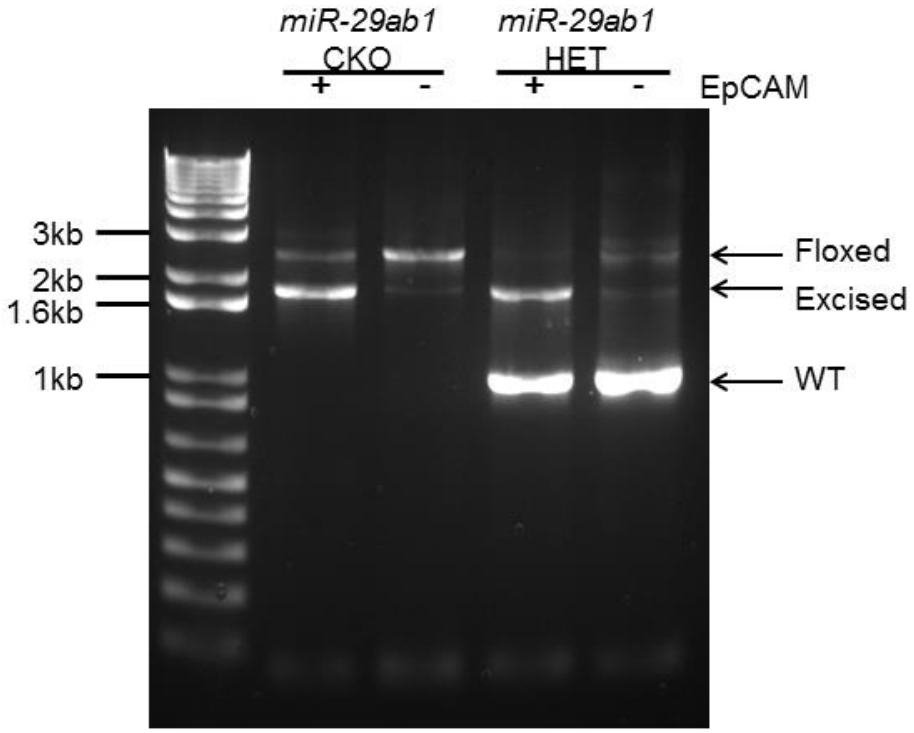

Figure 2-6.1. Deletion of *miR-29ab1* in Mouse Lung Epithelium Confirmed by PCR. Mouse fetal lungs at E18.5 were dissected, genotyped, and pooled within same genotype. Genomic DNA was isolated from EpCAM^{+} and EpCAM^{-} sorted cells at E18.5. Agarose gel electrophoresis showed that the excised allele was only apparent in EpCAM^{+} cells.

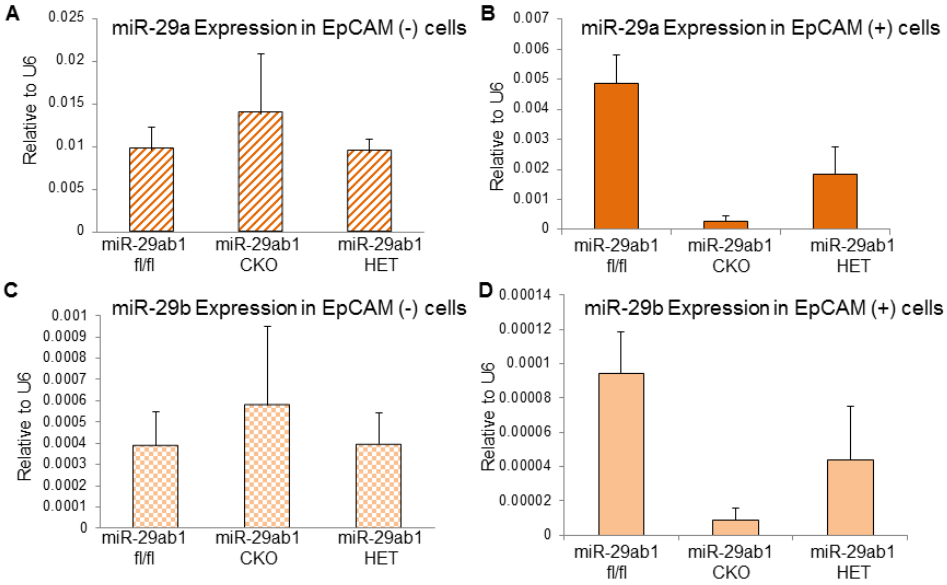

Figure 2-6.2. Levels of miR-29a and miR-29b are Significantly Decreased in Epithelial Cells Isolated from *miR-29ab1* Conditional Knockout Mice compared to *miR-29ab1*$^{fl/fl}$ Mice. Epithelial cells (EpCAM^{+}) and non-epithelial cells (EpCAM^{-}) were sorted based on EpCAM expression at E18.5. Expression of miR-29a (A and B) and miR-29b (C and D) was examined by RT-qPCR. In EpCAM^{-} cells, no significant difference in levels of miR-29a (A) or miR-29b (C) was observed between 3 groups of mouse fetal lung; in EpCAM^{+} cells, miR-29a (B) and miR-29b (D) expression was remarkably lower in *miR-29ab1* conditional knockout mice than *miR-29ab1*$^{fl/fl}$ mice.

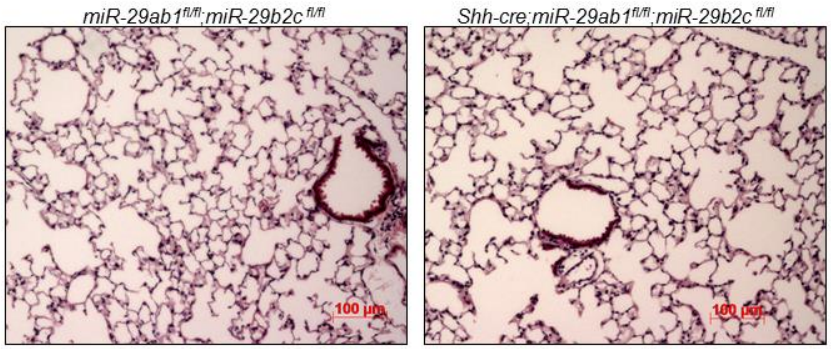

Figure 2-6.3 Histologic Examination of Adult Lungs from *Shh-cre;miR-29ab1*$^{fl/fl}$*;miR-29b2c*$^{fl/fl}$ *vs. 29ab1*$^{fl/fl}$*;miR-29b2c*$^{fl/fl}$ Mice. Hematoxylin and eosin staining of *Shh-cre;miR-29ab1*$^{fl/fl}$*;miR-29b2c*$^{fl/fl}$ and *miR-29ab1*$^{fl/fl}$*;miR-29b2c*$^{fl/fl}$ lungs at 4 months of age.

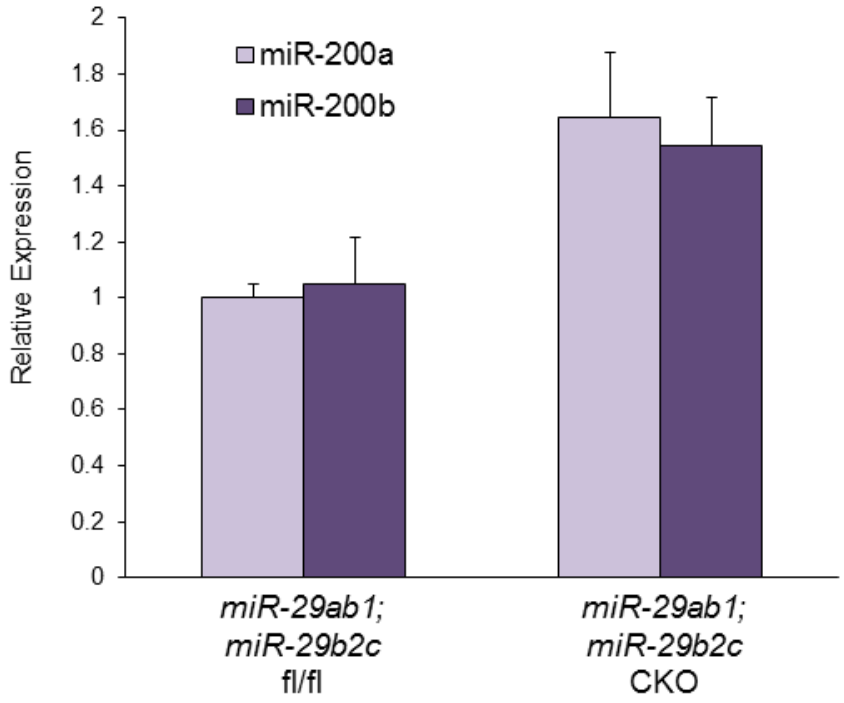

Figure 2-6.4. miR-200a&b are Upregulated in *miR-29ab1;miR-29b2c* Conditional Knockout Mouse Fetal Lung. Whole mouse fetal lungs from conditional double knockout (N=4) and control (N=5) groups at E18.5 were analyzed for miR-200 expression.

Discussion

These findings demonstrate the successful deletion of *miR-29* floxed alleles in lung epithelium of mice using targeted excision with *Shh-cre*. The data obtained thus far suggest that targeted deletion of both *miR-29ab1* and *miR-29b2c* from lung epithelium had no discernible effect on fetal lung epithelial cell differentiation and function. Preliminary histological examination failed to indicate any abnormalities in alveolarization in conditional *miR-29* double knockout mice. One possible reason for this apparent lack of phenotypic effect is the compensatory upregulation of miR-200 family expression in *miR-29* double knockout mice (Figure 2-6.4). As mentioned previously, the miR-200 family, which also increases in expression during fetal lung development and type II cell differentiation, shares several important targets and signaling pathways with miR-29. Moreover, compensatory upregulation of the miR-200 family was only studied in whole lung RNA isolated from the *double-deficient vs. double-floxed* embryos. In ongoing studies, we will analyze miR-200 expression in $EpCAM^+$ *vs.* $EpCAM^-$ cells to determine whether miR-200 is upregulated to a greater extent in epithelial cells of the double-deficient embryos. Moreover, further analysis of the roles of miR-29 family members in lung epithelium differentiation might uncover unknown functions that were studied over the course of this project.

It is noticed that many miRNAs appear to be dispensable for development or viability when studied under standard laboratory conditions (Bushati and Cohen, 2007; Leaman et al., 2005; Miska et al., 2007), however, emerging evidence suggests that many miRNA mutants manifest severe defects when they are subjected to stress, injury or changing environments. For instance, *miR-7* knockout flies manifest impaired eye development when the

environment around developing *Drosophila* larvae was perturbed by fluctuating the environmental temperature between 31°C and 18°C every ~1.5 hr (Li et al., 2009a); mice deficient in miR-208 cannot respond properly to cardiac pressure overload (van Rooij et al., 2007); deletion of miR-143/145 in mice results in a dramatic impairment of intestinal epithelial regeneration after dextran sulfate sodium injury (Chivukula et al., 2014), and; inactivation of miR-8 renders zebrafish incapable of responding to osmotic stress (Flynt et al., 2009). Thus, miRNA mutants could developmentally appear normal but exhibit phenotypic crisis under stress conditions (Leung and Sharp, 2010). Interestingly, sleeping beauty (SB)-mediated *miR-29* gene transfer into mouse lung tissues is capable of preventing and treating pulmonary fibrosis associated with inflammatory macrophage infiltration induced by bleomycin (Xiao et al., 2012b). This raises the possibility that lung epithelium-specific deletion of *miR-29* may render mutant mice susceptible to injuries including bleomycin-induced lung fibrosis and neonatal bronchopulmonary dysplasia caused by hyperoxia exposure.

CHAPTER THREE
Methodology

Isolation and Culture of HFL Epithelial Cells and Type II Cells

Lung tissues from mid-trimester human abortuses (17 to 22 weeks gestation) were obtained from Advanced Bioscience Resources ABR, Inc. (Alameda, CA), in accordance with the Donors Anatomical Gift Act of the State of Texas. The protocols were approved by the Institutional Review Board of the University of Texas Southwestern Medical Center. Human fetal lung epithelial cells and type II cells were isolated, as described previously (42, 113). In short, to isolate HFL type II cells, fetal lung tissues were minced and rinsed in serum-free Waymouth's MB752/1 medium (GIBCO). Explants were placed in 35-mm sterile culture dishes on lens paper supported by stainless steel grids. The explants were cultured in serum-free Waymouth's medium for 5 days to enrich the population of differenti¬ated type II cells. Cells were dispersed from the explants by digestion with collagenases type I (0.5 mg/ml; Sigma) and type IA (0.5 mg/ml; Sigma). The resulting cell suspension was depleted of fibroblasts via incubation with diethylaminoethyl (DEAE)-dextran (250 μg/ml) for 30 min at 37°C, followed by centrifugation at 400 ×g for 5 min. The cell pellet was resuspended in Waymouth's MB752/1 medium containing 10% (vol/vol) fetal bovine serum (FBS), and loaded onto a 90%-60% Percoll gradient. The type II cells were collected and plated onto culture dishes coated with extracellular matrix prepared from Madin-Darby canine kidney cells (MDCKs) (113) and incubated overnight. Cells were then washed three times with

medium to eliminate dead and nonadherent cells and cultured in Waymouth's medium without FBS.

To isolate undifferentiated epithelial cells, freshly obtained mid-gestation HFL was minced into 1- to 2-mm^3 fragments and dissociated by collagenase digestion, followed by DEAE-dextran treatment and Percoll gradient centrifugation (described above) without prior explant culture. After isolation, epithelial or type II cells were plated onto 60-mm dishes and cultured overnight in Waymouth's medium containing 10% FBS. The next day, cells were washed 3 times in serum-free Waymouth's medium and cultured in serum-free medium ± dibutyryl cAMP (Bt_2cAMP) (1 mM) alone or in combination for up to 72 h. Cells were harvested at different time points for extraction of RNA and protein and for morphological analysis.

Mouse Lung Tissue Preparation

All animal protocols were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center. Eight-week-old, timed-pregnant mice (ICR/CD1) were obtained from Harlan Laboratories and housed in the Animal Resources Center starting from 14 dpc. At 15.5 to 18.5 dpc, mouse fetal lungs were collected, as described previously (Benlhabib and Mendelson, 2011). Briefly, pregnant mice were euthanized by isoflurane inhalation and cervical dislocation. The fetuses were removed from their amniotic sacs and subjected to hypothermia for several minutes before decapitation. The fetal lungs were harvested and either frozen in liquid nitrogen for subsequent RNA and

protein isolation or placed in Waymouth's MB752/1 medium on ice for epithelial cell isolation.

Isolation of Epithelial Cells from Mouse Fetal Lung

Mouse fetal lung epithelial cell cultures were prepared, as described previously (Benlhabib and Mendelson, 2011). To collect mouse fetal lung, timed-pregnant ICR mice were dissected, uteri were collected and placed in petri dish on ice containing PBS. Embryos were released from uterus, exposed to hypothermia, and euthanized by decapitation. Mouse fetal lung tissues were dissected from the embryos, separated from the heart, trachea and esophagus, minced and dissociated by collagenase digestion, as described above. The dissociated cells were washed, resuspended in Waymouth's medium containing 10% fetal bovine serum, and plated onto tissue culture dishes. After 10 min of culture at 37°C, the medium (containing suspended epithelial cells and some fibroblasts) was removed; these were plated onto a second set of culture dishes. The cells that adhered to the first set of dishes during the 10-min culture period were considered to be a relatively "pure" fibroblast population. These were flash-frozen for subsequent RNA analysis. The cells plated onto the second set of culture dishes were incubated at 37°C for an additional 35 min. The cells that adhered to these dishes (a mixture of fibroblasts and epithelial cells) were discarded, while the non-adherent cells in the supernatant were considered to be a relatively "pure" population of epithelial cells. These were flash-frozen for subsequent RNA analysis.

Immunoblot Analysis

Immunoblotting was conducted on whole-cell extracts of HFL epithelial cells or type II cells or on homogenates of mouse fetal lung tissues. Whole-cell extracts were isolated from cell and tissue samples using RIPA buffer (Cell Signaling). Protein concentrations were determined by a BCA protein assay (Pierce cat# 23225). Equivalent amounts of protein were added to 4X LDS sample buffer (Invitrogen) and samples were heated to 95C for 10 min, loaded on 4-12% Bis-Tris protein gels (Invitrogen), run at 150-200 volts and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated in blocking PBST buffer, containing 3% nonfat dry milk for 60 min at room temperature before addition of primary antibodies. Antibodies directed against SP-A (Odom et al., 1987), SP-C (Santa Cruz), LPCAT1 (Abcam), TGF-β2 (Abcam), p-SMAD2 (Cell Signaling), SMAD2 (Cell Signaling), ZEB1 (Santa Cruz) or ZEB2 (Santa Cruz) were diluted in blocking buffer. Endogenous β-actin (antibody from Abcam), and β-tubulin (antibody from Cell Signaling) were analyzed as loading controls. Primary antibodies were incubated with membranes overnight at 4C with rocking. Membranes were washed 4 times with PBST for 5 min each at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit IgG, used as a secondary antibody (Zymed Invitrogen cat# 62-1820) was diluted in blocking buffer (1:6,000) and incubated with membranes for 60 min. Membranes were washed 4 times in PBST for 5 min each time and visualized using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. Quantitative measurement of immunoblots was performed using Image J (http://rsb.info.nih.gov/ij/index.html).

Immunofluorescence

Isolated MFL epithelial cells were cultured on extracellular matrix-coated chamber slides overnight. The epithelial cells were fixed in 4% formaldehyde in PBS for 15 min. The chamber slides were then blocked with normal goat serum for 30 min. Incubation with a primary antibody for E-cadherin (1:200 dilution, Cell Signaling) was performed overnight at 4C in the dark. The sections were then incubated with anti-goat fluorophore-labeled secondary antibody (1:500 dilution, Jackson ImmunoResearch) for one hour at room temperature. Incubation with another fluorochrome-conjugated primary antibody for Vimentin (1:200, Cell Signaling) was performed overnight at 4C in the dark. Nuclei were counterstained with 4,6-diamino-2-phenylindole (DAPI, 1:100,000 dilution, Sigma) for 5 minutes at room temperature and slides were mounted with fluorescence mounting medium (Dako). A fluorescence microscope (Carl Zeiss Microscopy) was used to capture images of the slides.

Quantitative Reverse Transcriptase PCR (RT-qPCR)

For gene expression analysis, total RNA isolated from cells and tissues was treated with deoxyribonuclease (Invitrogen), reverse-transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad), and subsequently amplified using Sybr Green (Applied Biosystems) using the Bio-Rad CFX 384 realtime system. Expression of genes of interest was determined using forward and reverse primers detailed in Table 3.1. Real-time PCR (qPCR) cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and

60°C for 1 min. The cycle threshold was set at a level at which the exponential increase in PCR amplification was approximately parallel between all samples. All primer sets produced amplicons of the expected size and sequence. Relative gene expression was calculated using the comparative cycle threshold ($^{\Delta\Delta}$Ct) method. Expression of miRNAs was determined using TaqMan primers (ABI) (Table 3.2) and TaqMan miRNA reverse transcription kit (Applied Biosystems). Relative gene expression was calculated using the comparative cycle threshold ($^{\Delta\Delta}$Ct) method.

Table 3.1. Primer sets used in Sybr Green qRT-PCR

Human	
18S-F	ACCGCAGCTAGGAATAATGGA
18S-R	GCCTCAGTTCCGAAAAACA
SP-A-F	GGGCAGTGGAATGACAGGAA
SP-A-R	CTGAAGGCCAGACAGGATCC
SP-C-F	CACTGGTCTAGGACCCGAGAAG
SP-C-R	GGTGCCTCTGGAGATTTTCG
Mouse	
36B4-F	CACTGGTCTAGGACCCGAGAAG
36B4-R	GGTGCCTCTGGAGATTTTCG
TGF-β2-F	TCGACATGGATCAGTTTATGCG
TGF-β2-R	CCCTGGTACTGTTGTAGATGGA
SP-A-F	TCCAGGGTTTCCAGCTTACCT
SP-A-R	GACAGCATGGATCCTTGCAAG

Table 3.2. Assay IDs for TaqMan primers used to analyze the expression of miR-29 and miR-200 family

Taqman Assay	Assay ID
hsa-miR-29a	002112
hsa-miR-29b	000413
hsa-miR-29c	000587
hsa-miR-200a	000502
hsa-miR-200b	002251
U6	001973

miR-29 Family Inhibitor Transfection

Freshly isolated HFL epithelial cells or type II cells were cultured in Waymouth's MB752/1 medium (Gibco) and transfected with HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. Briefly, 1.2×10^6 cells in 1 mL of culture medium were plated onto 60 mm tissue culture dishes, and transfected with miR-29 family inhibitor (Exiqon) at a final concentration of 20 nM. Cells were allowed to adhere to the culture dishes in the presence of anti-miR and treated ± Bt_2cAMP (1 mM) 24 h post transfection. Cells were harvested for subsequent RT-qPCR, immunoblot and morphological analysis after 48 h of treatment.

Osmium Tetroxide Staining of Lamellar Bodies in HFL Type II cells in Primary Culture

To assess the presence of lamellar bodies in cultured HFL type II cells, osmium tetroxide staining was used as previously described (Alcorn et al., 1997). In brief, the cells were plated onto ECM-coated dishes, transfected with anti-miRs, and cultured in serum-free medium containing Bt_2cAMP. Prior to analysis, the cells were rinsed in phosphate-buffered saline (PBS, pH 7.3) and fixed with glutaraldehyde (1.5% in PBS) for 15 min. The cells were then washed twice and treated with osmium tetroxide (1.0%; Sigma Chemical Co.) in PBS for 30 min at room temperature. The cells were subsequently washed twice and incubated in tannic acid (1.0% in PBS, pH 6.8) for 30 min. After washing with PBS, the cells were examined and photographed under light microscopy.

Plasmid Construction

MiR-29ab1 promoter-luciferase reporter constructs were made in pGL3-basic vector (Promega). The transcription start site of *miR-29b1/miR-29a* was previously identified (Chang et al., 2008), and a 539bp fragment (-481bp to +58bp) was cloned and inserted into pGL3-basic vector. Human TTF-1 expression plasmid pcDNA3.1(+)-wt-TTF-1 (Addgene plasmid # 49989) and a TTF-1 expression plasmid lacking the homeodomain (pcDNA3.1(+)-TTF-1-HDD; Addgene plasmid # 52063) was a gift from Dr. David Mu (Runkle et al., 2012). pcDNA3.1 and pRL-TK (Promega) vectors were used to normalize DNA and transfection efficiency, respectively. All subcloned DNAs were confirmed by sequencing.

Cell Line Culture and Transient Transfection

A549 cells were cultured in Waymouth's MB752/1 medium (Gibco) with 10% fetal bovine serum. A549 cells were checked for viability by Trypan blue staining, counted, and seeded at in 24-well plates. *MiR-29ab1* promoter-luciferase plasmid was used in transient transfection assays using Fugene HD at a DNA/Fugene ratio of 1:3 according to the manufacturer's instructions (Promega). Briefly, 24-well plates at about 70% confluence were transfected with a fixed amount (50 ng) of *miR-29ab1* promoter-luciferase plasmid and various amounts of TTF-1 expression plasmids. The *miR-29ab1* promoter-luciferase plasmid was transfected at 50ng/well. Transfection efficiency was normalized to Renilla luciferase activity using 5 ng/well of pRL-TK. Two days after transfection, cells from each treatment group were then harvested in 100 µl of 1X Passive lysis buffer (Promega). Firefly luciferase

and Renilla luciferase activities were assayed using Dual-Luciferase assay system (Promega). To correct for transfection efficiencies, relative luciferase activity was calculated by normalizing Firefly luciferase activity to Renilla luciferase activity obtained from the same sample. pCDNA3 empty vector was also used as a control.

Chromatin Immunoprecipitation (ChIP)

Human fetal lung type II cells were cultured as described above, and ChIP was conducted using a Magna ChIPTM A/G kit (Millipore) according to the manufacturer's instructions. Cells were incubated for 10 min using 1% formaldehyde to cross-link proteins and DNA. Glycine (0.125 M) was then added to stop crosslinking. Cells were lysed with cell lysis buffer to remove cytoplasm and then resuspended with nuclear lysis buffer containing 1 mM PMSF and 1x protease inhibitor to obtain soluble chromatin. The lysates were sheared to ~200-1000 base pairs in length by sonication. Lysates containing sheared chromatin were diluted and incubated with antibodies against TTF-1 (Santa Cruz) at 4 C overnight. An aliquot incubated with nonimmune IgG was used as a control. Protein A/G magnetic beads were added to each tube and the immune complexes collected by magnetic separator. The beads containing the immunoprecipitated complexes were washed sequentially for 5 min in Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer, and TE buffer. The beads were eluted with 100 µl freshly prepared elution buffer and incubated at 62°C to reverse crosslinking. The immunoprecipitated DNA was collected using spin columns and amplified by qPCR using

primers that flanked the TTF-1-binding element. Primers used for ChIP analysis are: forward- CTGTTGACCAGACACGCCTA; reverse- GCACCGAAGGTCTAAGCATT.

Animal Housing and Mouse Generation

Targeting strategies and procedures for generation of both floxed *miR-29ab1* and floxed *miR-29b2c* mice have been described in detail previously (Smith et al., 2012). Lung epithelium specific deletions for single and double alleles were achieved by breeding *miR-29ab1*$^{flox/flox}$ mice or *miR-29ab1*$^{flox/flox}$;*miR-29b2c*$^{flox/flox}$ mice to Shh-cre mice (Purchased from Jackson Labs) (Harfe et al., 2004). This allows creation of conditional knockout mice for single and double alleles. After heterozygous crosses, all mice were maintained on a mixed C57BL/6 and 129/SvJ genetic background. To genotype the mice, tails were clipped and lysed using a KAPA mouse genotyping kit (KAPA Biosystems). Sequences of primers used for genotyping and size of PCR products are:

miR-29ab1	Forward-1: 5'-TGTGTTGCTTTGCCTTTGAG-3'	wild-type band, 450bp
	Reverse-1: 5'-CCACCAAGAACACTGATTTCAA-3'	floxed band, 600bp
	Forward-2: 5'-TGTGTTGCTTTGCCTTTGAG-3'	wild-type band, 1000bp
	Reverse-2: 5'-CCACCAAGAACACTGATTTCAA-3'	floxed band, 2500bp ko band, 2000bp
miR-29b2c	Forward-1: 5'-CCTACAGGGTCATGGGATAGAGA-3'	wild-type band, 550bp
	Reverse-1: 5'-TTGATTTCTCGAAGGAGAGAGCC-3'	floxed band, 670bp

For histological examination of adult lungs, mice were anesthetized using ketamine/xylazine/atropine, the trachea was exposed and separated from salivary glands, connective tissue and muscle covering the trachea using blunt dissection. To inflate the lungs, 4% paraformaldehyde in phosphate-buffered saline (PBS) was slowly instilled into the trachea catheter. Once the lungs were maximally inflated with fixative, they were tied off tightly with suture and incubated in 4% paraformaldehyde in PBS overnight. Tissues were embedded in paraffin, sectioned at 8–12 μm using a Motorized Rotary Microtome (Leica), mounted on glass slides, and stained with hematoxylin and eosin.

Fluorescence-activated Cell Sorting (FACS)

To isolate $EpCAM^+$ cells from E18.5 lungs using FACS, uteri were removed from timed-pregnant mice, individual lungs were dissected from each embryos and put in a tube with ice cold Dulbecco's phosphate-buffered saline (DPBS). The tail of each embryo was used for genotyping. Mouse fetal lungs of the same genotype were transferred from DPBS to a clean petri dish, finely minced with a razor blade, and digested in a collagenase mixture (1 mg collagenase/lung in 500ul DPBS, 0.2 g glucose/liter, 200U/ml DNase) at 37°C for 30 min while shaking at 165 rpm. The cell suspensions were centrifuged and washed with 20 mL of DPBS containing 2% FBS, followed by red blood cells lysis with lysis buffer (BD Pharm Lyse Lysing buffer) at 37 C for 3 min. Cells were filtered through 40 μm cell strainer, and resuspended in blocking solution (2% FBS and 1 μg anti-FcR antibody in DPBS) and incubated on ice for 10 min. Anti-mouse EpCAM antibody (Biolegend, at a dilution of 1:200) staining was performed on ice for 30 min and kept from light. The cells were then

washed three times with DPBS containing 2% FBS and resuspended in Propidium Iodide (PI) solution (2% FBS + 1.0 μg/ml PI + 0.5 μM EDTA in DPBS). Cells were sorted using ARIA sorter (Beckton Dickinson), and separated cells were used for RNA analysis and PCR analysis of genotyping.

CHAPTER FOUR
Closing Remarks & Future Directions

Closing Remarks

Gas exchange after birth in mammals is entirely dependent on the well-established 3D configuration of the alveolus, whose formation and function are controlled by a diversity of signaling and transcriptional networks. Alveoli are formed from peripheral saccules during late gestation and postnatal periods. During late gestation and after birth, septation and continued lung growth creates the extensive alveolar surfaces mediating efficient gas exchange (Whitsett and Weaver, 2015). Synthesis and secretion of surfactant lipids and proteins into the airspaces at birth by type II cells lining the peripheral lung prepares neonates for survival at birth. The lack of lung maturation and the associated lack of pulmonary surfactant in preterm infants cause respiratory distress syndrome, a common cause of morbidity and mortality associated with premature birth (Whitsett and Weaver, 2015).

The molecular mechanisms that regulate alveolar epithelium differentiation and surfactant synthesis during fetal lung development remain incompletely understood. Based on extensive research from our laboratory and others, it is apparent that the formation and function of the alveolus are mediated by a diversity of signaling and transcription factor networks, some of which play dual roles in regulation of cell differentiation and surfactant protein gene expression (Mendelson, 2000). Specifically, we previously observed that type II cell-specific, developmental, and cAMP induction of *SP-A* gene expression are mediated by

the binding of transcription factors and coregulators to response elements within an 300-bp region upstream of the SP-A gene (Mendelson, 2000). Moreover, cAMP and IL-1 stimulation of SP-A expression in HFL type II cells is dependent upon a critical O_2 tension (Acarregui et al., 1993; Islam and Mendelson, 2006). One of the key response elements required for cAMP/IL-1 induction of *SP-A* expression in human fetal type II cells, the TBE, recruits TTF-1, NF-κB, and HAT coactivators in an O_2-dependent manner (Islam and Mendelson, 2006).

MicroRNAs are small molecules that have been shown to be involved in a number of complex biological processes (Bartel, 2009). Since miRNAs are evolutionarily conserved across vertebrate species, their roles in lower mammals, such as the mouse, are often preserved in higher organisms, such as the human (Lee et al., 2007). Despite our growing understanding of the involvement and contribution of different clusters and families of miRNAs to lung development, including branching morphogenesis and lung epithelial progenitor cell differentiation, the functions of miRNAs has not been elucidated in HFL type II cell differentiation until recently (Benlhabib et al., 2015).

In previous studies, using a microarray based approach and cultured primary HFL epithelial cell model, we uncovered a role for the miR-200 family and its targets, transcription factors ZEB1 and ZEB2, as mediators of developmental regulation of type II cell differentiation and function in HFL (Benlhabib et al., 2015). We observed that during early to mid-gestation when the fetal lung is relatively hypoxic, cAMP signaling is inhibited, allowing increased expression of TGF-β and ZEB1/2, which exist in a double-positive feedback loop. This maintains suppression of miR-200s and permits further up-regulation of ZEB1/2 and TGF-β family members. The increased TGF-β and ZEB1/2 suppress TTF-1

transcriptional activity and promote increased plasticity of epithelial cells and their capacity to acquire invasive/mesenchymal properties without entirely committing to a mesenchymal phenotype. During the third trimester of gestation, increased vascularization of the fetal lung and elevated local O_2 tension promote increased cAMP signaling. This causes suppression of TGF-β and ZEB1 and increased TTF-1 binding and transcriptional activation of genes involved in lung epithelial cell differentiation (Benlhabib et al., 2015).

In the present study, we demonstrated that members of the miR-29 family, which were also found to be upregulated during HFL epithelial cell differentiation by miRNA microarray (Benlhabib et al., 2015), was markedly upregulated in HFL epithelial cells cultured in medium containing Bt_2cAMP in association with the pronounced upregulation of SP-A expression. Interestingly, we found that miR-29 family members were developmentally upregulated in epithelial cells isolated from mouse fetal lung tissues at 17.5 dpc and 18.5 dpc compared to 15.5 dpc. The developmental upregulation of miR-29 family members in fetal lung epithelial cells is of particular interest, as this family was reported to directly target TGF-β2 (Chou et al., 2013), which is established as a negative regulator of HFL type II cell differentiation (Beers et al., 1998; Benlhabib et al., 2015). Moreover, we found that expression of the miR-29 family increased markedly in HFL epithelial cells cultured in medium containing Bt_2cAMP in association with a significant upregulation of SP-A expression and downregulation of the miR-29 target, TGF-β2.

Previous studies from our lab showed that cAMP enhances type II cell differentiation by inhibiting ZEB1/2 expression and function, in part, through inhibition of TGF-β signaling (Benlhabib et al., 2015). TGF-β2 expression was found to be downregulated in HFL

epithelial cells cultured with Bt_2cAMP, which in turn decreased expression of ZEB1 and ZEB2 (Benlhabib et al., 2015). In the present study, we further examined the expression of TGF-β2 and its downstream mediators including p-SMAD2 and ZEB1/2 in mouse fetal lung during late gestation. Similar to what we observed in HFL epithelial cells during differentiation in culture, we found that TGF-β2 mRNA was significantly downregulated in mouse fetal lung epithelial cells at 17.5 dpc and 18.5 dpc compared to 16.5 dpc.

In addition to defining the regulation of miR-29 family members and its target, TGF-β2, we also wished to discern the functional role(s) of these factors in HFL epithelial cells. Through use of miR-29 inhibitors in cultured HFL epithelial cells, we obtained evidence that knockdown of all miR-29 family members resulted in a pronounced de-repression of TGF-β2 and remarkable loss of expression of surfactant proteins, SP-A and SP-C. Knockdown of miR-29 family also inhibited expression of LPCAT1, a key enzyme in surfactant dipalmitoylphosphatidylcholine synthesis, and blocked lamellar body accumulation in HFL type II cells, suggesting their physiological relevance.

Our findings from previous studies suggest that increased O_2 availability to HFL type II cells in culture plays a permissive role in cAMP and cytokine induction of *SP-A* expression by facilitating the binding of TTF-1 and NF-κB to the TBE as well as in promoting the recruitment of essential coregulators, which in turn mediate changes in histone posttranslational modifications and the opening of the chromatin structure (Islam and Mendelson, 2006). Our current findings indicate that Bt_2cAMP treatment of HFL type II cells also increased miR-29 family expression by enhancing binding of endogenous TTF-1 to the *miR-29ab1* promoter.

Collectively, our findings reveal that increased miR-29 family expression in the fetal lung near term serves a key role in promoting type II cell differentiation and function through repression of TGF-β2 signaling. During early to mid-gestation, the fetal lung is relatively hypoxic. This impairs cAMP signaling (Acarregui et al., 1993; Benlhabib and Mendelson, 2011; Islam and Mendelson, 2006), allowing increased expression of TGF-β and ZEB1/2 (Benlhabib et al., 2015), which inhibit type II cell differentiation. The increased TGF-β and ZEB1/2 levels maintain suppression of miR-29 family members, inhibit TTF-1 transcriptional activity, and promote increased plasticity of epithelial cells and their invasive/ mesenchymal properties without entirely committing to a mesenchymal phenotype (Benlhabib et al., 2015). Suppressed TTF-1 transcriptional activity further decreases miR-29 family expression. Near term, increased vascularization of the fetal lung and elevated local O_2 tension increases cAMP signaling and stimulates TTF-1 DNA-binding and transcriptional activity, resulting in increased miR-29 expression. This, in turn, suppresses miR-29 targets, such as TGF-β2, resulting in increased type II cell differentiation and surfactant lipoprotein synthesis.

Future Directions

In future experiments, we will use gene targeted mice to further explore the roles of the miR-29 family in lung branching morphogenesis, alveologenesis, epithelial cell differentiation and response to various injuries *in vivo*. For these studies, we have created mice carrying an epithelial cell-specific double-knockout of *miR-29ab1* and *miR-29b2c* clusters. Effects of knockout of the miR-29 family on lung development and pathogenesis are

currently being studied. We also plan to challenge these *miR-29* double knockout mice by subjecting them during the neonatal period to hyperoxia, which has been observed to induce bronchopulmonary dysplasia (BPD). The *miR-29* double-KO mice will be assessed together with double-floxed controls to compare their susceptibility to hyperoxia-induced broncho-pulmonary dysplasia (BPD).

BPD was first described as a disease of preterm infants that received prolonged mechanical ventilation and oxygen supplementation for acute respiratory distress syndrome (Northway et al., 1967). BPD, a common pulmonary complication seen in preterm infants and characterized by alveolar/capillary hypoplasia and a long-term reduction in total number of alveoli, has a multifactorial pathogenesis (Jobe, 2011). Although the pathogenesis of BPD is largely unknown, hyperoxic injury was shown to influence critical signaling pathways including an increased number of inflammatory cells and enhanced TGF-β signaling (Alejandre-Alcazar et al., 2007; Gien and Kinsella, 2011; Warner et al., 1998). Based on the findings of the current study, we believe that miR-29 family members are critical factors that repress TGF-β signaling during fetal lung epithelial cell differentiation. We, therefore, hypothesize that mice lacking miR-29 alleles in lung epithelium will manifest enhanced susceptibility when exposed to hyperoxia as compared to littermate floxed controls. However, because of the possible compensatory effects of the miR-200 family in conditional *miR-29* double-knockout mice, obtaining a clear understanding of the roles of the miR-29 family in the physiology of lung development and the pathogenesis of BPD may be difficult to ascertain.

As an alternative to the BPD model, we also may test the susceptibility of the miR-29 double-knockout mice to development of lung fibrosis after treatment with bleomycin. Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive parenchymal lung disease with a median survival time of 3–5 years in the absence of lung transplantation following diagnosis, which predominately affects elderly people (Mouratis and Aidinis, 2011). The most widely used animal model of lung fibrosis is bleomycin-treated mouse model (Ref for mouse model). It was previously demonstrated that miR-29 family expression was reduced in the lungs of bleomycin-treated mice (Cushing et al., 2011); however, effects of induced miR-29 deficiency on susceptibility and pathogenesis of bleomycin-induced lung fibrosis has never been studied. We hypothesize that lung epithelium-specific *miR-29* double knockout mice exposed to bleomycin may develop an earlier onset or more severe lung fibrosis. Establishing the role of miR-29 family in the pathogenesis of BPD and IPF would be of particular importance, since this could suggest a direct contribution of lung epithelial cells to the pathological process. In addition, our findings may reveal the utility of the miR-29 family as a potential predictive marker or therapeutic modality for this devastating disease.

In addition, the role of TGF-β signaling in lung epithelial cell differentiation and pulmonary pathogenesis has yet to be fully explored. Previous studies demonstrated that all three isoforms of TGF-β are expressed during early lung development, where they serve an important role in branching morphogenesis. Low levels of TGF-β expression are still evident in the adult lung, where it possibly is involved in normal tissue repair following lung injury (Bartram and Speer, 2004). Injury of lung tissue by chemical, bacteriologic, or immunologic noxious agents leads to an induction of TGF-β that restrains some of the inflammatory

reactions and mediates tissue remodeling and repair (Ahuja et al., 1993; Kehrl et al., 1986). If the repair processes are exaggerated and not adequately localized, lung pathology with fibrosis can ensue (Bartram and Speer, 2004). Investigations of the mediators of TGF-β signaling in lung development and pathogenesis may provide novel insight into the regulation of TGF-β expression in lung development and disease.

Specifically, the roles and molecular mechanisms underlying the actions of ZEB1 and ZEB2 in lung cell differentiation and pathogenesis have yet to be fully understood. ZEB1 and 2 are known to exist in a positive feedback loop with TGF-β (Gregory et al., 2011) and serve critical roles in EMT via targeting and repressing several genes involved in polarity and adhesion of epithelial cells (Korpal et al., 2008). Our previous findings indicate that ZEB1/2 expression declined markedly in HFL epithelial cells when cultured with Bt_2cAMP, whereas, knockdown of ZEB1 resulted in elevated *SP-A* expression suggesting a repressive function of ZEB1 in surfactant protein expression (Benlhabib et al., 2015). Therefore, it would be of interest to generate transgenic mice inducibly overexpressing ZEB1 or ZEB2 in lung epithelium and determine effects on lung morphology and disease. We hypothesize that mice overexpressing ZEB1 and/or ZEB2 may manifest reduced surfactant protein expression, impaired alveologenesis, enhanced expression of EMT markers and susceptibility to lung fibrosis.

In addition to the miR-29 and miR-200 families, we also noted changes in the expression of other miRNAs in our original microarray of HFL epithelial cells during differentiation. Among them, the miR-199a cluster is of particular interest. Our preliminary data suggest that menbers of the miR-199a cluster, which includes miR-199a-5p, miR-199a-3p, and miR-214,

were significantly decreased in HFL epithelial cells cultured with Bt_2cAMP as compared to control. Interestingly, miR-199a-5p (He et al., 2014), miR-199a-3p (Chakrabarty et al., 2007) and miR-214 (Williams et al., 2012) are known to target the mRNA encoding cyclooxygenase-2 (COX-2), a key regulatory enzyme in synthesis of prostaglandins acting through cAMP, which serve an important role in the upregulation of *SP-A* gene expression in human fetal lung (Mendelson et al., 1991). In addition, miR-199a-5p was reported to target NF-kB p50 and p65 (Hassan et al., 2014), as well as IkappaB kinase-beta (IKKβ) (Chen et al., 2008) and predicted to target Sp1 by TargetScan analysis (http://www.targetscan.org/), which are critical transcriptional activators of *SP-A* transcription (Islam and Mendelson, 2002; Young and Mendelson, 1997). Therefore, in ongoing experiments, we are investigating the roles of members of the miR-199a cluster on COX-2, NF-kB p50, p65, IKKβ, Sp1 and SP-A expression. In this manner, the decline in miR-199a cluster during late gestation could lead to increased expression of COX-2, which would enhance the synthesis of prostaglandins and cAMP, resulting an increase in SP-A expression. Confirmation of direct regulation of miR-199a cluster on their gene targets would provide further evidence for the role of miRNAs in surfactant protein expression and fetal lung epithelium differentiation.

In conclusion, considering the abundance of potential and known targets of the miR-29 family, miR-199a-5p/miR-199a-3p/miR-214 cluster, miR-200 family that are relevant to *SP-A* expression and HFL epithelial cell differentiation , we propose that these factors may collectively play an extensive and immensely important role in regulating fetal lung epithelium differentiation and pulmonary pathogenesis.

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