ALVEOLAR EPITHELIAL TRANS-DIFFERENTIATION, LUNG DEVELOPMENT AND DISEASE – ROLE OF TGF ß1 AND MicroRNAs

By

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INTRODUCTION

1.1 ALVEOLAR EPITHELIUM

The lung consists of functional respiratory units called alveoli and are lined by two distinct and specialized epithelial cell types, namely, Alveolar Epithelial Type I cells (AEC I) and Alveolar Epithelial Type II (AEC II) cells. AEC I are squamous. Their thin and flat morphology enables them to cover almost 95% of the total surface area of the alveoli. This large surface area makes them an effective air-blood barrier through which the actual gaseous exchange takes place. They also play a role in maintaining fluid homeostasis in the lung (Dobbs et al., 1998; Borok et al., 2002; Johnson et al., 2002; Ridge et al., 2003). Recently, it has been discovered that AEC I protects lung epithelium from oxidative injury (Chen et al., 2006). Also, new studies have shown that AEC I contains molecular machinery that has the potential for diverse functions including lung remodeling, angiogenesis matrix deposition and host defense (Qiao et al., 2003; Dahlin et al., 2004). These cells are considered to be terminally differentiated. Their architecture also make them prone to any kind of acute lung injury during which they are damaged significantly when compared to the AEC II cells.

AEC II are cuboidal cells and are limited to the corners of the alveoli. They cover about 5% of the surface area of the alveoli even though their number is greater than AEC I. They secrete the lung surfactant which reduces the normal surface tension at the alveolar

surface. These cells also have a role in fluid homeostasis (Jin et al., 2006). Most importantly they are considered to be progenitors of AEC I (Warburton & Bellusci, 2004). When the AEC II population is damaged in the alveoli during acute lung injury or insult, the AEC II lose their quiescence, proliferate and differentiate into AEC I, thus restoring the AEC I population and thereby the air-blood barrier (Adamson & Bowden, 1971; Evans et al., 1973; Uhal, 1997; Fehrenbach, 2001; Narasaraju et al., 2006). This trans-differentiation process is of high importance in maintaining the normal functionality of alveolar epithelium.

Molecular mechanisms that govern this trans-differentiation process largely remain unclear. Keratinocyte Growth Factor (KGF) has been implicated in preventing this trans-differentiation process in vitro by signaling mediated through JNK pathway (Qiao et al., 2008). It has been thought that commitment of AEC I and II lineages requires continuous regulatory inputs and the fate can be altered by differential signaling (Danto et al., 1995). Some studies have looked at the genes and proteins that are differentially expressed in AEC II and trans-differentiated AEC I (Campbell et al., 1999; Qiao et al., 2003). This transformation has also been shown to be increased by exposure to nitric oxide (Evans et al., 1975). IL-1β expression mediated by EGF/TGFα signaling pathways have been implicated in helping with the trans-differentiation process following lung injury (Geiser, 2003). IGF-1 signaling and the expression of an AEC I specific protein, RT140 are also thought to have a role in modulating this process (Maitre et al., 1995; Clegg et al., 2005).

I.2. TRANSFORMING GROWTH FACTOR BETA (TGF ß) PATHWAY

Transforming Growth Factor beta (TGF β) superfamily consists of structurally related cytokines and is known to have important biological functions. The members of this family include isoforms of TGF β, bone morphogenetic proteins, inhibins and activins (Massague, 2000; Moustakas et al., 2001; Ten Dijke et al., 2002; Li et al., 2006). They mediate biological processes like proliferation, differentiation, migration and apoptosis in various cell types (Roberts & Sporn, 1993; Schuster & Krieglstein, 2002; Huang & Huang, 2005). TGF β has five isoforms namely TGF β1 (Derynck et al., 1985), TGF β2 (Miller et al., 1989), TGF β3 (Derynck et al., 1988; Miller et al., 1989), TGF β4 (Jakowlew et al., 1988) and TGF β5 (Kondaiah et al., 1990). Only TGF β1, TGF β2 and TGF β3 have been cloned from mammalian sources so far (Fitzpatrick et al., 1990).

TGF β1 can act both in autocrine and paracrine manner and initiates signaling by binding to specific type II receptors (TβRII) receptors on the cell surface. (Shi & Massague, 2003). This, in turn, brings type I receptors (TβRI) closer to TβRII forming a hetero-tetrameric receptor complex (Massague, 1998). TβRII receptor gets autophosphorylated, the mechanism of which is still unclear. TβRII receptor kinase induce phosphorylation of multiple serine threonine residues of the cytoplasmic region of TβRI and in turn the phosphorylation of the receptor regulated Smads (R-Smads), Smad2 and Smad3 (Massague, 1998). The phosphorylation causes Smads to dissociate from the Smad anchor for receptor activation (Tsukazaki et al., 1998) and increases their affinity for the common partner Smad4 (Chacko et al., 2001). This Smad heteromeric complex then translocates into the nucleus by an importin mediated mechanism (Xiao et al., 2000; Xiao et al., 2001) or by binding to components of the nuclear pore complex (Xu et al., 2002).

The complex can bind directly or indirectly through specific DNA binding proteins, resulting in positive or negative regulation of the transcription of various target genes (Feng & Derynck, 2005; Massague et al., 2005). The inhibitory Smads, Smads6 and 7, can competitively bind to the activated TβRI and inhibit signaling through this pathway (Hayashi et al., 1997; Imamura et al., 1997). Once the response is achieved, all Smads except Smad4 are turned off mainly by ubiquitin mediated degradation (Izzi & Attisano, 2004).

TGF β1 was initially thought to be a mitogen (Moses et al., 1981; Roberts et al., 1981; Ridley et al., 1989) and was later found to have inhibitory effect on cell growth in different cell cultures (Shipley et al., 1985). It acts as either a positive or negative regulator of cell differentiation depending on the cell type and the physiological state (Huang & Huang, 2005). TGF β1 induces squamous differentiation in human bronchial epithelial cells (Masui et al., 1986) and also play a role in the differentiation of rat kidney fibroblasts to cartilage-like cells (Seyedin et al., 1986). TGF β1 is also shown to mediate differentiation in pregnant mouse mammary glands (Yamamoto et al., 1994). On the other hand it can act as an inhibitor of differentiation in various systems. For example, it is an inhibitor of myogenic differentiation (Massague et al., 1986) and adipogenic differentiation of 3T3 fibroblasts (Ignotz & Massague, 1985).

The anti-proliferative effect of TGF β1 is mediated generally by its ability to modulate the expression levels of various proteins that play an important role in the G1 to S phase transition during the cell cycle progression. The actual proteins affected might vary with the particular cell type (Ten Dijke et al., 2002). Up regulation of cyclin dependent kinases

(CDKs) is important in the progression of cell cycle and TGF β1 is shown to up regulate the expression of 2 CDK inhibitors namely p15 (p15Ink4b) (Hannon & Beach, 1994) and p21 (p21Cip1) (Datto et al., 1995). It can also alter the transcription of genes like c-Myc (Pietenpol et al., 1990; Chen et al., 2001; Frederick et al., 2004), CDK activating phosphatase, cdc25a (Iavarone & Massague, 1997). Also, TGF β can signal through Smad independent pathways that recruit phosphatidylinositol 3-kinase and members of mitogen-activated protein kinase (MAPK) (Atfi et al., 1997; Verrecchia et al., 2001; Derynck & Zhang, 2003).

In the lung, the studies on TGF β1 have so far focused on its role in the pathogenesis of idiopathic pulmonary fibrosis (Border & Noble, 1994; Corrin et al., 1994; Selman & Pardo, 2003; Xu et al., 2003; Venkatesan et al., 2004) and in lung development (Serra et al., 1994). The activation of TGF β signaling increases the deposition of extracellular matrix components (ECM), which is considered to be the hallmark of fibrosis (Keane et al., 2005). Up regulation of TGF β1 mediated through integrin αvβ6 is reported in bleomycin-induced pulmonary fibrosis (Sheppard, 2001). In cultured AEC II cells, TGF β1 can inhibit the proliferative effects of KGF (Zhang et al., 2004). AEC II exposed to hyperoxia undergoes a proliferative phase with concurrent down regulation of TGF β3 (Buckley et al., 1996). Recently, TGF β1 has been shown to inhibit alveolar development in new born mouse lung exposed to hypoxia (Ambalavanan et al., 2008), but the presence of TGF β1 and Smads in alveolar epithelial cells and their potential role in controlling the AEC II- AEC I trans-differentiation process have not yet been reported.

1.3 FETAL LUNG DEVELOPMENT

Fetal lung development, like the development of any other organ, is a tightly regulated and complex process. The development of lung can be divided into five different stages namely, the embryonic stage, pseudoglandular stage, canalicular stage, saccular stage and alveolar stage (Burri, 1984)

After the primitive gut is formed, lung develops as an outpouch from the ventral wall of the future esophagus initiating the embryonic phase of lung development (days 0-13 in rat). The epithelial component of the lung is therefore derived from the fore gut endoderm and the surrounding connective tissue evolve from the mesoderm. The separation of the bud from the gut and the fusion of laryngeotracheal groove enable these buds to grow rapidly into the mesenchyme by successive dichotomous divisions distal to the gut. Subsequently, lobar and segmental portions of the airway are formed as tubular structures lined by columnar epithelium. The vascular connections begin to develop during this stage with pulmonary arteries forming a vascular plexus which initially connects to the systemic veins and then the capillaries are formed which connects to the pulmonary veins. By the end of the embryonic stage four distinct lung veins open to the right auricle of the lung. The lung begins to resemble a tubulo-acinar gland and enters the next stage, namely, the pseudo glandular stage (days 13-18 in rat). Two main events occur during this stage: formation of all the prospective conducting airways and appearance of acinar tubules (Kitaoka et al., 1996). Airway tubes proximally become lined by the large columnar cells and distally by cuboidal cells. Differentiated cell types like ciliated, non-ciliated, basel and goblet cells appear during this stage. Cartilage and smooth muscle cells begin to appear around the trachea. Close relationships develop

between the airways and the blood vessels to facilitate future gaseous exchange (Burri, 1984).

The canalicular stage (days 18-20 in rats) is characterized by the early development of pulmonary parenchyma. The acinus from the earlier stage seems to be newly delineated and future terminal and respiratory bronchioles with clusters of small tubules and buds are seen during this period. There is lengthening of each tubular branch and widening of the distal airspaces. Capillaries begin to form close contact with overlying cuboidal epithelium in each acinus. There is appearance of lamellar bodies in AEC II cells Subsequently at these contact points the cells become flatter, leading to the first appearance of an air-blood barrier. This phenomenon is seen peripherally as the terminal epithelium is essential for further branching. This trans-differentiation of cuboidal cells to the flat AEC I cells ensures that the air blood barrier undergoes proper development and is ready by the time of birth for gaseous exchange (Burri, 1984)

Saccular stage (day 20-full term) is characterized by the formation of thin walled terminal sac. These, in turn, branch into alveolar ducts and alveolar sacs. During this period, a significant increase in future respiratory air spaces with subsequent decrease in the interstitial tissue is seen. This facilitates the proper orientation of capillaries around each developing air space. Elastin deposition around these airways is also initiated and is indicative of the beginning of formation of alveoli (Mitchell et al., 1990). The blood vessels become more organized and increase both in diameter and length (Burri, 1984).

At the beginning of the alveolar stage (from full term to postnatal) the walls of the air spaces (primary septa) become thicker with a central sheet of connective tissue flanked

on both sides by capillary networks. This is followed by the formation of the secondary septa which partitions the saccules into alveolar ducts and sacs (Burri et al., 1974). There is remarkable and considerable reduction in the interstitial tissue separating airspaces. The alveoli develop their classical appearance with ridges and crests evolving from the previously smooth linings of the airspace. The lung progressively prepare for efficient gaseous exchange during this period (Burri, 1984; Massaro & Massaro, 1996).

Spatial and temporal cascades of signaling through a multitude of pathways ensure that appropriate, co-ordinated fetal lung development takes place. Even though many of these pathways and their components are studied in lung development, the precise mechanisms that fine tune the multiple events during development are still not fully understood. For example, the trigger for primary bud induction from foregut endoderm is poorly understood (Cardoso, 2001). Also factors that determine the left-right asymmetry of the lung which leads to more number of lobes being formed in the right lung than the left is poorly understood. Remarkably, this fate is determined even before the lung buds are formed.

Mesenchymal-epithelial interactions are important events in fetal lung development. Various signaling pathways have been studied in this context. Fibroblast growth factor family members (FGF) are known mediators of not only this interaction but also in branching morphogenesis and cellular differentiation process (Shannon, 1994; Shannon et al., 1998). The most thoroughly studied signaling molecule in lung development, arguably, is FGF-10 (Bellusci et al., 1997). The signaling by FGF-10 from the mesoderm is critical for the formation of bronchi as evident from a targeted deletion study where

FGF-10 knock out mice had trachea, but no bronchi or lung parenchyma (Min et al., 1998). Another member of this family namely FGF-7 (KGF, keratinocyte growth factor), is known to act as a proliferation factor for lung epithelium (Post et al., 1996).

In the distal branching morphogenesis, a secreted protein named sonic hedgehog (Shh) is found to have an important role (Hogan, 1999). Shh increases mesenchymal proliferation and alters the expression of FGF-10. Knockout of the Shh gene results in a lung remaining as a rudimentary sac with reduced mesenchymal proliferation and increased FGF-10 expression. However, the proximal-distal differentiation seems to be, remarkably, unaffected (Pepicelli et al., 1998; de Vries et al., 2005).

The members of TGF-β family play an important role in lung morphogenesis. TGFβ 1 reduces branching and saccular bud formation in in vitro lung cultures (Serra et al., 1994). Knockout of TGFβ3 delays lung development and decreases the SP-C expression, an indication of defective AEC II formation and surfactant synthesis (Kaartinen et al., 1995). Another member of this family, BMP4 influences type II cell formation and the rate of epithelial cell proliferation during development (Bellusci et al., 1996).

A variety of transcription factors are also involved in lung development (Mendelson, 2000). The members of the Gli family, Gli1, Gli2 and Gli3 (Grindley et al., 1997), retinoid receptors, members of the N-myc family (Stanton et al., 1992), thyroid transcription factor (TTF1/Nkx2.1) (Kimura et al., 1996), hepatocyte nuclear factor 3β (HNF-3β) (Chen et al., 1998), glucocorticoid receptor (GR) (Odom et al., 1988), cyclic AMP response element-binding protein (CREB) (Botas et al., 1998) are all known to modulate various events in branching and differentiation of fetal lung.

The importance of most of these factors was established by loss-of-function studies where the knockout of a specific gene resulted in developmental abnormalities. But the regulatory mechanisms that govern the spatial and temporal regulation of the majority of these signaling molecules are still poorly understood. To untangle the intricate web of signaling and to decipher the mechanisms of various levels of control that are put in place to orient organ development in the right direction is a challenging task in itself. This makes solving the puzzle of development regulation a more complex and dynamic field of study.

In many diseases that affect the normal development of lung, many of these pathways or their control mechanisms might be affected. This, in turn, can lead to numerous defects that affect the normal functioning of both prenatal and postnatal lung. Dysregulation of developmental pathways can either be a cause or an effect for the manifestation of diseases. This can also act in tandem with other contributing factors, leading to the disease process.

1.4 Bronchopulmonary dysplasia

Bronchopulmonary dysplasia (BPD) is the most common form of chronic lung disease in infants. The cause of BPD is multifactorial and involves factors like the immaturity of lung due to pre-term birth, complications due to mechanical ventilation leading to barotrauma and volutrauma, oxidant injury and treatment using pro-inflammatory mediators (Northway et al., 1967; Groneck & Speer, 1995; Van Marter et al., 2000; Jobe & Ikegami, 2001; Turunen et al., 2006). The steps taken to help extremely low birth weight neonates like positive pressure ventilation actually contribute to the manifestation

of this disease. The infants suffering from severe forms of this disease are prone to serious respiratory infections and lower lung functions from early childhood. In many cases this can have some affect on proper neural development (Vohr et al., 2004; Ehrenkranz et al., 2005). The risk of developing BPD increases with a decrease in birth weight and gestational age. For neonates with birth weights between 700-900 gram, the chance of developing BPD is almost 50% while that for neonates with weight over 1250 gram is less than 5% (Avery et al., 1987). In earlier days, BPD was characterized as a severe and chronic disease with significant fibrosis and cellular proliferation (Kunig et al., 2005). The lesions in this 'old' BPD pathology were manifested due to hyperoxia resulting from positive pressure ventilation on an immature and surfactant deficient lung (Coalson, 2006). This included damage to airway epithelia, smooth muscle hyperplasia, areas of atelectasis or over inflation, fibrosis and vascular lesions (Bonikos et al., 1976). The advent of new treatment methodologies like exogeneous surfactant administration, improved ventilation strategies, better nursing techniques in neonatal intensive care units and new antenatal treatment regimes using steroids have altered the clinical progression and lung histology of BPD (Coalson, 2006). This 'new' BPD pathology is less severe and terms like 'neonatal chronic lung disease' has been coined to include this new less severe form of BPD so as to encompass the complete spectrum of 'new' BPD (Hyde et al., 1989).

In this new and milder form of BPD which manifests in more immature infants, the primary cause for the pathogenesis is not oxidative injury and mechanical ventilation, but the immaturity of the developing lung that has been forced to undergo gaseous exchange without completing proper development. The 'new'BPD pathology is characterized by

decreased, large and simplified alveoli, decreased dysmorphic capillaries, negligible airway epithelial lesions, variable interstitial fibro-proliferation and variable airway smooth muscle hyperplasia (Coalson, 2003). The only consistent change in the 'new' BPD pathology is the alveolar simplification and enlargement of alveoli (Coalson, 2003). This results from the impairment in postnatal alveolarization in an immature lung due to preterm birth and not due to the arrest of development (Husain et al., 1998; Coalson, 2003). The 'new' BPD model may also include abnormal distribution of capillaries, stunted secondary septation and abnormal microvasculature (De Paepe et al., 2006). Also experimental treatments such as hyperoxia, hypoxia, lack of nutrition and glucocorticoid administration are shown to decrease septation as seen in 'new'BPD (Massaro & Massaro, 1996). Elastogenesis, an essential process in alveolar septation is also thought to be hampered in BPD (Bruce et al., 1992; Bourbon et al., 2005). The molecular mechanisms that cause the stunted lung growth and abnormal alveolar septation in BPD are poorly understood. Disturbing the normal angiogenesis using anti-angiogenic agents results in the formation of lung structures as seen in 'new' BPD (Jakkula et al., 2000; Kunig et al., 2005). Inhibition of signaling through vascular endothelial growth factor (VEGF) receptor also shows the same kind of lesion (Jakkula et al., 2000; Le Cras et al., 2002). Also hyperoxia causes impaired VEGF signaling and is thought to contribute to the pathology of BPD (Lassus et al., 2001; Afshar et al., 2003). Bombesin-like peptides (BLP) produced by neuroendocrine cells are increased during experimental induction of BPD in primate models and are also thought to mediate the disease process (Sunday et al., 1998). Gene expression profiling in experimental BPD has been done in an animal model that shows 'old' BPD pathology (Wagenaar et al., 2004). As a result there were

more changes in the inflammatory mediators like interleukins. But in stages where reduction in secondary septation and alveolar enlargement were observed, there was downregulation of fibroblast growth factor receptor-4 (FGF-4) (Wagenaar et al., 2004). This is consistent with the data from FGF-4 (-/-) knock out mice which are normal at birth but have complete lack of alveogenesis and formation of secondary septum (Weinstein et al., 1998). There was a decrease in VEGF receptor-2 expression which is known to cause alveolar septal cell apoptosis and defective alveolar development (Kasahara et al., 2000). Abnormal expression of other developmentally important factors like FGF-7 (KGF) (Das & Ravi, 2004) and insulin like growth factors (IGF) I and II (Veness-Meehan et al., 1997) have also been reported in BPD.

Several animal models have been used to study BPD. Each model has given us different insights into the development of BPD. The lamb model developed by Bland et al has given valuable insights into changes in pulmonary circulation and fluid imbalance (Bland et al., 2000). The baboon model was developed to study the 'old'BPD (Coalson et al., 1982; Escobedo et al., 1982). Many surfactant treatment studies were standardized in this model as it closely resembles the humans. Also there are rat and mouse models for BPD (Bonikos et al., 1975; Han et al., 1996). Like in baboons and unlike in lambs, there is postnatal alveolarization which continues for 14 days after birth in both rat and mice (Coalson, 2006). Lesions corresponding to both 'old' and 'new' BPD can be manifested by changing the concentration of oxygen to which they are exposed and the stage at which they are exposed (Bonikos et al., 1975; Pappas et al., 1983; Han et al., 1996; Warner et al., 1998). The data from all these models have been compared and used to

draw conclusions about the common factors and lesions associated with both forms of BPD even though much remains unknown.

1.5 MICRORNAS

MicroRNAs are small RNAs ~21-24 nucleotides in length and endogeneous in origin. They are transcribed from genes, but not translated to any proteins. They suppress protein expression by cleavage of mRNA or translational suppression (Bartel, 2004). miRNAs were discovered in 1993 by Ambros and his colleagues in the nematode *Caenorhabditis elegans* (Lee et al., 1993). The down-regulation of LIN-14 protein is essential for the progression from 1st larval stage (L1) to the next (L2) in this organism. It was found that the transcription of lin-4 gene was essential for this downregulation to occur. More importantly the transcription of lin-4 did not result in a protein, but in two small RNAs, one ~21 nucleotide in length and the other ~61 nucleotide in length. The longer one could form a stem-loop structure and act as the precursor for the shorter one. Later this group along with Wightman et al found that the smaller RNA had anti-sense complimentarity to multiple sites in the 3'UTR of lin-14 mRNA (Lee et al., 1993; Wightman et al., 1993). It was also observed that binding between these complimentary regions decreased the expression of LIN-14 protein without significant change in the level of lin-4 mRNA (Wightman et al., 1993). These two studies together brought forth a model where there is base pairing between multiple lin-4 RNAs to the complimentary sites in the 3'UTR of lin-14 mRNA thereby causing translational repression of lin-14 and subsequent progression from L1 to L2 during *C. elegans* development. This new kind of regulation was first thought to be a phenomenen exclusive to *C. elegans*. In 2000, two separate groups discovered another small RNA, let-7, which was essential for the development of

a later larval stage to adult (Reinhart et al., 2000; Slack et al., 2000). More importantly, homologues of this gene were discovered in many other organisms including human beings (Pasquinelli et al., 2000). Within a shorter period of time, multiple labs cloned small RNAs from humans, flies and worms. These RNAs were non coding , ~ 22 nucleotides in length and seemed to derive from a longer precursor with a stem-loop structure (Bartel, 2004). Many of them were conserved evolutionarily and exhibited cell type specificity. The recognition and confirmation of the existence of these small RNAs, termed microRNAs (miRNAs), led to intensive research to identify new members of this family. This has resulted in the discovery of multiple miRNAs across different species of both plants and animals. A miRNA registry was also set up where all the newly identified miRNAs. Named miRBase, this registry presently contains 6396 entries of potential miRNAs (Griffiths-Jones, 2004). Many of these miRNAs are still unverified at a functional level.

A majority of the miRNAs are transcribed from regions distant from the previously known genes (Lagos-Quintana et al., 2003). Earlier studies indicated that many miRNA genes were located in the introns of pre-mRNAs, did not have their own promoters and were in the same orientation as the predicted mRNAs (Lagos-Quintana et al., 2003; Lai et al., 2003; Lim et al., 2003). New studies have revealed that almost 70% of mammalian miRNA genes are located in specific and defined transcription units (Rodriguez et al., 2004). Clusters of miRNA genes have also been discovered with a potential to be transcribed as a single unit (Lau et al., 2001; Lagos-Quintana et al., 2003) although this a very small minority among all known miRNA genes. Since this is a relatively new and rapidly evolving field, there is potential for new insights to significantly overhaul the

current knowledge. From the current body of knowledge, it can be said that miRNAs can be present on diverse locations within a genome. This paves the way for a wide array of miRNA expression scenarios which greatly enhances the potential for gene regulation in multiple ways.

The biogenesis of miRNAs is initiated by the transcription of the primary miRNA (pri-miRNA) which is hundreds of nucleotides long. The pri-miRNA is then processed into ~70 nucleotide precursor RNA (pre-miRNA) by a nuclear RNase III enzyme called drosha (Lee et al., 2003). This 160 kDa protein is conserved in animals and not in plants (Wu et al., 2000). It needs a cofactor known as DiGeorge syndrome critical region gene 8 (DGCR8) in humans or Pasha in insects and worms (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). This pre-miRNA which has a typical 5' phosphate and ~2 nt 3' overhang is then exported into the cytoplasm through Ran-dependent nuclear transport receptor named exportin-5 (Exp5) (Yi et al., 2003; Lund et al., 2004). In the cytoplasm the pre-miRNAs are processed into mature ~22 nucleotide long duplexes by another RNase III enzyme called dicer (Lee et al., 2002; Yi et al., 2003). Like drosha, dicer is highly conserved and needs binding with a partner to elicit its effect (Chendrimada et al., 2005). The mature miRNAs are then taken up into micro ribonuclear protein complexes (miRNPs) also called RNA induced silencing complexes (RISC). The strand with loose pairing at the 5' end remains incorporated while the other is degraded (Schwarz et al., 2003).

The mechanism by which miRNAs downregulate gene expression is thought to vary depending upon the degree of complementarity between the miRNA and the target

regions in the mRNA. Sufficient complementarity is believed to degrade mRNA while a lower complementarity leads to translational suppression (Hutvagner & Zamore, 2002). But there are exceptions where even near perfect complementarity leads to translational suppression but not cleavage (Aukerman & Sakai, 2003). Recently, another mechanism of action has been proposed where binding of miRNAs lead to faster deadenylation of mRNAs thereby accelerating their degradation (Wu et al., 2006).

miRNAs are now believed to be major players in regulating various biological processes ranging from development of organs to virus invasion. Embryonic stem cell differentiation, a cardinal event in the development of any organ or organ system, is now thought to be modulated by miRNAs. The knock down of dicer in fruit flies caused embryonic lethality with abnormal morphology and significant lack of stem cells (Kloosterman & Plasterk, 2006). In mice, even though dicer deficiency did not stop the formation of stem cell colonies, it severely impaired the differentiation capability of embryonic stem cells and caused defect in the cellular morphology (Kanellopoulou et al., 2005). Even in somatic stem cells, this holds true, where dicer deficiency has led to developmental defects in limb and hair follicles (Kloosterman & Plasterk, 2006). Even though the roles of individual miRNAs are still not clearly defined, these studies demonstrate that right from early development the miRNAs might be playing an important role in determining the developmental fate of an organism.

Recent studies have identified several miRNAs that play a major role in the proper development of various organ systems. miR-273 has been found to be important in establishing left-right assemmetry during neuronal development (Hobert, 2006). A

remarkable observation was seen in heart, where even partial deletion of miR-1-2 caused severe and fatal defects in cardiac morphology (Zhao et al., 2007). miR-208 deletion caused abnormal response of heart to stress (van Rooij et al., 2007). In immune system, miR-155 down regulation caused defects in adaptive immunity response with increased fibrosis and infiltration in lung (Rodriguez et al., 2007) while miR-150 deletion altered normal lymphocyte production (Xiao et al., 2007). Another important functional role was seen in insulin secretion where miR-375 expression in pancreatic islets directly altered exocytosis of insulin from pancreatic beta cells (Poy et al., 2004). Various miRNAs have now been implicated in modulation of a range of functions like adipocyte differentiation (Esau et al., 2004), balancing proliferation and apoptosis (Brennecke et al., 2003; Cheng et al., 2005) and lipid metabolism (Esau et al., 2006). Progression of various forms of cancers have also been found to be modulated by miRNAs (Takamizawa et al., 2004; He et al., 2005b; Yanaihara et al., 2006; Blenkiron & Miska, 2007). They can either act as oncogenes or tumor supressors (Cowland et al., 2007). Examples of miRNAs as tumor suppressors include miR-15, miR-16 (Calin et al., 2002) and let-7 family members (Johnson et al., 2005). Oncogenic miRNAs include miR-155 (Eis et al., 2005; Yanaihara et al., 2006) and the miRNA cluster miR-17-92 (He et al., 2005a). Another interesting role for miRNAs is seen in virus invasion where viruses like HIV-1 supress silencing mediated by miRNAs during their replication in host cells (Triboulet et al., 2007). Many viruses are also thought to make miRNAs that faciliate their invasion into host cells. Many of the knock out studies that were done to study the function of various genes would have invariably included deletion of introns. Since a significant number of miRNAs is known to be present in introns, the loss of miRNAs

might also be a contributing factor to the phenotypic consequences seen in these individual knockout studies (Osokine et al., 2008). Taken together, the current knowledge in this rapidly advancing area of research reiterates the notion that miRNAs are major players in the regulation of a wide array of biological processes ranging from development to disease.

In both development and diseases in lung, miRNA studies have been very limited. Dicer knockout has been shown to impair lung morphogenesis (Lu et al., 2005; Harris et al., 2006). miRNA let-7 has been implicated in the progression of lung cancer (Takamizawa et al., 2004). The only study in lung development, to date, has shown that overexpression of miR-17-92 cluster resulted in enhanced proliferation and reduced differentiation of epithelial progenitor cells in the developing lung (Lu et al., 2007).

1.6 OBJECTIVES

The objectives of the present dissertation are as follows:

1. **To elucidate the role of TGF β1 mediated signaling in alveolar epithelial trans-differentiation**

   The current study examines in detail the role of TGF β1 mediated signaling in the trans-differentiation of AEC II to AEC I and to see whether the signaling is Smad-dependent. The study also proposes a probable pathway through which their action might be elicited.

2. **To identify and characterize miRNAs that modulate fetal lung development**

   Since miRNAs are now regarded as important mediators of development and disease, profiling the expression pattern of miRNAs during lung development will give us better insights into new levels of regulation of this process. This study examines miRNA expression pattern during fetal lung development and validates the expression profile of selected miRNAs. miRNAs which show significant and interesting trends of expression were chosen for further studies so as to assign functionality to them

3. **To establish a rat model of BPD and profile the miRNAs those are significantly changed in BPD**

   This study also aims at establishing a rat model for BPD where the pathology corresponds to that described for'new' BPD. Using this model, the miRNAs that are significantly changed in BPD will be profiled and the changes confirmed. This will give valuable insights into the role of miRNAs in the development of this disease.

REFERENCES

1. Adamson IY & Bowden DH. (1971). Sialomucin and pulmonary cell injury. Ultrastructural and cytochemical studies in mice exposed to oxygen. *Arch Pathol* **92,** 273-278.

2. Afshar S, Gibson LL, Yuhanna IS, Sherman TS, Kerecman JD, Grubb PH, Yoder BA, McCurnin DC & Shaul PW. (2003). Pulmonary NO synthase expression is attenuated in a fetal baboon model of chronic lung disease. *Am J Physiol Lung Cell Mol Physiol* **284,** L749-758.

3. Ambalavanan N, Nicola T, Hagood J, Bulger A, Serra R, Murphy-Ullrich JE, Oparil S & Chen YF. (2008). Transforming growth factor-beta signaling mediates hypoxia-induced pulmonary arterial remodeling and inhibition of alveolar development in newborn mouse lung. *Am J Physiol Lung Cell Mol Physiol (article in press)*.

4. Atfi A, Djelloul S, Chastre E, Davis R & Gespach C. (1997). Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor beta-mediated signaling. *J Biol Chem* **272,** 1429-1432.

5. Aukerman MJ & Sakai H. (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. *Plant Cell* **15,** 2730-2741.

6. Avery ME, Tooley WH, Keller JB, Hurd SS, Bryan MH, Cotton RB, Epstein MF, Fitzhardinge PM, Hansen CB, Hansen TN & et al. (1987). Is chronic lung disease in low birth weight infants preventable? A survey of eight centers. *Pediatrics* **79,** 26-30.

7. Bartel DP. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116,** 281-297.

8. Bellusci S, Grindley J, Emoto H, Itoh N & Hogan BL. (1997). Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. *Development* **124,** 4867-4878.

9. Bellusci S, Henderson R, Winnier G, Oikawa T & Hogan BL. (1996). Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development* **122,** 1693-1702.

10. Bland RD, Albertine KH, Carlton DP, Kullama L, Davis P, Cho SC, Kim BI, Dahl M & Tabatabaei N. (2000). Chronic lung injury in preterm lambs: abnormalities of the pulmonary circulation and lung fluid balance. *Pediatr Res* **48,** 64-74.

11. Blenkiron C & Miska EA. (2007). miRNAs in cancer: approaches, aetiology, diagnostics and therapy. *Hum Mol Genet* **16 Spec No 1,** R106-113.

12. Bonikos DS, Bensch KG, Ludwin SK & Northway WH, Jr. (1975). Oxygen toxicity in the newborn. The effect of prolonged 100 per cent O2 exposure on the lungs of newborn mice. *Lab Invest* **32,** 619-635.

13. Bonikos DS, Bensch KG, Northway WH, Jr. & Edwards DK. (1976). Bronchopulmonary dysplasia: the pulmonary pathologic sequel of necrotizing bronchiolitis and pulmonary fibrosis. *Hum Pathol* **7,** 643-666.

14. Border WA & Noble NA. (1994). Transforming growth factor beta in tissue fibrosis. *N Engl J Med* **331,** 1286-1292.

15. Borok Z, Liebler JM, Lubman RL, Foster MJ, Zhou B, Li X, Zabski SM, Kim K-J & Crandall ED. (2002). Alveolar Epithelial Ion and Fluid Transport: Na transport proteins are expressed by rat alveolar epithelial type I cells. *Am J Physiol Lung Cell Mol Physiol* **282,** L599-608.

16. Botas C, Poulain F, Akiyama J, Brown C, Allen L, Goerke J, Clements J, Carlson E, Gillespie AM, Epstein C & Hawgood S. (1998). Altered surfactant homeostasis and alveolar type II cell morphology in mice lacking surfactant protein D. *Proc Natl Acad Sci U S A* **95,** 11869-11874.

17. Bourbon J, Boucherat O, Chailley-Heu B & Delacourt C. (2005). Control mechanisms of lung alveolar development and their disorders in bronchopulmonary dysplasia. *Pediatr Res* **57,** 38R-46R.

18. Brennecke J, Hipfner DR, Stark A, Russell RB & Cohen SM. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell* **113,** 25-36.

19. Bruce MC, Schuyler M, Martin RJ, Starcher BC, Tomashefski JF, Jr. & Wedig KE. (1992). Risk factors for the degradation of lung elastic fibers in the ventilated neonate. Implications for impaired lung development in bronchopulmonary dysplasia. *Am Rev Respir Dis* **146,** 204-212.

20. Buckley S, Bui KC, Hussain M & Warburton D. (1996). Dynamics of TGF-beta 3 peptide activity during rat alveolar epithelial cell proliferative recovery from acute hyperoxia. *Am J Physiol* **271,** L54-60.

21. Burri PH. (1984). Fetal and postnatal development of the lung. *Annu Rev Physiol* **46,** 617-628.

22. Burri PH, Dbaly J & Weibel ER. (1974). The postnatal growth of the rat lung. I. Morphometry. *Anat Rec* **178,** 711-730.

23. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F & Croce CM. (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* **99,** 15524-15529.

24. Campbell L, Hollins AJ, Al-Eid A, Newman GR, von Ruhland C & Gumbleton M. (1999). Caveolin-1 expression and caveolae biogenesis during cell transdifferentiation in lung alveolar epithelial primary cultures. *Biochem Biophys Res Commun* **262,** 744-751.

25. Cardoso WV. (2001). Molecular regulation of lung development. *Annu Rev Physiol* **63,** 471-494.

26. Chacko BM, Qin B, Correia JJ, Lam SS, de Caestecker MP & Lin K. (2001). The L3 loop and C-terminal phosphorylation jointly define Smad protein trimerization. *Nat Struct Biol* **8,** 248-253.

27. Chen CR, Kang Y & Massague J. (2001). Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. *Proc Natl Acad Sci U S A* **98,** 992-999.

28. Chen J, Chen Z, Chintagari NR, Bhaskaran M, Jin N, Narasaraju T & Liu L. (2006). Alveolar type I cells protect rat lung epithelium from oxidative injury. *J Physiol* **572,** 625-638.

29. Chen J, Knowles HJ, Hebert JL & Hackett BP. (1998). Mutation of the mouse hepatocyte nuclear factor/forkhead homologue 4 gene results in an absence of cilia and random left-right asymmetry. *J Clin Invest* **102,** 1077-1082.

30. Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K & Shiekhattar R. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436,** 740-744.

31. Cheng AM, Byrom MW, Shelton J & Ford LP. (2005). Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res* **33,** 1290-1297.

32. Clegg GR, Tyrrell C, McKechnie SR, Beers MF, Harrison D & McElroy MC. (2005). Coexpression of RTI40 with alveolar epithelial type II cell proteins in lungs following injury: identification of alveolar intermediate cell types. *Am J Physiol Lung Cell Mol Physiol* **289,** L382-390.

33. Coalson JJ. (2003). Pathology of new bronchopulmonary dysplasia. *Semin Neonatol* **8,** 73-81.

34. Coalson JJ. (2006). Pathology of bronchopulmonary dysplasia. *Semin Perinatol* **30,** 179-184.

35. Coalson JJ, Kuehl TJ, Escobedo MB, Hilliard JL, Smith F, Meredith K, Null DM, Jr., Walsh W, Johnson D & Robotham JL. (1982). A baboon model of bronchopulmonary dysplasia. II. Pathologic features. *Exp Mol Pathol* **37,** 335-350.

36. Corrin B, Butcher D, McAnulty BJ, Dubois RM, Black CM, Laurent GJ & Harrison NK. (1994). Immunohistochemical localization of transforming growth factor-beta 1 in the lungs of patients with systemic sclerosis, cryptogenic fibrosing alveolitis and other lung disorders. *Histopath* **24,** 145-150.

37. Cowland JB, Hother C & Gronbaek K. (2007). MicroRNAs and cancer. *Apmis* **115,** 1090-1106.

38. Dahlin K, Mager EM, Allen L, Tigue Z, Goodglick L, Wadehra M & Dobbs L. (2004). Identification of genes differentially expressed in rat alveolar type I cells. *Am J Respir Cell Mol Biol* **31,** 309-316.

39. Danto SI, Shannon JM, Borok Z, Zabski SM & Crandall ED. (1995). Reversible transdifferentiation of alveolar epithelial cells. *Am J Respir Cell Mol Biol* **12,** 497-502.

40. Das KC & Ravi D. (2004). Altered expression of cyclins and cdks in premature infant baboon model of bronchopulmonary dysplasia. *Antioxid Redox Signal* **6,** 117-127.

41. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y & Wang XF. (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci U S A* **92,** 5545-5549.

42. De Paepe ME, Mao Q, Powell J, Rubin SE, DeKoninck P, Appel N, Dixon M & Gundogan F. (2006). Growth of pulmonary microvasculature in ventilated preterm infants. *Am J Respir Crit Care Med* **173,** 204-211.

43. de Vries FA, de Boer E, van den Bosch M, Baarends WM, Ooms M, Yuan L, Liu JG, van Zeeland AA, Heyting C & Pastink A. (2005). Mouse Sycp1 functions in synaptonemal complex assembly, meiotic recombination, and XY body formation. *Genes Dev* **19,** 1376-1389.

44. Denli AM, Tops BB, Plasterk RH, Ketting RF & Hannon GJ. (2004). Processing of primary microRNAs by the Microprocessor complex. *Nature* **432,** 231-235.

45. Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB & Goeddel DV. (1985). Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* **316,** 701-705.

46. Derynck R, Lindquist PB, Lee A, Wen D, Tamm J, Graycar JL, Rhee L, Mason AJ, Miller DA, Coffey RJ & et al. (1988). A new type of transforming growth factor-beta, TGF-beta 3. *Embo J* **7,** 3737-3743.

47. Derynck R & Zhang YE. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425,** 577-584.

48. Dobbs LG, Gonzalez R, Matthay MA, Carter EP, Allen L & Verkman AS. (1998). Highly water-permeable type I alveolar epithelial cells confer high water permeability between the airspace and vasculature in rat lung. *Proc Natl Acad Sci U S A* **95,** 2991-2996.

49. Ehrenkranz RA, Walsh MC, Vohr BR, Jobe AH, Wright LL, Fanaroff AA, Wrage LA & Poole K. (2005). Validation of the National Institutes of Health consensus definition of bronchopulmonary dysplasia. *Pediatrics* **116,** 1353-1360.

50. Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, Lund E & Dahlberg JE. (2005). Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A* **102,** 3627-3632.

51. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, Subramaniam A, Propp S, Lollo BA, Freier S, Bennett CF, Bhanot S & Monia BP. (2006). miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* **3,** 87-98.

52. Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, Ravichandran LV, Sun Y, Koo S, Perera RJ, Jain R, Dean NM, Freier SM, Bennett CF, Lollo B & Griffey R. (2004). MicroRNA-143 regulates adipocyte differentiation. *J Biol Chem* **279,** 52361-52365.

53. Escobedo MB, Hilliard JL, Smith F, Meredith K, Walsh W, Johnson D, Coalson JJ, Kuehl TJ, Null DM, Jr. & Robotham JL. (1982). A baboon model of bronchopulmonary dysplasia. I. Clinical features. *Exp Mol Pathol* **37,** 323-334.

54. Evans MJ, Cabral LJ, Stephens RJ & Freeman G. (1973). Renewal of alveolar epithelium in the rat following exposure to NO2. *Am J Pathol* **70,** 175-198.

55. Evans MJ, Cabral LJ, Stephens RJ & Freeman G. (1975). Transformation of alveolar type 2 cells to type 1 cells following exposure to NO2. *Exp Mol Pathol* **22,** 142-150.

56. Fehrenbach H. (2001). Alveolar epithelial type II cell: defender of the alveolus revisited. *Respir Res* **2,** 33-46.

57. Feng XH & Derynck R. (2005). Specificity and versatility in tgf-beta signaling through Smads. *Annu Rev Cell Dev Biol* **21,** 659-693.

58. Fitzpatrick DR, Denhez F, Kondaiah P & Akhurst RJ. (1990). Differential expression of TGF beta isoforms in murine palatogenesis. *Development* **109,** 585-595.

59. Frederick JP, Liberati NT, Waddell DS, Shi Y & Wang XF. (2004). Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element. *Mol Cell Biol* **24,** 2546-2559.

60. Geiser T. (2003). Mechanisms of alveolar epithelial repair in acute lung injury--a translational approach. *Swiss Med Wkly* **133,** 586-590.

61. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N & Shiekhattar R. (2004). The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432,** 235-240.

62. Griffiths-Jones S. (2004). The microRNA Registry. *Nucleic Acids Res* **32,** D109-111.

63. Grindley JC, Bellusci S, Perkins D & Hogan BL. (1997). Evidence for the involvement of the Gli gene family in embryonic mouse lung development. *Dev Biol* **188,** 337-348.

64. Groneck P & Speer CP. (1995). Inflammatory mediators and bronchopulmonary dysplasia. *Arch Dis Child Fetal Neonatal Ed* **73,** F1-3.

65. Han J, Lee Y, Yeom KH, Kim YK, Jin H & Kim VN. (2004). The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* **18,** 3016-3027.

66. Han RN, Buch S, Tseu I, Young J, Christie NA, Frndova H, Lye SJ, Post M & Tanswell AK. (1996). Changes in structure, mechanics, and insulin-like growth factor-related gene expression in the lungs of newborn rats exposed to air or 60% oxygen. *Pediatr Res* **39,** 921-929.

67. Hannon GJ & Beach D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* **371,** 257-261.

68. Harris KS, Zhang Z, McManus MT, Harfe BD & Sun X. (2006). Dicer function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci U S A* **103,** 2208-2213.

69. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Jr., Wrana JL & Falb D. (1997). The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* **89,** 1165-1173.

70. He H, Jazdzewski K, Li W, Liyanarachchi S, Nagy R, Volinia S, Calin GA, Liu CG, Franssila K, Suster S, Kloos RT, Croce CM & de la Chapelle A. (2005a). The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A* **102,** 19075-19080.

71. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ & Hammond SM. (2005b). A microRNA polycistron as a potential human oncogene. *Nature* **435,** 828-833.

72. Hobert O. (2006). Architecture of a microRNA-controlled gene regulatory network that diversifies neuronal cell fates. *Cold Spring Harb Symp Quant Biol* **71,** 181-188.

73. Hogan BL. (1999). Morphogenesis. *Cell* **96,** 225-233.

74. Huang SS & Huang JS. (2005). TGF-beta control of cell proliferation. *J Cell Biochem* **96,** 447-462.

75. Husain AN, Siddiqui NH & Stocker JT. (1998). Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. *Hum Pathol* **29,** 710-717.

76. Hutvagner G & Zamore PD. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297,** 2056-2060.

77. Hyde I, English RE & Williams JD. (1989). The changing pattern of chronic lung disease of prematurity. *Arch Dis Child Fetal Neonatal Ed* **64,** 448-451.

78. Iavarone A & Massague J. (1997). Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. *Nature* **387,** 417-422.

79. Ignotz RA & Massague J. (1985). Type beta transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts. *Proc Natl Acad Sci U S A* **82,** 8530-8534.

80. Imamura T, Takase M, Nishihara A, Oeda E, Hanai J-i, Kawabata M & Miyazono K. (1997). Smad6 inhibits signalling by the TGF-[beta] superfamily. *Nature* **389,** 622-626.

81. Izzi L & Attisano L. (2004). Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. *Oncogene* **23,** 2071-2078.

82. Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF & Abman SH. (2000). Inhibition of angiogenesis decreases alveolarization in the developing rat lung. *Am J Physiol Lung Cell Mol Physiol* **279,** L600-607.

83. Jakowlew SB, Dillard PJ, Sporn MB & Roberts AB. (1988). Nucleotide sequence of chicken transforming growth factor-beta 1 (TGF-beta 1). *Nucleic Acids Res* **16,** 8730.

84. Jin N, Kolliputi N, Gou D, Weng T & Liu L. (2006). A novel function of ionotropic gamma -aminobutyric acid receptors involving alveolar fluid homeostasis. *J Biol Chem***,** M606895200.

85. Jobe AH & Ikegami M. (2001). Prevention of bronchopulmonary dysplasia. *Curr Opin Pediatr* **13,** 124-129.

86. Johnson MD, Widdicombe JH, Allen L, Barbry P & Dobbs LG. (2002). Alveolar epithelial type I cells contain transport proteins and transport sodium, supporting an active role for type I cells in regulation of lung liquid homeostasis. *Proc Natl Acad Sci U S A* **99,** 1966-1971.

87. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D & Slack FJ. (2005). RAS is regulated by the let-7 microRNA family. *Cell* **120,** 635-647.

88. Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N & Groffen J. (1995). Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* **11,** 415-421.

89. Kanellopoulou C, Muljo SA, Kung AL, Ganesan S, Drapkin R, Jenuwein T, Livingston DM & Rajewsky K. (2005). Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev* **19,** 489-501.

90. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J & Voelkel NF. (2000). Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* **106,** 1311-1319.

91. Keane MP, Strieter RM & Belperio JA. (2005). Mechanisms and mediators of pulmonary fibrosis. *Crit Rev Immunol* **25,** 429-463.

92. Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM & Gonzalez FJ. (1996). The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev* **10,** 60-69.

93. Kitaoka H, Burri PH & Weibel ER. (1996). Development of the human fetal airway tree: analysis of the numerical density of airway endtips. *Anat Rec* **244,** 207-213.

94. Kloosterman WP & Plasterk RH. (2006). The diverse functions of microRNAs in animal development and disease. *Dev Cell* **11,** 441-450.

95. Kondaiah P, Sands MJ, Smith JM, Fields A, Roberts AB, Sporn MB & Melton DA. (1990). Identification of a novel transforming growth factor-beta (TGF-beta 5) mRNA in Xenopus laevis. *J Biol Chem* **265,** 1089-1093.

96. Kunig AM, Balasubramaniam V, Markham NE, Morgan D, Montgomery G, Grover TR & Abman SH. (2005). Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats. *Am J Physiol Lung Cell Mol Physiol* **289,** L529-535.

97. Lagos-Quintana M, Rauhut R, Meyer J, Borkhardt A & Tuschl T. (2003). New microRNAs from mouse and human. *Rna* **9,** 175-179.

98. Lai EC, Tomancak P, Williams RW & Rubin GM. (2003). Computational identification of Drosophila microRNA genes. *Genome Biol* **4,** R42.

99. Landthaler M, Yalcin A & Tuschl T. (2004). The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. *Curr Biol* **14,** 2162-2167.

100. Lassus P, Turanlahti M, Heikkila P, Andersson LC, Nupponen I, Sarnesto A & Andersson S. (2001). Pulmonary vascular endothelial growth factor and Flt-1 in fetuses, in acute and chronic lung disease, and in persistent pulmonary hypertension of the newborn. *Am J Respir Crit Care Med* **164,** 1981-1987.

101. Lau NC, Lim LP, Weinstein EG & Bartel DP. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* **294,** 858-862.

102. Le Cras TD, Markham NE, Tuder RM, Voelkel NF & Abman SH. (2002). Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal lung structure. *Am J Physiol Lung Cell Mol Physiol* **283,** L555-562.

103. Lee RC, Feinbaum RL & Ambros V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75,** 843-854.

104. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S & Kim VN. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425,** 415-419.

105. Lee Y, Jeon K, Lee JT, Kim S & Kim VN. (2002). MicroRNA maturation: stepwise processing and subcellular localization. *Embo J* **21,** 4663-4670.

106. Li MO, Wan YY, Sanjabi S, Robertson AK & Flavell RA. (2006). Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* **24,** 99-146.

107. Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB & Bartel DP. (2003). The microRNAs of Caenorhabditis elegans. *Genes Dev* **17,** 991-1008.

108. Lu J, Qian J, Chen F, Tang X, Li C & Cardoso WV. (2005). Differential expression of components of the microRNA machinery during mouse organogenesis. *Biochem Biophys Res Commun* **334,** 319-323.

109. Lu Y, Thomson JM, Wong HY, Hammond SM & Hogan BL. (2007). Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. *Dev Biol* **310,** 442-453.

110. Lund E, Guttinger S, Calado A, Dahlberg JE & Kutay U. (2004). Nuclear export of microRNA precursors. *Science* **303,** 95-98.

111. Maitre B, Clement A, Williams MC & Brody JS. (1995). Expression of insulin-like growth factor receptors 1 and 2 in the developing lung and their relation to epithelial cell differentiation. *Am J Respir Cell Mol Biol* **13,** 262-270.

112. Massague J. (1998). TGF-beta signal transduction. *Annu Rev Biochem* **67,** 753-791.

113. Massague J. (2000). How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* **1,** 169-178.

114. Massague J, Cheifetz S, Endo T & Nadal-Ginard B. (1986). Type beta transforming growth factor is an inhibitor of myogenic differentiation. *Proc Natl Acad Sci U S A* **83,** 8206-8210.

115. Massague J, Seoane J & Wotton D. (2005). Smad transcription factors. *Genes Dev* **19,** 2783-2810.

116. Massaro GD & Massaro D. (1996). Formation of pulmonary alveoli and gas-exchange surface area: quantitation and regulation. *Annu Rev Physiol* **58,** 73-92.

117. Masui T, Wakefield LM, Lechner JF, LaVeck MA, Sporn MB & Harris CC. (1986). Type beta transforming growth factor is the primary differentiation-inducing serum factor for normal human bronchial epithelial cells. *Proc Natl Acad Sci U S A* **83,** 2438-2442.

118. Mendelson CR. (2000). Role of transcription factors in fetal lung development and surfactant protein gene expression. *Annu Rev Physiol* **62,** 875-915.

119. Miller DA, Lee A, Matsui Y, Chen EY, Moses HL & Derynck R. (1989). Complementary DNA cloning of the murine transforming growth factor-beta 3 (TGF beta 3) precursor and the comparative expression of TGF beta 3 and TGF beta 1 messenger RNA in murine embryos and adult tissues. *Mol Endocrinol* **3,** 1926-1934.

120. Min H, Danilenko DM, Scully SA, Bolon B, Ring BD, Tarpley JE, DeRose M & Simonet WS. (1998). Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. *Genes Dev* **12,** 3156-3161.

121. Mitchell JJ, Reynolds SE, Leslie KO, Low RB & Woodcock-Mitchell J. (1990). Smooth muscle cell markers in developing rat lung. *Am J Respir Cell Mol Biol* **3,** 515-523.

122. Moses HL, Branum EL, Proper JA & Robinson RA. (1981). Transforming Growth Factor Production by Chemically Transformed Cells. *Cancer Res* **41,** 2842-2848.

123. Moustakas A, Souchelnytskyi S & Heldin CH. (2001). Smad regulation in TGF-beta signal transduction. *J Cell Sci* **114,** 4359-4369.

124. Narasaraju TA, Chen H, Weng T, Bhaskaran M, Jin N, Chen J, Chen Z, Chinoy MR & Liu L. (2006). Expression profile of IGF system during lung injury and recovery in rats exposed to hyperoxia: a possible role of IGF-1 in alveolar epithelial cell proliferation and differentiation. *J Cell Biochem* **97,** 984-998.

125. Northway WH, Jr., Rosan RC & Porter DY. (1967). Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. *N Engl J Med* **276,** 357-368.

126. Odom MJ, Snyder JM, Boggaram V & Mendelson CR. (1988). Glucocorticoid regulation of the major surfactant associated protein (SP-A) and its messenger ribonucleic acid and of morphological development of human fetal lung in vitro. *Endocrinology* **123,** 1712-1720.

127. Osokine I, Hsu R, Loeb GB & McManus MT. (2008). Unintentional miRNA ablation is a risk factor in gene knockout studies: a short report. *PLoS Genet* **4,** e34.

128. Pappas CT, Obara H, Bensch KG & Northway WH, Jr. (1983). Effect of prolonged exposure to 80% oxygen on the lung of the newborn mouse. *Lab Invest* **48,** 735-748.

129. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E & Ruvkun G. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* **408,** 86-89.

130. Pepicelli CV, Lewis PM & McMahon AP. (1998). Sonic hedgehog regulates branching morphogenesis in the mammalian lung. *Curr Biol* **8,** 1083-1086.

131. Pietenpol JA, Stein RW, Moran E, Yaciuk P, Schlegel R, Lyons RM, Pittelkow MR, Munger K, Howley PM & Moses HL. (1990). TGF-beta 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* **61,** 777-785.

132. Post M, Souza P, Liu J, Tseu I, Wang J, Kuliszewski M & Tanswell AK. (1996). Keratinocyte growth factor and its receptor are involved in regulating early lung branching. *Development* **122,** 3107-3115.

133. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P & Stoffel M. (2004). A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* **432,** 226-230.

134. Qiao R, Yan W, Clavijo C, Mehrian-Shai R, Zhong Q, Kim KJ, Ann D, Crandall ED & Borok Z. (2008). Effects of KGF on alveolar epithelial cell transdifferentiation are mediated by JNK signaling. *Am J Respir Cell Mol Biol* **38,** 239-246.

135. Qiao R, Zhou B, Liebler JM, Li X, Crandall ED & Borok Z. (2003). Identification of three genes of known function expressed by alveolar epithelial type I cells. *Am J Respir Cell Mol Biol* **29,** 98-105.

136. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR & Ruvkun G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* **403,** 901-906.

137. Ridge KM, Olivera WG, Saldias F, Azzam Z, Horowitz S, Rutschman DH, Dumasius V, Factor P & Sznajder JI. (2003). Alveolar type 1 cells express the alpha2 Na,K-ATPase, which contributes to lung liquid clearance. *Circ Res* **92,** 453-460.

138. Ridley AJ, Davis JB, Stroobant P & Land H. (1989). Transforming growth factors-beta 1 and beta 2 are mitogens for rat Schwann cells. *J Cell Biol* **109,** 3419-3424.

139. Roberts AB, Anzano MA, Lamb LC, Smith JM & Sporn MB. (1981). New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc Natl Acad Sci U S A* **78,** 5339-5343.

140. Roberts AB & Sporn MB. (1993). Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). *Growth Factors* **8,** 1-9.

141. Rodriguez A, Griffiths-Jones S, Ashurst JL & Bradley A. (2004). Identification of mammalian microRNA host genes and transcription units. *Genome Res* **14,** 1902-1910.

142. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, van Dongen S, Grocock RJ, Das PP, Miska EA, Vetrie D, Okkenhaug K, Enright AJ, Dougan G, Turner M & Bradley A. (2007). Requirement of bic/microRNA-155 for normal immune function. *Science* **316,** 608-611.

143. Schuster N & Krieglstein K. (2002). Mechanisms of TGF-beta-mediated apoptosis. *Cell Tissue Res* **307,** 1-14.

144. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N & Zamore PD. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115,** 199-208.

145. Selman M & Pardo A. (2003). The epithelial/fibroblastic pathway in the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* **29,** S93-97.

146. Serra R, Pelton RW & Moses HL. (1994). TGF beta 1 inhibits branching morphogenesis and N-myc expression in lung bud organ cultures. *Development* **120,** 2153-2161.

147. Seyedin SM, Thompson AY, Bentz H, Rosen DM, McPherson JM, Conti A, Siegel NR, Galluppi GR & Piez KA. (1986). Cartilage-inducing factor-A. Apparent identity to transforming growth factor-beta. *J Biol Chem* **261,** 5693-5695.

148. Shannon JM. (1994). Induction of alveolar type II cell differentiation in fetal tracheal epithelium by grafted distal lung mesenchyme. *Dev Biol* **166,** 600-614.

149. Shannon JM, Nielsen LD, Gebb SA & Randell SH. (1998). Mesenchyme specifies epithelial differentiation in reciprocal recombinants of embryonic lung and trachea. *Dev Dyn* **212,** 482-494.

150. Sheppard D. (2001). Integrin-mediated activation of transforming growth factor-beta(1) in pulmonary fibrosis. *Chest* **120,** 49S-53S.

151. Shi Y & Massague J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113,** 685-700.

152. Shipley GD, Tucker RF & Moses HL. (1985). Type beta transforming growth factor/growth inhibitor stimulates entry of monolayer cultures of AKR-2B cells into S phase after a prolonged prereplicative interval. *Proc Natl Acad Sci U S A* **82,** 4147-4151.

153. Slack FJ, Basson M, Liu Z, Ambros V, Horvitz HR & Ruvkun G. (2000). The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. *Mol Cell* **5,** 659-669.

154. Stanton BR, Perkins AS, Tessarollo L, Sassoon DA & Parada LF. (1992). Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop. *Genes Dev* **6,** 2235-2247.

155. Sunday ME, Yoder BA, Cuttitta F, Haley KJ & Emanuel RL. (1998). Bombesin-like peptide mediates lung injury in a baboon model of bronchopulmonary dysplasia. *J Clin Invest* **102,** 584-594.

156. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T & Takahashi T. (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Canc Res* **64,** 3753-3756.

157. Ten Dijke P, Goumans MJ, Itoh F & Itoh S. (2002). Regulation of cell proliferation by Smad proteins. *J Cell Physiol* **191,** 1-16.

158. Triboulet R, Mari B, Lin YL, Chable-Bessia C, Bennasser Y, Lebrigand K, Cardinaud B, Maurin T, Barbry P, Baillat V, Reynes J, Corbeau P, Jeang KT & Benkirane M. (2007). Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* **315,** 1579-1582.

159. Tsukazaki T, Chiang TA, Davison AF, Attisano L & Wrana JL. (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* **95,** 779-791.

160. Turunen R, Nupponen I, Siitonen S, Repo H & Andersson S. (2006). Onset of mechanical ventilation is associated with rapid activation of circulating phagocytes in preterm infants. *Pediatrics* **117,** 448-454.

161. Uhal BD. (1997). Cell cycle kinetics in the alveolar epithelium. *Am J Physiol* **272,** L1031-1045.

162. Van Marter LJ, Allred EN, Pagano M, Sanocka U, Parad R, Moore M, Susser M, Paneth N & Leviton A. (2000). Do clinical markers of barotrauma and oxygen toxicity explain interhospital variation in rates of chronic lung disease? The Neonatology Committee for the Developmental Network. *Pediatrics* **105,** 1194-1201.

163. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J & Olson EN. (2007). Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **316,** 575-579.

164. Veness-Meehan KA, Moats-Staats BM, Price WA & Stiles AD. (1997). Re-emergence of a fetal pattern of insulin-like growth factor expression during hyperoxic rat lung injury. *Am J Respir Cell Mol Biol* **16,** 538-548.

165. Venkatesan N, Pini L & Ludwig MS. (2004). Changes in Smad expression and subcellular localization in bleomycin-induced pulmonary fibrosis. *American journal of physiology* **287,** L1342-1347.

166. Verrecchia F, Chu ML & Mauviel A. (2001). Identification of novel TGF-beta /Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. *J Biol Chem* **276,** 17058-17062.

167. Vohr BR, Wright LL, Dusick AM, Perritt R, Poole WK, Tyson JE, Steichen JJ, Bauer CR, Wilson-Costello DE & Mayes LC. (2004). Center differences and outcomes of extremely low birth weight infants. *Pediatrics* **113,** 781-789.

168. Wagenaar GT, ter Horst SA, van Gastelen MA, Leijser LM, Mauad T, van der Velden PA, de Heer E, Hiemstra PS, Poorthuis BJ & Walther FJ. (2004). Gene expression profile and histopathology of experimental bronchopulmonary dysplasia induced by prolonged oxidative stress. *Free Radic Biol Med* **36,** 782-801.

169. Warburton D & Bellusci S. (2004). The molecular genetics of lung morphogenesis and injury repair. *Paed Resp Rev* **5,** S283-S287.

170. Warner BB, Stuart LA, Papes RA & Wispe JR. (1998). Functional and pathological effects of prolonged hyperoxia in neonatal mice. *Am J Physiol* **275,** L110-117.

171. Weinstein M, Xu X, Ohyama K & Deng CX. (1998). FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. *Development* **125,** 3615-3623.

172. Wightman B, Ha I & Ruvkun G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* **75,** 855-862.

173. Wu H, Xu H, Miraglia LJ & Crooke ST. (2000). Human RNase III is a 160-kDa protein involved in preribosomal RNA processing. *J Biol Chem* **275,** 36957-36965.

174. Wu L, Fan J & Belasco JG. (2006). MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci U S A* **103,** 4034-4039.

175. Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, Rajewsky N, Bender TP & Rajewsky K. (2007). MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell* **131,** 146-159.

176. Xiao Z, Liu X, Henis YI & Lodish HF. (2000). A distinct nuclear localization signal in the N terminus of Smad 3 determines its ligand-induced nuclear translocation. *Proc Natl Acad Sci U S A* **97,** 7853-7858.

177. Xiao Z, Watson N, Rodriguez C & Lodish HF. (2001). Nucleocytoplasmic Shuttling of Smad1 Conferred by Its Nuclear Localization and Nuclear Export Signals. *J Biol Chem* **276,** 39404-39410.

178. Xu L, Kang Y, Col S & Massague J. (2002). Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus. *Mol Cell* **10,** 271-282.

179. Xu YD, Hua J, Mui A, O'Connor R, Grotendorst G & Khalil N. (2003). Release of biologically active TGF-beta1 by alveolar epithelial cells results in pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* **285,** L527-539.

180. Yamamoto T, Komura H, Morishige K, Tadokoro C, Sakata M, Kurachi H & Miyake A. (1994). Involvement of autocrine mechanism of transforming growth factor-beta in the functional differentiation of pregnant mouse mammary gland. *Eur J Endocrinol* **130,** 302-307.

181. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM & Harris CC. (2006). Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Canc Cell* **9,** 189-198.

182. Yi R, Qin Y, Macara IG & Cullen BR. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17,** 3011-3016.

183. Zhang F, Nielsen LD, Lucas JJ & Mason RJ. (2004). Transforming growth factor-beta antagonizes alveolar type II cell proliferation induced by keratinocyte growth factor. *Am J Respir Cell Mol Biol* **31,** 679-686.

184. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ & Srivastava D. (2007). Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell* **129,** 303-317.

CHAPTER II

TRANS-DIFFERENTIATION OF ALVEOLAR EPITHELIAL TYPE II CELLS TO TYPE I CELLS INVOLVES AUTOCRINE SIGNALING BY TGF ß1 THROUGH THE SMAD PATHWAY

ABSTRACT

Type II Alveolar Epithelial Cells (AEC II) proliferate and trans-differentiate into Type I Alveolar Epithelial Cells (AEC I) when the normal AEC I population is damaged in the lung alveoli. We hypothesized that signaling by Transforming Growth Factor β1 (TGF β1), through its down stream Smad proteins, is involved in keeping AEC II quiescent in normal cells and its altered signaling may be involved in the trans-differentiation of AEC II to AEC I. In the normal lung, TGF β1 and Smad4 were highly expressed in AEC II. Using an *in vitro* cell culture model, we demonstrated that the trans-differentiation of AEC II into AEC I-like cells began with a proliferative phase, followed by a differentiation phase. The expression of TGF β1, Smad2 and Smad3 and their phosphorylated protein forms, and cell cycle inhibitors, p15Ink4b and p21Cip1 was lower during the proliferative phase but higher during the differentiation phase. Furthermore, cyclin dependent kinases 2, 4 and 6 showed an opposite trend of expression. TGF β1 secretion into the media increased during the differentiation phase, indicating an autocrine regulation. The addition of TGF β1 neutralizing antibody after the proliferative phase and silencing of Smad4 by RNA interference inhibited the trans-differentiation process. In summary, our results suggest that the trans-differentiation of AEC II to AEC I

is modulated by signaling through the Smad-dependent TGF β1 pathway by altering the expression of proteins that control the G1 to S phase entry in the cell cycle.

INTRODUCTION

Alveolar Epithelial cells (AEC II) have a multifunctional role in the lung including secretory, synthetic and progenitor capacities. AEC II serve as remodeling reservoirs for lung epithelium (Warburton and Bellusci, 2004). They are the progenitors for AEC I. By virtue of their squamous shape, AEC I cover the major surface area of alveoli and thus are the main epithelial component of the thin air-blood barrier. In contrast, AEC II are limited to the corners of the alveoli. The progenitor function of AEC II is activated when the lung epithelium encounters a variety of disease conditions including acute lung injury (Fehrenbach, 2001). AEC II cell proliferation and hyperplasia, followed by trans-differentiation into AEC I is a hallmark of alveolar epithelial injury. This helps to restore the normal air-blood barrier. However, the molecular mechanisms involved in the trans-differentiation process are not clear. Questions regarding the causes of AEC II proliferation and trans-differentiation into AEC I during lung injury and prevention of this in normal alveoli largely remain unanswered.

The Transforming Growth Factor β (TGF β) superfamily consists of cytokines that are implicated in the regulation of a variety of biological responses (Li et al., 2006; Massague, 2000; Moustakas et al., 2001; ten Dijke et al., 2002). Its members include isoforms of TGF β, bone morphogenetic proteins, Mullerian inhibiting substance, inhibins and activins. A member of this family, TGF β1, regulates cell proliferation, differentiation and migration in many cell and tissue systems. This cytokine is secreted

by various cell types and acts both in autocrine and paracrine manners. The binding of TGF β1 to its specific type II receptor (TβRII) induces the formation of a hetero tetrameric complex with the type I receptor (TβRI). This complex phosphorylates the TβRI. The activated TβRI in turn phosphorylates the receptor-regulated Smads (R-Smads), Smad2 and Smad3. The phosphorylated R-Smads disassociate with the Smad anchor for receptor activation (SARA) (Tsukazaki et al., 1998) and assemble with the common partner Smad (Co-Smad) named Smad4. The Smad heteromeric complex translocates into the nucleus, binds to DNA directly or indirectly through specific DNA binding proteins, and regulates the transcription of target genes (Feng and Derynck, 2005; Massague et al., 2005) Also there are inhibitory Smads (I-Smads), Smad6 and Smad7, which are antagonistic to the R-Smads and act by competitively binding to the activated TβRI (Hayashi et al., 1997; Imamura et al., 1997). Once the response is achieved, R-Smads and I-Smads are turned off mainly by ubiquitin-mediated degradation (Izzi and Attisano, 2004).

The mechanism by which TGF β inhibits cell proliferation varies in different epithelial cells (ten Dijke et al., 2002). Generally, the action is elicited by altering the levels of cell cycle proteins that are involved in the G1 to S phase entry before the restriction point in the cell cycle. TGF β increases the expression of two cyclin dependent kinase inhibitors, p15 ($p15I^{nk4b}$) (Hannon and Beach, 1994) and p21 ($p21^{Cip1}$) (Datto et al., 1995), which in turn inhibit Cyclin Dependent Kinases (CDKs) 2, 4, and 6. TGF β can also inhibit the transcription of genes needed for the cell cycle progression. This includes c-Myc (Chen *et al.*, 2001; Frederick *et al.*, 2004; Pietenpol et al., 1990) and CDK activating phosphatase, cdc25A (Iavarone and Massague, 1997). Additionally, TGF β can initiate signaling

through pathways other than those involving Smads, such as PI3 kinase and various members of the mitogen activated protein kinase (MAPK) (Atfi et al., 1997; Derynck and Zhang, 2003; Verrecchia et al., 2001).

Although several studies have shown that TGF β1 has a role in modulating abnormal lung conditions such as idiopathic pulmonary fibrosis (IPF) (Corrin et al., 1994; Selman and Pardo, 2003; Venkatesan et al., 2004; Xu et al., 2003), whether it controls AEC II proliferation and trans-differentiation into AEC I has not been examined in detail. Studies on the effect of TGF β1 on AEC II so far have been limited to its anti-proliferative effects on culture systems that maintain the AEC II phenotype (Zhang et al., 2004). Here, we report the role of Smad-dependent TGF β1 pathway in the trans-differentiation of AEC II to AEC I using a model that mimics the differentiation process that takes place during lung injury. Through the addition of TGF β1 and its neutralizing antibody, RNA interference (RNAi)-mediated silencing of Smad4 and by showing differential expression of the components of TGF β pathway and cell cycle proteins during the trans-differentiation process, we have demonstrated a possible mechanism of controlling this process in the alveolar epithelium.

MATERIALS AND METHODS

Rat IgG, deoxyribonuclease 1, n-propyl gallate, 5-bromo-2'-deoxy uridine and recombinant human TGF β1 were obtained from Sigma Aldrich (St. Louis, MO). Elastase was from Worthington Biochemical Corporation (Lakewood, NJ). Mouse monoclonal anti-Smad4, goat polyclonal anti-p15, anti-p21, anti-CDK2, anti-CDK4, anti-CDK6, goat polyclonal anti-purinergic receptor P2X7 (P2X7) and anti-surfactant protein C (SP-C)

antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-Smad2, rabbit polyclonal anti-pSmad2 and anti-pSmad3 antibodies were from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal anti-Smad3 was from Abcam (Cambridge, MA). Monoclonal anti-TGF β1 antibody and mouse/rat/porcine TGF β1 Quantikine ELISA Kit were from R & D systems (Minneapolis, MN). Mouse anti-LB-180 antibody was from Covance (Berkeley, CA). Alexa 568- and 488-conjugated anti-rabbit and anti-mouse secondary antibodies were from Molecular Probes (Eugene, OR). Cy3-conjugated affini pure goat anti-mouse IgG was from Jackson Immuno Research Laboratories (West Grove, PA). The Dc protein assay kit and horse-radish peroxidase (HRP)-conjugated anti-mouse antibodies were from BioRad (Hercules,CA). The enhanced chemiluminescence detection system was from Amersham Biotech (Piscataway, NJ). Anti-rat and anti-mouse IgG-conjugated magnetic beads were from Dynal Biotech (Lake success, NY). The TRI reagent was from Molecular Research Center Inc. (Cincinnati, OH). M-MLV Reverse transcriptase was from Invitrogen (Carlsbad, CA). DNA polymerase was from New England Biolabs (Beverly, MA). Quantitect SYBR Green PCR kit was from Qiagen (Valencia, CA). Penicillin, streptomycin and fetal bovine serum (FBS) were from Gibco (Grand Island, NY). Dulbecco's Modified Eagles Medium Hams Nutrient Mixture F-12 (DMEM/F-12) in 1:1 ratio was from ATCC (Manassas, VA).

Isolation and culture of AEC II

AEC II were isolated from pathogen free male Sprague-Dawley rats (200-250g) by an improved method as previously described (Chen et al., 2004a). In brief, adult rat lungs were perfused, lavaged and digested with elastase (3 units/ml). The cell mixture was

filtered through 160- and 37-µm nylon mesh once and 15-µm nylon mesh twice, and plated on rat IgG-coated plates twice for 45 and 30 min to remove macrophages. The unattached cells were further incubated with anti-Leukocyte Common Antigen (anti-LC, 40 µg/ml) and rat IgG (70 µg/ml) antibodies for 30 min at 40C. This was followed by incubation for 20 min with anti-rat and anti-mouse IgG-conjugated magnetic beads. A magnetic field was applied to remove the cells attached to the magnetic beads. The purity of AEC II was above 95% as determined by modified Papanicolaou staining and the viability was above 98%.

AEC II were seeded onto 35 mm tissue culture-treated plastic dishes at a density of 1.3 x 10^6 cells per dish in Minimum Essential Medium (MEM) with 10% FBS and cultured for 1 to 7 days. The media were changed after the first 24 h and thereafter on alternate days. In this culture system, the trans-differentiation of AEC II to AEC I-like cells was evident from day 3 onwards and was nearly complete by day 5. For the sandwich ELISA experiment to quantitate TGF β1 secreted into media, modified serum free medium (MDSF) was used. The MDSF contained 1:1 ratio of DMEM/F 12 media, bovine serum albumin (1.5 mg/ml), 10 mM HEPES, 0.1 mM non-essential amino acids, 2 mM glutamine, 1000 U/mL penicillin G and 100 µg/ml streptomycin.

Immunohistochemistry and Immunohistochemistry

Paraffin-embedded tissue sections of perfused rat lungs were de-waxed using xylene and rehydrated using descending grades of ethanol. They were then washed in phosphate-buffered saline (PBS, pH 7.4). Antigen retrieval was done by boiling the slides for 15 min in 20 mM citrate buffer (pH 6.0). Cytospinned cells or cells cultured in 35 mm plastic

dishes were fixed in 4% paraformaldehyde. The tissue sections or cells were permeabilized with 0.4% Triton X-100 for 20 min and blocked for 1 h in 10% FBS. The slides were then incubated with mouse anti-Smad4 (1:50), mouse anti-TGF β1 (1:10), LB-180 (1:200), SP-C (1:100) or P2X7(1:50) antibodies at 40C overnight. Slides were washed and incubated with Alexa 568-conjugated anti-rabbit/anti-goat and Alexa 488-conjugated anti-mouse/anti-rabbit secondary antibodies or Cy-3-conjugated Affini pure anti-mouse IgG (1:250 dilution). Finally, slides were then washed and mounted on an anti-fade medium (5% n-propyl gallate and 80% glycerol in PBS) and viewed through a Nikon Eclipse E600 fluorescence microscope or Nikon Eclipse TE 2000 U inverted fluorescent microscope.

Real-time PCR

Total RNA (1 μg) was isolated using TRI reagent and reverse-transcribed into cDNA using 200 U of M-MLV reverse transcriptase and a mixture of random and oligo dT primers. Gene sequence information was obtained using nucleotide databases (www.ncbi.nlm.nih.gov/Entrez). All primer sequences (Table 1) were designed using the primer express 2.0 software and confirmed for specificity by using the non-redundant basic local alignment search.

Table 1 Primers used for real time PCR

GenBank ID	Gene name	Primers	
BC076380	TGF β1	Forward	AATTCCTGGCGTTACCTTGGT
		Reverse	TGTATTCCGTCTCCTTGGTTCA
AB010147	Smad2	Forward	ATGGTCGTCTTCAGGTGTCTCA
		Reverse	TGAAAGCGTATTCGCAGTTCTC
BC064437	Smad3	Forward	TTCAACGGAACTTGGGAATGAG
		Reverse	TTGCTGACCACCCTGCTCTT
AF056002	Smad4	Forward	GCAGTTGGTAACAGGTAGTTTGGAT
		Reverse	TCCCACGCTTCTACTTCAACGT
NM_008542	Smad6	Forward	AGGCCACCAACTCCCTCATC
		Reverse	GTGTCCGGTGCTCCCAGTAT
BC086995	p15	Forward	AAGGCAAAGCCAATCAGAAATAA
		Reverse	ATAGAGGGTCCCAGAACTTCATACA
NM_080782	p21	Forward	GCAAAGTATGCCGTCGTCTGT
		Reverse	CGTCTCAGTGGCGAAGTCAA
M11188	18S	Forward	TCCCAGTAAGTGCGGGTCATA
		Reverse	CGAGGGCCTCACTAAACCATC

The reverse transcribed cDNA was further diluted 5 times and quantitative real time PCR was done using an ABI prism 7700 system (PE Applied Biosystems, Foster City, CA). The reactions were carried out on 96 well plates. 18S rRNA was amplified on the same plates and used to normalize the data. The reaction volume was 10 µl containing 5 µl of the SYBR master mix, 0.4 µl of 25 mM MgCl2, 0.3 µl each of 5 µM forward and reverse primers, 2 µl of RNase free water and 2 µl of the cDNA samples. Each sample was prepared in duplicate and at least 3 different sets of cell preparations were used. The thermal cycling conditions used were: 95ºC for 15 min followed by 40 cycles at 95ºC for

20 sec, 60ºC for 30 sec and 72ºC for 30 sec. The data acquisition temperature varied from 76 to 78ºC depending on the melting temperature of the individual PCR products. Dissociation curve analysis was performed for each gene to ensure the specificity of PCR products. The relative abundance of each gene was calculated by subtracting the CT value of each sample of an individual gene from the corresponding value for 18S gene (delta CT). Freshly isolated AEC II (Day 0) were used as the reference point. Delta delta CT were obtained by subtracting CT of the reference point. These values were raised to the power of 2 ($2^{\text{delta delta CT}}$) to give expression levels relative to day 0 expression.

Western blot

Cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM EDTA and 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotonin and 10 μg/ml leupeptin). The protein concentration was determined by the Dc protein assay kit. 20 μg of protein was separated on 12% SDS polyacrylamide gels, transferred to a nitrocellulose membrane using the semidry transfer apparatus (BioRad) at 17 mA for 60 min. The membrane was stained with Ponceau S to ensure proper transfer and blocked overnight with 5% dry skim milk power in 100 mM Tris-buffered saline plus 0.1% Tween-20 (TBS-T). The membranes were incubated with anti-Smad2, anti-Smad3, anti-Smad4, anti-TGF β1, anti-pSmad2, anti-pSmad3, anti-p15, anti-p21, anti-CDK2, anti-CDK4 or anti-CDK6 antibodies at 1:100 dilutions overnight at 4ºC. After being washed in TBS-T 3 times, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse, goat or rabbit IgG's (1:2000) for 1 h. The blots were washed again. The individual target proteins were visualized using the enhanced chemiluminescence detection system.

5-Bromo-2'-deoxy uridine (BrdU) labeling

To determine cell proliferation in the *in vitro* AEC trans-differentiation model, BrdU was added at a concentration of 10 μM into the culture media and incubated for 12 - 14 h. After being washed with PBS (pH 7.4), the cells were fixed with 75% methanol containing 5 mM glycine at -200C for 20 min. The cells were washed again and treated with 2 M HCl to denature the DNA strands followed by neutralization using borate buffer (pH 9.4). The cells then were washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked in 10% FBS in PBS-T for 2 h. BrdU was detected using monoclonal anti-BrdU Antibody (1:100 dilution) and FITC-conjugated rabbit anti mouse antibody (1:250 dilution).

Sandwich ELISA

To measure the TGF β1 secretion into the media, AEC II were cultured in MDSF on plastic dishes for 0 -5 days. The use of MDSF avoids potential contamination from endogenous TGF β1 in FBS. The amount of TGF β1 secreted into the medium was assayed using an ELISA kit. Briefly, monoclonal anti-TGF β1 antibody was precoated on a microplate. The latent TGF β1 in the media was activated by the addition of 1 N HCl, followed by neutralization using 1.2 N NaOH/0.5M HEPES. Serially diluted standards, controls and activated samples were incubated on the microplate for 2 h at room temperature on an orbital shaker. This was followed by thorough washing and the addition of HRP-conjugated polyclonal antibody specific to TGF β1. After 2 h of incubation and subsequent washing, the substrate solution was added to each well. The plate was protected from light and incubated for 30 min at room temperature so that color developed in proportion to the amount of TGF β1 bound in the initial step. The reaction

was terminated by adding the stop solution. The optical density was measured within 30 min using a microplate reader set to 450 nm with a wavelength correction set at 540 nm.

Construction of adenovirus vectors

A new pK4-shRNA vector, developed in our lab, was used to silence Smad4. The single vector expresses four short hairpin RNA (shRNA) driven by four different promoters after transfecting into cells. The details for the construction of the new vector will be published elsewhere. The four small interfering RNA (siRNA) sequences for Smad4 were:

5'-GGTGGAGAGAGTGAGACATT-3' (85-104),

5'-GCGTCTGTGTGAACCCATATC-3'(374-394),

5'-GGAATTGATCTCTCTGGATTA-3'(418-428)

5'-GGAGTGCAGTTGGAGTGTAA A-3' (1156-1176).

A vector contain four non-relevant siRNA sequences was used as a control. The sequences were:

5’-ACGTGACAC GTTCGGAGAATT-3’,

5’-GATCGTGATAACC TAGCTGTC-3’,

5’-ATGCTTGACGCAGCTAAC GCA-3’,

5’-GGCTAACTACGCACAGTAAGT-3’.

The expression cassettes were ligated into pENTR vectors (Invitrogen) using the Invitrogen Gateway® technology. They were switched to the adenoviral vectors by LR recombinations. The viral vectors were transfected into 293A cells to produce the first generation of virus. After amplification, the second generation of viruses was used to

infect target cells. The virus with Smad4 silencing sequence was named AdSmad4Si and the control with the four non-relevant sequences AdCon.

Statistical analysis

Data was expressed as means ± SE. Statistical analysis was performed by using one way ANOVA using the GraphPad Prism software, followed by Dunnett's Multiple Comparison Test. The significance was assigned at $p < 0.05$.

RESULTS

TGF β1 and Smad4 were highly expressed in AEC II.

To identify cellular localization of TGF β1 and Smad4, double-immunolabeling was performed on perfused normal rat lung tissue sections by using monoclonal antibodies for TGF β1 or Smad4, and goat polyclonal antibody for SP-C (an AEC II marker). The results revealed a strong co-localization of both proteins with SP-C in AEC II (Fig. 1). The negative controls with only secondary antibodies did not show any signals. TGF β1 and Smad4 were also present to a lesser extent in AEC I. A high expression of TGF β1 was also seen in alveolar macrophages and bronchial epithelial cells.

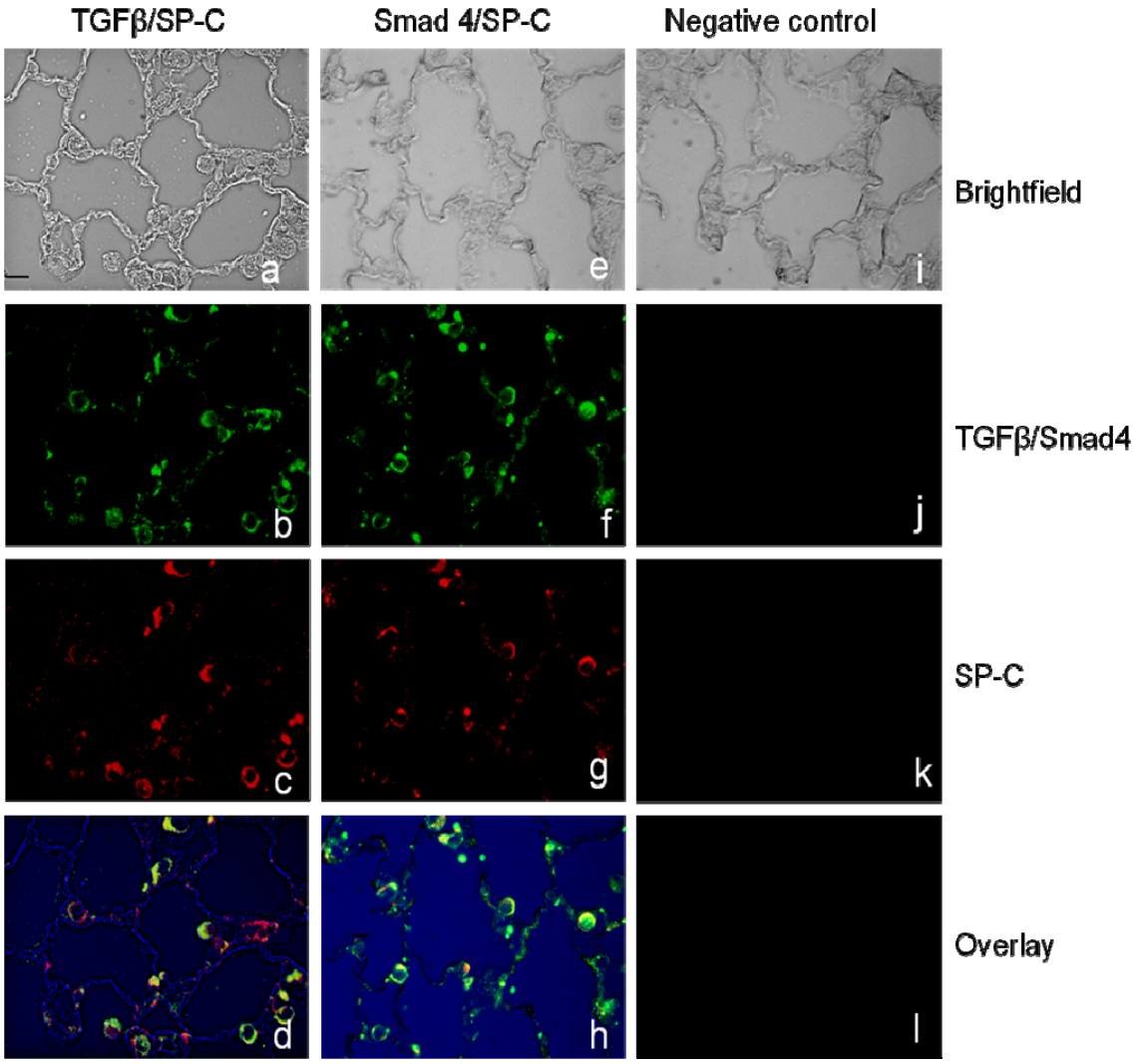

Figure 1 - Immunohistochemistry of rat lung tissue sections for TGF β1 and Smad4.

Double labeling of rat lung tissue sections was done using anti-TGF β1 and anti-SP-C antibodies *(Panels a-d)* or anti-Smad4 and anti-SP-C antibodies *(Panels e-h)*. This was followed by incubation with the corresponding Alexa 488- or 568-conjugated secondary antibodies. Panel *d* shows the overlay between TGF β1 and SP-C signals while *panel h* shows the overlay between Smad4 and SP-C. The negative control was without primary antibodies *(Panels i -l)*. Scale bars: 5 µm.

AEC II proliferated and then differentiated to AEC I-like cells in the *in vitro* trans-differentiation model.

It is well known that AEC II gradually trans-differentiate into AEC I-like cells when cultured on plastic dishes (Paine and Simon, 1996; Williams, 2003). However, the kinetics of cell proliferation and differentiation during this process has not been examined. The trans-differentiation of AEC II to AEC I-like cells was confirmed by immunostaining with AEC II and AEC I markers, LB-180 for AEC II and P2X7 for AEC I (Chen et al., 2004b; Zen et al., 1998). On day 1, LB-180 expression was strong with no expression of P2X7, but it disappeared on day 3 and day 5 along with the concurrent appearance of P2X7 (Fig. 2A). This indicates that the trans-differentiation occurs between day 3 and day 5. Some day 3 cells showed the expression of both AEC II and AEC I markers, demonstrating the possibility of an intermediate cell type. The BrdU staining experiments showed that AEC II proliferation increased from day 1 to day 2 and decreased thereafter (Fig. 2B). This suggests that there is a proliferation phase (day 0 to day 2) which precedes the differentiation phase (day 3 to day 5) in the *in vitro* model.

Figure 2

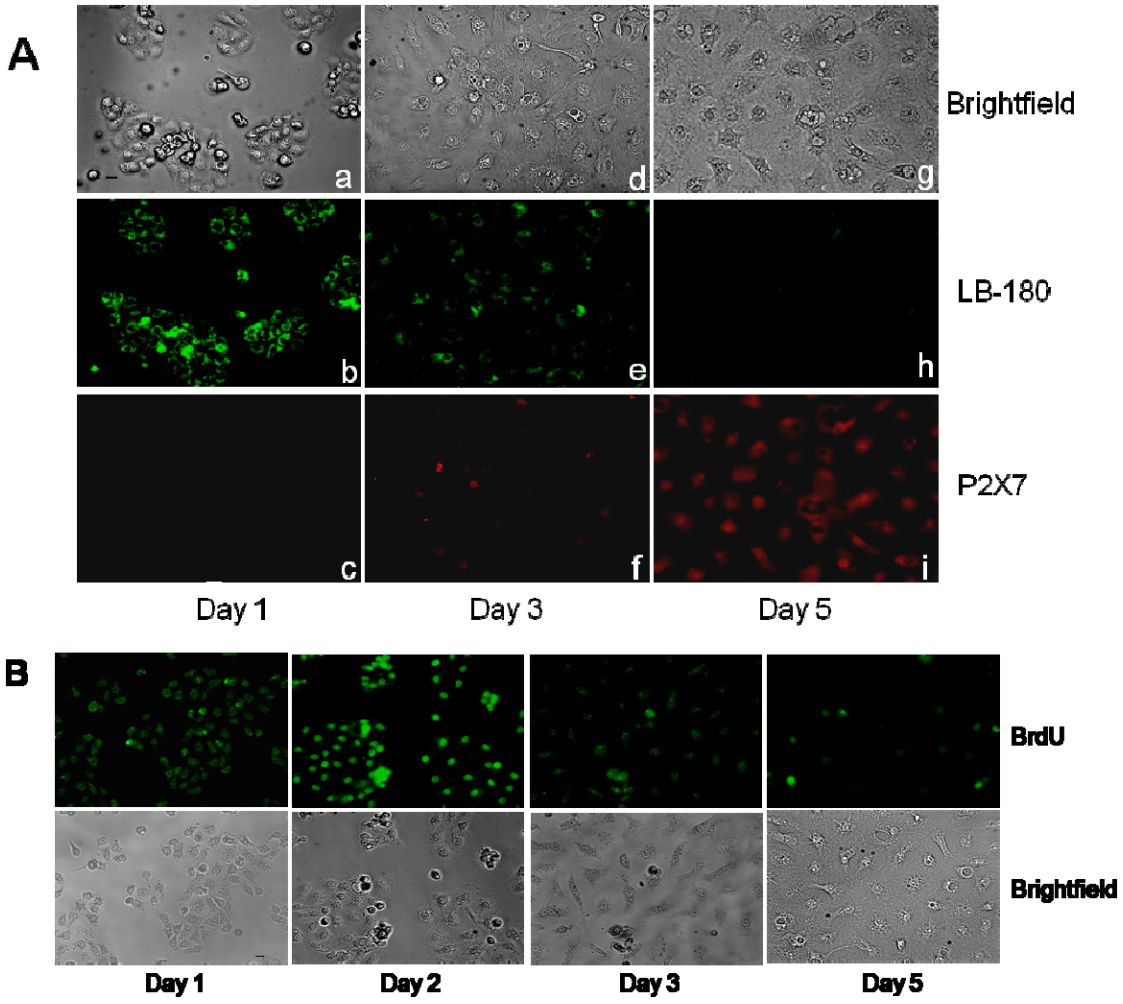
A
a
d
g
Brightfield
b
e
h
LB-180
c
f
i
P2X7
Day 1
Day 3
Day 5
B
BrdU
Brightfield
Day 1
Day 2
Day 3
Day 5

Figure 2 - Expression of AEC I and AEC II markers and BrdU labeling during the trans-differentiation

A. Double labeling of the cultured cells on days 1, 3 and 5 was done using anti-LB-180 (panels b, e and h) and anti-P2X7 antibodies (panels c, f and i). The protein expression was visualized using corresponding Alexa 488- or 568-conjugated secondary antibodies.

B. BrdU labeling: BrdU was added at a concentration of 10 μM to the culture media 12-14 hours before cell fixing. Immunostaining was done on the cells cultured for 1-5 days using anti-BrdU antibody and Alexa 488-conjugated secondary antibody. Scale bar: 5 μm.

mRNA and protein expression levels of TGF β1 and Smads were altered during the trans-differentiation from AEC II to AEC I-like cells.

Since there is a proliferation phase immediately following AEC II isolation and culture, we hypothesized that the expression of TGF β1 and its down stream components, R-Smads would be lower during the proliferation phase, but increase during the differentiation phase so as to facilitate the cell cycle exit. We therefore examined the mRNA and protein expression levels of TGF β1 and Smads in the *in vitro* trans-differentiation model by real time PCR and Western blotting. (The TGF β1 mRNA expression level was low during proliferation, but increased significantly during the later stage of differentiation (Fig. 3A). The same trend was observed at the protein level (Fig. 3B).

Figure 3

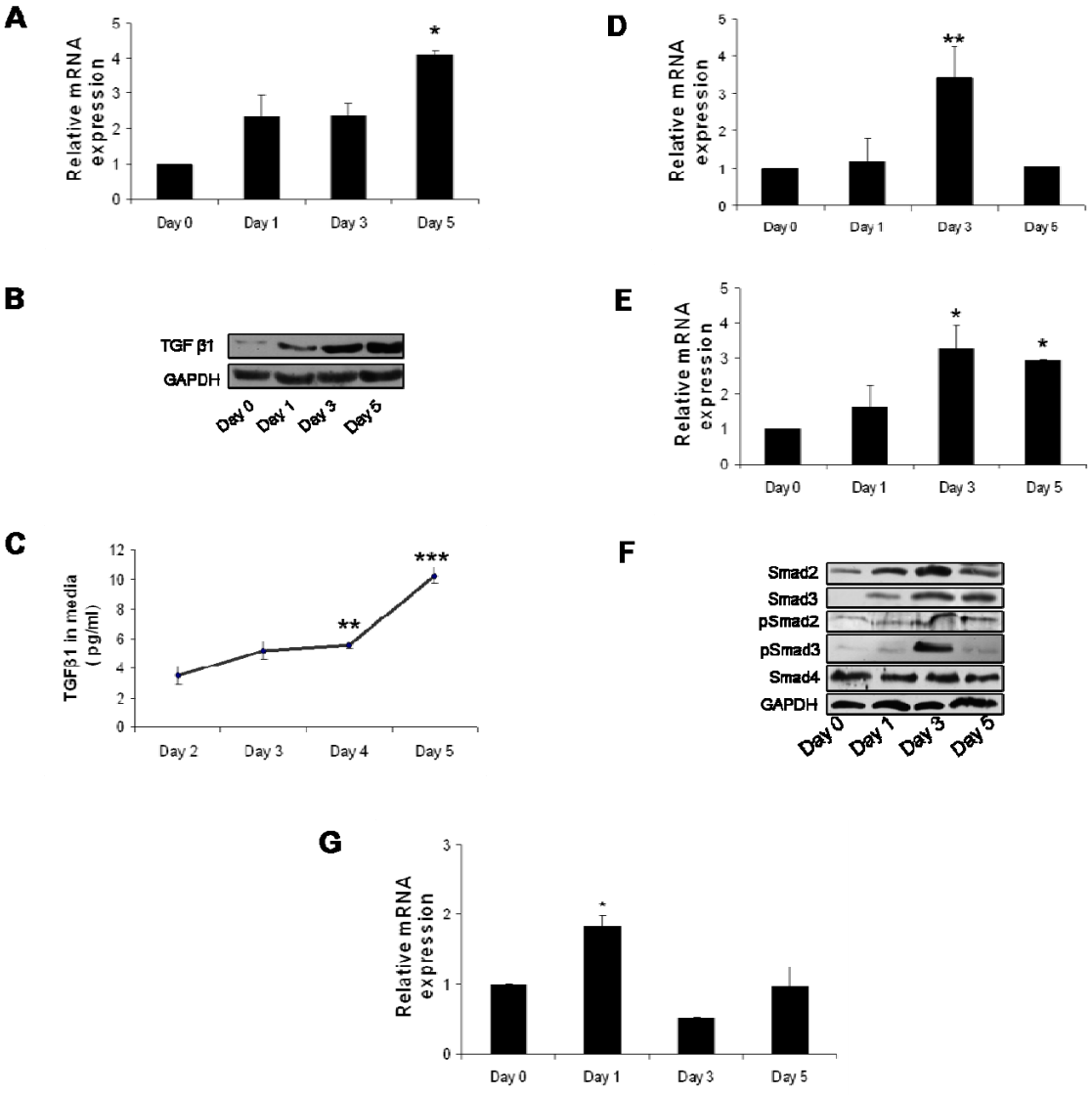
A
Relative mRNA expression
Day 0
Day 1
Day 3
Day 5
B
TGF β1
GAPDH
C
TGFβ1 in media (pg/ml)
Day 2
Day 3
Day 4
Day 5
D
E
F
Smad2
Smad3
pSmad2
pSmad3
Smad4
GAPDH
G

Figure 3 - The expression and secretion of TGF β1 during trans-differentiation. AEC II were seeded on plastic dishes and cultured for 1-5 days. Total RNA was extracted and reverse-transcribed to cDNA. Real-time PCR was done to determine the mRNA abundance of TGF β1 (*Panel A*), Smad2 (*Panel D*), Smad3 (*Panel E*) and Smad6 (Panel G). Data was normalized to 18S RNA and expressed as a ratio to the day 0 value. The error bars represent standard errors. Absence of an error bar denotes very low standard error value. *P<0.05 v.s. day 0, **P<0.01 v.s. day 0, n=3 independent cell preparations, each assay performed in duplicates. The protein levels of TGF β1 (*Panel B*), Smad2, Smad3, Smad4, and phosphorylated Smad2 and Smad3 (pSmad2 and pSmad3) (Panel F) were detected by Western blot using corresponding antibodies. GAPDH was used as the loading control. The TGF β1 secretion into the media during a 24 h period was determined by sandwich ELISA (*Panel C*). AEC II were cultured for 1-5 days. The medium was changed every day. At the indicated time points the media was collected and TGF β1 content was measured. The error bars represent standard errors.

**P<0.05 v.s. day 2, ***P<0.01 v.s. day 2, n=3 independent cell preparations, each assay performed in duplicates.

TGF β1 activates its signal transduction pathway by binding to the specific TβRII-TβRI receptor complex on the extracellular side of the cell membrane. We therefore investigated whether TGF β1 was secreted from the cultured cells as they trans-differentiated. AEC II were cultured in MDSF media, the media collected on days 2, 3, 4 and 5 and the TGF β1 concentration was determined by sandwich ELISA. TGF β1 secretion into the surrounding media increased significantly during trans-differentiation and corresponded with the increase in TGF β1 production within the cells (Fig. 3C). The same experiment, when repeated in MEM + 10% FBS, yielded similar results (data not shown).

The mRNA expression of Smad2 and Smad3 remained lower during the proliferation phase and increased on day 3 as differentiation became evident (Fig. 3 D and E). This almost corresponded to their protein levels (Fig. 3F). Since the presence of their phosphorylated form would be a better indicator of the activation of the Smad-dependent TGF β pathway, we further determined the phosphorylation of Smad2 and Smad3 using antibodies that only recognized the phosphorylated form of Smad2 or Smad3. As expected, the phosphorylation of both Smad2 and Smad3 were increased during differentiation (Fig. 3F), indicative of active TGF β1 signaling. The expression level of Co-Smad, Smad4 protein was constant from day 0 to day 5(Fig. 3F).

The mRNA expression levels of I-Smads, Smad6 and Smad7, were also examined. Smad7 mRNA level did not show significant changes (data not shown), but Smad6 expression was higher during the proliferation phase (Fig. 3G).

The expression of cell cycle related proteins, p15, p21, CDK2, CDK4 and CDK6 were altered during the trans-differentiation.

TGF β mediates its action by altering the levels of cell cycle proteins that are involved in the G1 to S phase transition before the restriction point. TGF β increases the expression of two cyclin-dependent kinase inhibitors, p15 (Hannon and Beach, 1994) and p21 (Datto et al., 1995). The latter, in turn, inhibit the activity of CDK2, CDK4 and CDK6, which are required for the progression of the cell cycle. We examined the relative mRNA expression of p15 and p21 by real time PCR. Their expression remained low from day 0 to day 3 and increased significantly as the differentiation progressed (Fig. 4 A, B). These results were verified at the protein level by western blot (Fig. 4C). The protein levels of CDK2, CDK4 and CDK6 showed an opposite trend of expression (Fig. 4C)

Figure 4

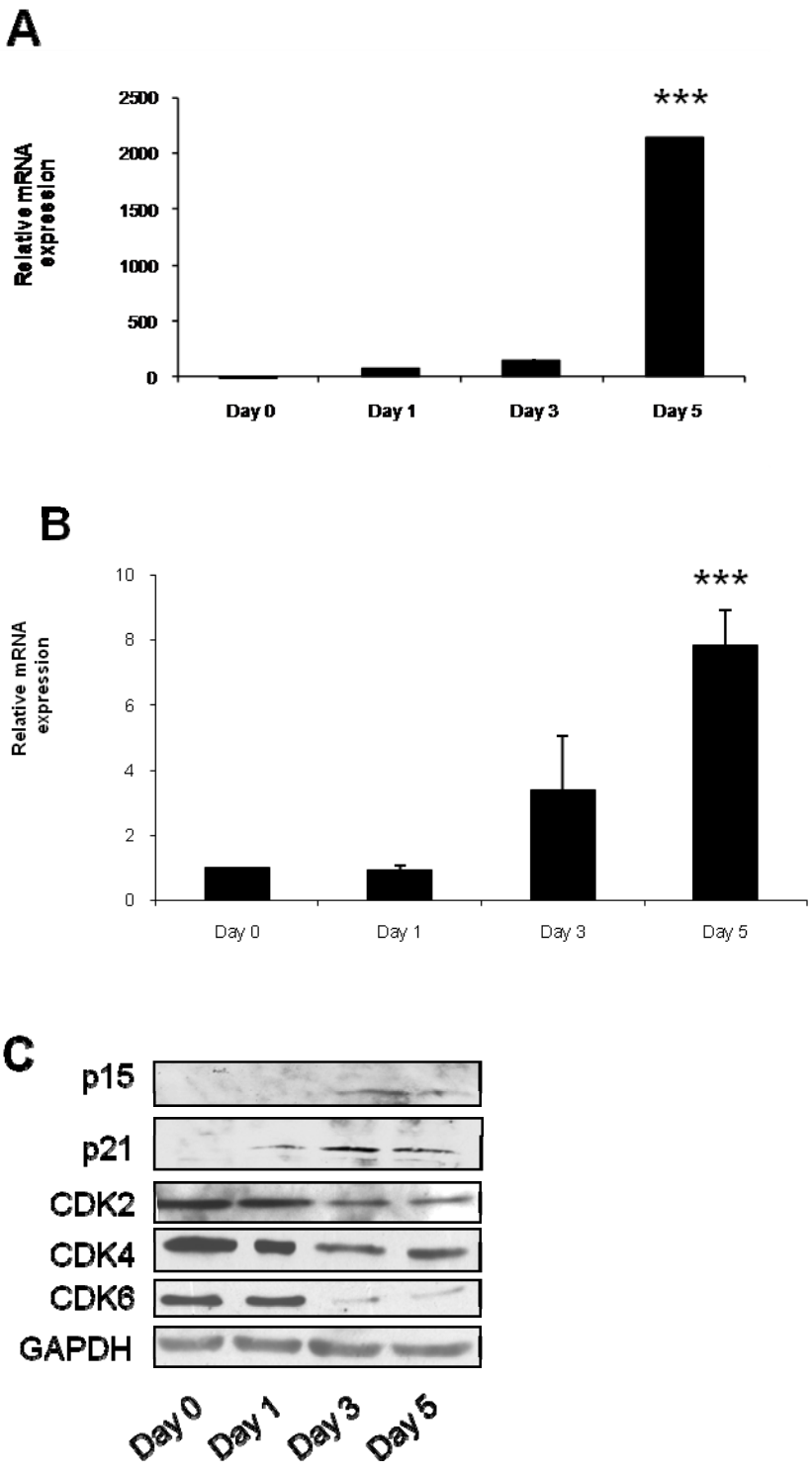
A
Relative mRNA expression
2500
2000
1500
1000
500
0
***
Day 0
Day 1
Day 3
Day 5
B
Relative mRNA expression
10
8
6
4
2
0
***
Day 0
Day 1
Day 3
Day 5
C
p15
p21
CDK2
CDK4
CDK6
GAPDH
Day 0
Day 1
Day 3
Day 5

Figure 4 - The expression of cell cycle inhibitors, p15 and p21, and CDK2, CDK4 and CDK6 during trans-differentiation.

A & B - RNA levels of p15 and p21: AEC II were seeded on plastic dishes and cultured for 1-5 days. Total RNA was extracted from the cultured cells collected on day 0, 1, 3 and 5 and reverse-transcribed to cDNA. Real-time PCR was done to determine the mRNA abundance of p15 (A) and p21. Data was normalized to 18S RNA and expressed as a ratio to the day 0 value. The error bars represent standard errors. Absence of an error bar denotes very low standard error value. *P<0.05 v.s day 0, ***P<.001 v.s. day 0, n=3 biological preparations, each assay performed in duplicates.

C - p15, p21, CDK2, CDK4 and CDK6 protein levels were detected by Western blot using corresponding antibodies. A representative GAPDH, used as the loading control, is shown in the lowest panel.

This low expression of cell cycle inhibitors during the proliferation and their increase during differentiation with an opposite expression trend of CDKs further strengthen our view that these two phases of cell events may be mediated by the endogenous TGF β1 released from cultured cells in this model.

Addition of exogenous TGF β1 prior to proliferative phase blocked both proliferation and trans-differentiation of AEC II while its addition after the proliferative phase did not affect the trans-differentiation.

The anti-proliferative effect of TGF β1 on AEC II has been documented previously (Zhang et al., 2004). We investigated the effects of addition of exogenous TGF β1 prior to and after the proliferation phase in our trans-differentiation culture model. For this purpose, AEC II were cultured for 5 days in the presence of recombinant TGF β1 at a concentration of 40 ng/ml from day 0 in one group (pre-proliferative addition) and from the end of day 2 in another group (post-proliferative addition). A control group with no additions was also prepared. The phenotypes of the cells on day 3 and day 5 were determined by double- labeling with AEC II and AEC I markers, LB 180 and P2X7, respectively (*Fig. 5*). When compared to the control (*Fig. 5 panels a-c and m-o*), the group with pre-proliferative addition of TGF β1 showed reduced proliferation and differentiation (Fig. 5 panels d-f and p- r). The cell counting revealed a 54% reduction in the cell number on day 3. The LB-180 expression in this group persisted even on day 5 showing that they retained the lamellar bodies and AEC II phenotype. Also, the AEC I marker (P2X7) expression was absent in this group indicating a failure to trans-differentiate to AEC I-like cells. A dose experiment (4, 10, 20, and 40 ng/ml) showed that TGF β1 as low as 4 ng/ml if added at the pre-proliferative phase can still retain AEC II

phenotype although the number of LB-180-positive cells appears to be higher at a higher concentration of TGF β1 (Data not shown). On the other hand, in the cells subjected to post-proliferative addition of TGF β1, normal trans-differentiation process was seen as in the untreated control with gradual loss of LB-180 expression and subsequent appearance of P2X7 expression (Fig. 5 panels g-I and s-u). The results indicate that for differentiation to take place there must be a preceding proliferative phase. Maybe that is the reason why TGF β1 and related Smad expression increase after the proliferative phase when cells are ready for differentiation. To further clarify this view, we added an excess of neutralizing antibody to TGF β1 at the end of day 2 (post-proliferative addition). The antibody binds with the endogenous TGF β1 and makes it unavailable to bind with the receptors and to initiate Smad-mediated signaling. The cells retained the AEC II phenotype and failed to trans-differentiate properly when the endogenous TGF β1 was blocked. They retained the LB-180 expression and failed to express P2X7 on day 3 and day 5 (Fig. 5 panels j-l and v-x). These experiments demonstrate that altered signaling through TGF β1 is important in the trans-differentiation of AEC II to AEC I and that the initial proliferation phase may be essential for subsequent differentiation process.

Figure 5

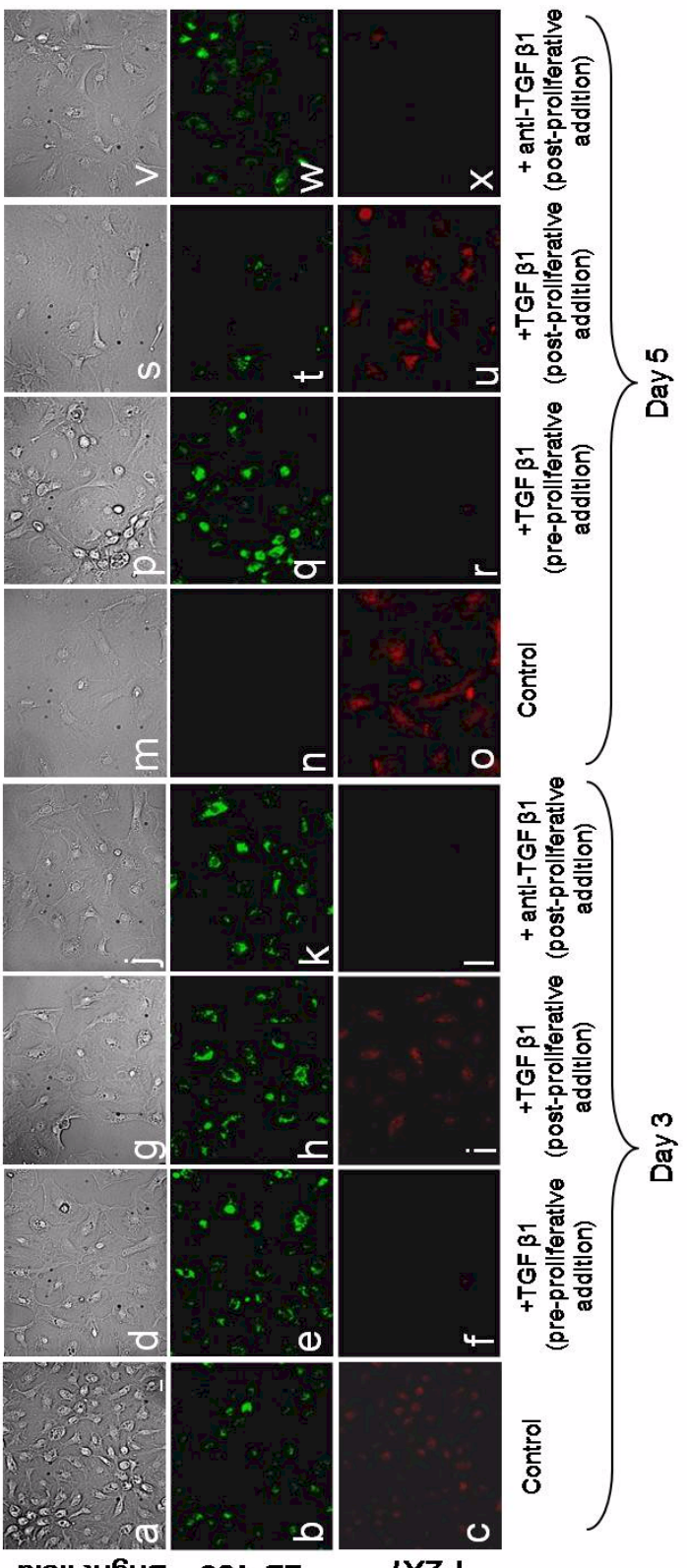
Bright field
LB-180
P2X7
Control
+TGF β1 (pre-proliferative addition)
+TGF β1 (post-proliferative addition)
+ anti-TGF β1 (post-proliferative addition)
Control
+TGF β1 (pre-proliferative addition)
+TGF β1 (post-proliferative addition)
+ anti-TGF β1 (post-proliferative addition)
Day 3
Day 5
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
w
x

Figure 5 - Effect of pre-proliferative and post-proliferative addition of exogenous TGF β1 and anti-TGF β1 antibody on AEC II trans-differentiation.

AEC II were cultured for 3 or 5 days. Recombinant TGF β1 (40 ng/ml) was added at day 0 (pre-proliferative addition) (panels d-f,and p-r) in one group and at the end of day 2 (post-proliferative addition) (panels g-I and s-u) in another. A third group was post-proliferative addition of anti-TGF β1 antibody at a concentration of (40 μg/ml) (panels j-l and v-x). The control was without any additions (panels a-c, m-o). Cells were fixed on days 3 and 5 and were double-labeled with anti-LB-180 (AEC II marker) and anti-P2X7 (AEC I marker). Scale bar: 7 μm.

Silencing of Smad4 by RNAi prevented the trans-differentiation.

Because the previous experiment involved exogenous addition of a higher quantity of TGF β1 which may not reflect the normal physiological conditions, we decided to block the Smad pathway within the cell by knocking down Smad4 expression using RNAi. Smad4 was chosen because it is the common partner Smad that binds with Smad2 and Smad3 and is an essential component of Smad-dependent TGF β signaling. Adenovirus-based RNAi targeted to Smad4 (AdSmad4Si) was used to silence Smad4 in AEC II. The viral control (AdCon) contained non-relevant siRNA sequences. AEC II were treated with the Adenoviruses on day 1 and Smad4 protein expression on day 3 and day 5 was determined using western blot. As seen in Fig. 6A, there was a significant reduction in the Smad4 expression by day 3 and complete silence was observed by day 5.

Figure 6

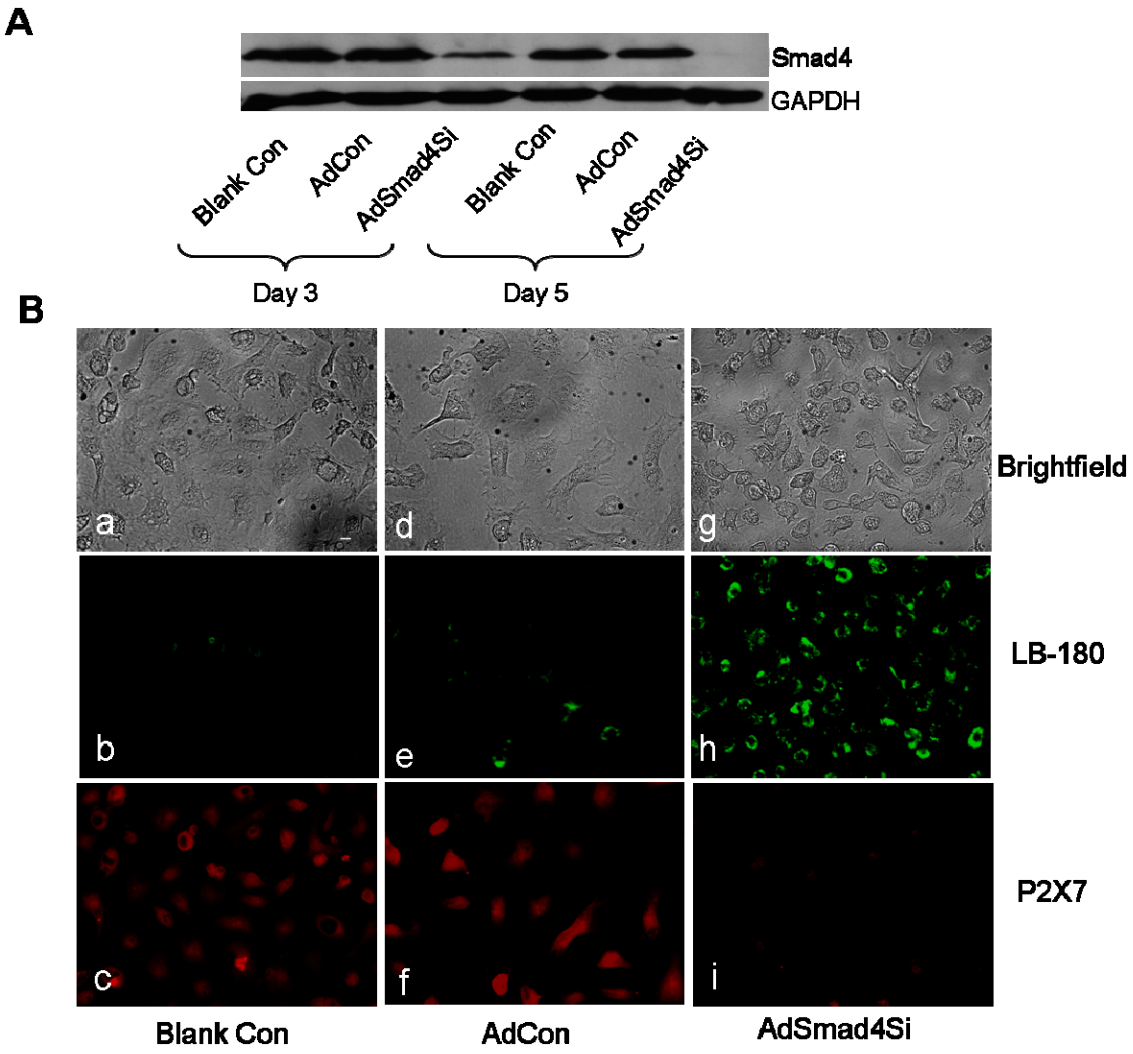
A
Smad4
GAPDH
Blank Con
AdCon
AdSmad4Si
Blank Con
AdCon
AdSmad4Si
Day 3
Day 5
B
a
d
g
Brightfield
b
e
h
LB-180
c
f
i
P2X7
Blank Con
AdCon
AdSmad4Si

Figure 6 - Effect of Smad4 silencing on trans-differentiation of AEC II to AEC I-like cells

A. After 24 h of plating, AEC II were transduced with Adenoviruses carrying siRNAs targeted to Smad4 (AdSmad4Si) or non-relevant siRNAs (AdCon). The blank control was without any additions. Multiplicity of Infection (MOI) used was 7 for Adsmad4Si and 36 for AdCon. Total protein was extracted on day 3 and day 5 and Western blots for Smad4 were performed. GAPDH was used as the loading control.

B. The effect of Smad4 silencing on trans-differentiation: Double labeling using anti-LB-180 and anti-P2X7 antibodies was done on AEC II treated for 5 days with AdSmad4si (*panels g, h and i*) and AdCon (*panels d, e and f*).

The consequences of silencing Smad4 on the trans-differentiation of AEC II were then examined by monitoring cell phenotype changes. The cells treated with AdSmad4Si retained the AEC II marker (LB-180) expression and failed to express the AEC I marker (P2X7) on day 5. The untreated and AdCon-treated cells trans-differentiated to AEC I-like cells and showed the P2X7 expression and concurrent disappearance of LB-180 (Fig. 6B). There was a visible difference in cell phenotypes; the AdSmad4Si-treated cells were rounder and smaller than the control cells.

DISCUSSION

In the cells possessing the capability to proliferate and differentiate, there is substantial coupling between these processes. The quiescence of AEC II in normal alveoli and their proliferation and trans-differentiation into AEC I in injured lung illustrates how differential signaling through the same TGF β1 pathway might be responsible for controlling both processes.

TGF β1 has been reported to be expressed in alveolar epithelial cells under pathological conditions. For example, TGF β1 has been observed in hypertrophic AEC II and in fibrotic areas immediately beneath them (Broekelmann et al., 1991; Corrin et al., 1994b; Khalil et al., 1996). However, TGF β1 expression in normal AEC II has not been reported previously. Our immunostaining results showed that TGF β1 and its down stream component, Smad4 were expressed in AEC II of normal lung. These proteins were also detectable in AEC I although to a less extent. TGF β1 signaling may play a role in keeping the AEC II quiescent in normal lung.

TGF β1 can be synthesized and secreted by isolated AEC II. The protein level of TGF β1 was increased as AEC II trans-differentiated to AEC I-like cells *in vitro* as determined by Western blot. The reasons for apparent low expression of TGF β1 in AEC I in the intact lung and its high expression in the trans-differentiated AEC I-like cells *in vitro* are unclear. However, there are several possibilities: (i) AEC I are very thin and squamous cells that cover ~95% of alveolar surface while AEC II are cubodial cells that occupy only ~5% of the surface even though the number of type II cells is twice as many as type I cells in the lung. Therefore, the signal for TGF β1 immunostaining is weak compared

to type II cells because of the shape of the cells; (ii) trans-differentiated AEC I *in vitro* may not reflect all the properties of the normal AEC I in vivo (Gonzalez et al., 2005). The changes in the cellular environment may alter its expression.

A novel finding in the current study is the identification of a proliferation phase preceding the differentiation phase in the *in vitro* trans-differentiation cell culture model. The appearance of AEC I-like cells by day 3 and subsequent differentiation was confirmed by differential labeling using established AEC II (LB-180) and AEC I (P2X7) markers. The previous studies have demonstrated the presence of various AEC I markers in these trans-differentiated cells, indicating that they are indeed similar to AEC I (Borok et al., 1998b; Borok et al., 1998a; Danto et al., 1995; Dobbs et al., 1988). Although this differentiation pattern has been observed previously, the initial proliferation phase has not been reported. BrdU labeling, which is indicative of active DNA synthesis preceding cell division, was found to be the highest on day 2. The expression pattern of the cell cycle inhibitors and CDKs, reflective of the cell cycle stages substantiated this biphasic cell proliferation and differentiation.

AEC I are the most susceptible to injury in alveolar epithelium. After lung injury, AEC I are destroyed and basement membrane denuded and AEC II proliferate and differentiate into AEC I to repair the epithelium (Adamson and Bowden, 1974; Evans et al., 1973; Narasaraju et al., 2006; Uhal, 1997). Therefore, this *in vitro* trans-differentiation model may closely relate to the process taking place in vivo during lung injury and repair. It should also be noted that isolating of AEC II from the lung and culturing them may mimic lung injury and repair. The loss of contact of AEC II with the surrounding cells

after isolation may initiate AEC II proliferation and then differentiate into AEC I-like cells.

TGF β can be a positive or a negative regulator of cell proliferation and differentiation (Huang and Huang, 2005). This depends on cell type and the physiological or pathological stage in which the cells are. The presence of other growth factors and signaling cascades also determines how TGF β acts at a particular stage of growth and differentiation. For example, TGF ß inhibits myogenic differentiation (Massague et al., 1986), but promotes differentiation in mammary epithelium (Yamamoto et al., 1994). TGF β1 can inhibit the proliferative effects of KGF on cultured AEC II (Zhang et al., 2004). During the recovery of hyperoxia-exposed AEC II, there is a proliferative phase marked by an escape from the negative autocrine regulation by TGF β3 (Buckley et al., 1996). However, whether TGF ß has the pro-differentiation effects on AEC is unknown.

The TGF β1, produced inside AEC, is secreted out as the trans-differentiation takes place and thus regulates this process in an autocrine manner. The secretion of TGF ß 1 was also observed in alveolar macrophages and fibroblasts from fibrotic lungs (Khalil et al., 1993; Venkatesan et al., 2002). An autocrine loop of TGF β1 in pregnant mouse mammary epithelium has been shown to aid the differentiation process (Yamamoto et al., 1994).

When AEC II trans-differentiated into AEC I-like cells *in vitro*, we found that the changes in expression of TGF β1 and R-Smads, Smad2 and Smad3 were biphasic: lower in the proliferation phase and higher in the differentiation phase. The increased phosphorylation of Smad2 and Smad3, a hallmark of activation of the TGF β pathway, showed a similar trend. However, an opposite trend of expression of Smad6, an inhibitory

Smad was observed. The high expression of Smad6 during the proliferation phase may further inhibit the TGF ß signaling and ensures the initial proliferation occur before the differentiation. Although Smad6 is a inhibitor of BMP signaling through its binding with the activated Smad1 (Hata et al., 1998), overexpression of Smad6 also blocked the TGF β signaling (Imamura et al., 1997). The results suggest that TGF ß signaling is depressed during the proliferation phase and activated during the differentiation phase.

TGF β can regulate cell cycle proteins involved in the G1 to S phase entry (Massague and Gomis, 2006). The activated Smad complexes caused by TGF β result in the transcriptional repression of the c-myc gene (Chen et al., 2001; Frederick et al., 2004; Pietenpol et al., 1990). Concurrent induction of two cell cycle inhibitors, p15 (Hannon and Beach, 1994) and p21 (Datto et al., 1995), inhibits the activity of CDK2, CDK4 and CDK6, which are required for the progression of the cell cycle. The expression profile of p15 and p21 during the trans-differentiation of AEC matched that of TGF β1 and R-Smads. However, protein levels of CDKs 2, 4 and 6 showed an opposite trend. These results suggest that the coordinated TGF ß signaling through cell cyle points plays a role in the temporal regulation of cell cycle entry and exit by the cells.

TGF β1, when added from day 0, stopped AEC II proliferation and later differentiation. The addition of TGF β1 must have aided in stopping the cell cycle during proliferative phase. This indicates that for differentiation to take place there must be a proliferative phase. This view was substantiated by the fact that the cells were refractory to the above stated effect of TGF β1 when it was added after the proliferative phase where normal differentiation ensued. Also the addition of excess of anti-TGF β1 antibody to block the

endogenous TGF β1 secreted caused the differentiation to stop, indicating that TGF β1 is essential for the differentiation process.

The effect of exogenous TGF β1 may be quite different from that which is secreted during a particular stage of growth and differentiation at normal physiological levels. So we wanted to block the pathway within the cell. Also since Smad-independent signaling has been reported for TGF β1, we wanted to make sure that signaling is indeed through the Smad pathway. To achieve these aims, the common mediator for Smad-mediated TGF β signaling, Smad4, was silenced using RNAi. The results have clearly demonstrated a halting of the trans-differentiation process in the silenced group. Additionally, these results validated our view that signaling through this pathway is essential for the differentiation of AEC II to AEC I to occur.

Our current studies suggest bifunctional roles of TGF β1 during the trans-differentiation of AEC II to AEC I: inhibit the proliferation of AEC II at the proliferative phase and promote their differentiation into AEC I at the differentiation phase. Once the proliferative phase is finished, endogenous TGF β1 induces the differentiation. This is supported by (i) the production of TGF β1 and the phosphorylation of Smad2/3 are induced at the differentiation phase; and (ii) TGF β1 antibodies if added at the end of the proliferative phase or silencing of Smad4 block the trans-differentiation of AEC II to AEC I.

Since our model closely mimics the proliferation and trans-differentiation process that takes place in the lung during acute injury, understanding the signaling mechanism

involved in this process may help to identify the components involved as potential targets for rational therapeutics.

REFERENCES

1. Adamson, I. Y. and Bowden, D. H. (1974). The type 2 cell as progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen. Lab Invest. 30, 35-42.

2. Atfi, A., Djelloul, S., Chastre, E., Davis, R. and Gespach, C. (1997). Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor beta-mediated signaling. J. Biol. Chem. 272, 1429-1432.

3. Borok, Z., Danto, S. I., Lubman, R. L., Cao, Y., Williams, M. C. and Crandall, E. D. (1998a). Modulation of t1alpha expression with alveolar epithelial cell phenotype *in vitro*. Am. J. Physiol. 275, L155-L164.

4. Borok, Z., Lubman, R. L., Danto, S. I., Zhang, X. L., Zabski, S. M., King, L. S., Lee, D. M., Agre, P. and Crandall, E. D. (1998b). Keratinocyte growth factor modulates alveolar epithelial cell phenotype *in vitro*: expression of aquaporin 5. Am. J. Respir. Cell Mol. Biol. 18, 554-561.

5. Broekelmann, T. J., Limper, A. H., Colby, T. V. and McDonald, J. A. (1991). Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. Proc. Natl. Acad. Sci. U S. A 88, 6642-6646.

6. Buckley, S., Bui, K. C., Hussain, M. and Warburton, D. (1996). Dynamics of TGF-beta 3 peptide activity during rat alveolar epithelial cell proliferative recovery from acute hyperoxia. Am J Physiol 271, L54-L60.

7. Chen, C. R., Kang, Y. and Massague, J. (2001). Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. Proc. Natl. Acad. Sci. U S. A 98, 992-999.

8. Chen, J., Chen, Z., Narasaraju, T., Jin, N. and Liu, L. (2004a). Isolation of highly pure alveolar epithelial type I and type II cells from rat lungs. Lab Invest. 84, 727-735.

9. Chen, Z., Jin, N., Narasaraju, T., Chen, J., McFarland, L. R., Scott, M. and Liu, L. (2004b). Identification of two novel markers for alveolar epithelial type I and II cells. Biochem. Biophys. Res. Commun. 319, 774-780.

10. Corrin, B., Butcher, D., McAnulty, B. J., Dubois, R. M., Black, C. M., Laurent, G. J. and Harrison, N. K. (1994). Immunohistochemical localization of transforming growth factor-beta 1 in the lungs of patients with systemic sclerosis, cryptogenic fibrosing alveolitis and other lung disorders. Histopathology 24, 145-150.

11. Danto, S. I., Shannon, J. M., Borok, Z., Zabski, S. M. and Crandall, E. D. (1995). Reversible transdifferentiation of alveolar epithelial cells. Am J Respir. Cell Mol Biol 12, 497-502.

12. Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y. and Wang, X. F. (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc. Natl. Acad. Sci. U S. A 92, 5545-5549.

13. Derynck, R. and Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425, 577-584.

14. Dobbs, L. G., Williams, M. C. and Gonzalez, R. (1988). Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. Biochim Biophys Acta 970, 146-156.

15. Evans, M. J., Cabral, L. J., Stephens, R. J. and Freeman, G. (1973). Renewal of alveolar epithelium in the rat following exposure to NO2. Am. J. Pathol. 70, 175-198.

16. Fehrenbach, H. (2001). Alveolar epithelial type II cell: defender of the alveolus revisited. Respir Res 2, 33-46.

17. Feng, X. H. and Derynck, R. (2005). Specificity and versatility in tgf-beta signaling through Smads. Annu. Rev. Cell Dev. Biol. 21, 659-693.

18. Frederick, J. P., Liberati, N. T., Waddell, D. S., Shi, Y. and Wang, X. F. (2004). Transforming growth factor beta-mediated transcriptional repression of c-myc is dependent on direct binding of Smad3 to a novel repressive Smad binding element. Mol Cell Biol. 24, 2546-2559.

19. Gonzalez, R., Yang, Y. H., Griffin, C., Allen, L., Tigue, Z. and Dobbs, L. (2005). Freshly-isolated rat alveolar type I cells, type II cells, and cultured type II cells have distinct molecular phenotypes. Am J Physiol Lung Cell Mol Physiol 288, L179-L189.

20. Hannon, G. J. and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature 371, 257-261.

21. Hata, A., Lagna, G., Massague, J. and Hemmati-Brivanlou, A. (1998). Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. Genes Dev. 12, 186-197.

22. Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y. Y., Grinnell, B. W., Richardson, M. A., Topper, J. N., Gimbrone, M. A., Jr., Wrana, J. L. et al. (1997). The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. Cell 89, 1165-1173.

23. Huang, S. S. and Huang, J. S. (2005). TGF-beta control of cell proliferation. J Cell Biochem. 96, 447-462.

24. Iavarone, A. and Massague, J. (1997). Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. Nature 387, 417-422.

25. Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J., Kawabata, M. and Miyazono, K. (1997). Smad6 inhibits signalling by the TGF-beta superfamily. Nature. 389, 622-626.

26. Izzi, L. and Attisano, L. (2004). Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. Oncogene 23, 2071-2078.

27. Khalil, N., O'Connor, R. N., Flanders, K. C. and Unruh, H. (1996). TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. Am. J. Respir. Cell Mol. Biol. 14, 131-138.

28. Khalil, N., Whitman, C., Zuo, L., Danielpour, D. and Greenberg, A. (1993). Regulation of alveolar macrophage transforming growth factor-beta secretion by corticosteroids in bleomycin-induced pulmonary inflammation in the rat. J. Clin. Invest. 92, 1812-1818.

29. Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A. K. and Flavell, R. A. (2006). Transforming growth factor-beta regulation of immune responses. Annu. Rev. Immunol. 24:99-146., 99-146.

30. Massague, J. (2000). How cells read TGF-beta signals. Nat. Rev. Mol. Cell Biol. 1, 169-178.

31. Massague, J., Cheifetz, S., Endo, T. and Nadal-Ginard, B. (1986). Type beta transforming growth factor is an inhibitor of myogenic differentiation. Proc. Natl. Acad. Sci. U S. A 83, 8206-8210.

32. Massague, J. and Gomis, R. R. (2006). The logic of TGFbeta signaling. FEBS Lett. 580, 2811-2820.

33. Massague, J., Seoane, J. and Wotton, D. (2005). Smad transcription factors. Genes Dev. 19, 2783-2810.

34. Moustakas, A., Souchelnytskyi, S. and Heldin, C. H. (2001). Smad regulation in TGF-beta signal transduction. J. Cell Sci. 114, 4359-4369.

35. Narasaraju, T., Chen, H., Weng, T., Bhaskaran, M., Jin, N., Chen, J. W., Chen, Z., Chinoy, M. R. and Liu, L. (2006). Expression profile of IGF system during lung injury and recovery in rats exposed to hyperoxia – a possible role of IGF-1 in alveolar epithelial cell proliferation and differentiation. J Cell Biochem 97, 984-998.

36. Paine, R. and Simon, R. H. (1996). Expanding the frontiers of lung biology through the creative use of alveolar epithelial cells in culture. Am. J. Physiol. 270, L484-L486.

37. Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, M. R., Munger, K., Howley, P. M. and Moses, H. L. (1990). TGF-beta 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell 61, 777-785.

38. Selman, M. and Pardo, A. (2003). The epithelial/fibroblastic pathway in the pathogenesis of idiopathic pulmonary fibrosis. Am J Respir. Cell Mol Biol. 29, S93-S97.

39. ten Dijke, P., Goumans, M. J., Itoh, F. and Itoh, S. (2002). Regulation of cell proliferation by Smad proteins. J. Cell Physiol. 191, 1-16.

40. Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L. and Wrana, J. L. (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell 95, 779-791.

41. Uhal, B. D. (1997). Cell cycle kinetics in the alveolar epithelium. Am. J. Physiol. 272, L1031-L1045.

42. Venkatesan, N., Pini, L. and Ludwig, M. S. (2004). Changes in Smad expression and subcellular localization in bleomycin-induced pulmonary fibrosis. Am. J. Physiol Lung Cell Mol. Physiol 287, L1342-L1347.

43. Venkatesan, N., Roughley, P. J. and Ludwig, M. S. (2002). Proteoglycan expression in bleomycin lung fibroblasts: role of transforming growth factor-beta(1) and interferon-gamma. Am. J. Physiol Lung Cell Mol. Physiol. 283, L806-L814.

44. Verrecchia, F., Chu, M. L. and Mauviel, A. (2001). Identification of novel TGF-beta /Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. J. Biol. Chem. 276, 17058-17062.

45. Warburton, D. and Bellusci, S. (2004). The molecular genetics of lung morphogenesis and injury repair. Paediatr. Respir. Rev. 5, S283-S287.

46. Williams, M. C. (2003). ALVEOLAR TYPE I CELLS: Molecular Phenotype and Development. Annu. Rev. Physiol 65, 669-695.

47. Xu, Y. D., Hua, J., Mui, A., O'Connor, R., Grotendorst, G. and Khalil, N. (2003). Release of biologically active TGF-beta1 by alveolar epithelial cells results in pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 285, L527-L539.

48. Yamamoto, T., Komura, H., Morishige, K., Tadokoro, C., Sakata, M., Kurachi, H. and Miyake, A. (1994). Involvement of autocrine mechanism of transforming growth factor-beta in the functional differentiation of pregnant mouse mammary gland. Eur. J. Endocrinol. 130, 302-307.

49. Zen, K., Notarfrancesco, K., Oorschot, V., Slot, J. W., Fisher, A. B. and Shuman, H. (1998). Generation and characterization of monoclonal antibodies to alveolar type II cell lamellar body membrane. Am J Physiol 275, L172-L183.

50. Zhang, F., Nielsen, L. D., Lucas, J. J. and Mason, R. J. (2004). Transforming growth factor-beta antagonizes alveolar type II cell proliferation induced by keratinocyte growth factor. Am. J. Respir. Cell Mol. Biol. 31, 679-686.

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CHAPTER III

MicroRNA-127 MODULATES FETAL LUNG DEVELOPMENT

ABSTRACT

MicroRNAs (miRNAs) are small endogenous RNAs and are widely regarded as one of the most important regulators of gene expression in both plants and animals. To define the roles of miRNAs in fetal lung development, we profiled the miRNA expression pattern during lung development using a miRNA microarray. We identified 21 miRNAs that showed significant changes in expression during lung development. These miRNAs were grouped into four distinct clusters based on their expression pattern. Cluster 1 contained miRNAs whose expression increased as development progressed while clusters 2 and 3 showed the opposite trend of expression. miRNAs in Cluster 4 including *miR-127* had the highest expression at the late stage of fetal lung development. Quantitative real-time PCR validated the microarray results of 6 selected miRNAs. *In situ* hybridization demonstrated that *miR-127* expression gradually shifted from mesenchymal cells to epithelial cells as development progressed. Over-expression of *miR-127* in fetal lung organ culture significantly decreased the terminal bud count, increased terminal and internal bud sizes and caused unevenness in bud sizes, indicating improper development. These findings suggest that *miR-127* may have an important role in fetal lung development.

Key words: lung development, microRNA, *in situ* hybridization, microarray, lung morphometry

INTRODUCTION

MicroRNAs (miRNAs) are a class of small RNAs (~21-24 nt) that regulate the expression of their target genes at the post-transcriptional level (Bartel, 2004; He and Hannon, 2004). In animal cells, they are first transcribed from miRNA genes in the genome as primary miRNAs (pri-miRNAs) and then processed by an RNase III enzyme, Drosha, into ~70 nt premature miRNAs (pre-miRNAs) with hairpin structures (Lee et al., 2003). With the help of Exportin 5, pre-miRNAs are then transported into the cytoplasm, where they are cleaved by another RNase III enzyme, Dicer (Lee et al., 2002; Yi et al., 2003). The cleavage results in double-stranded RNA duplexes. Usually, one strand of the duplex becomes mature miRNA (Khvorova et al., 2003). Mature miRNAs are then recruited into nucleoprotein complexes called RNA-induced silencing complexes (RISC). Based on the pairing of miRNAs and their target sites, the complexes can negatively regulate the expression of their genes in three ways (Filipowicz et al., 2008). They can cleave the messenger RNAs. They can inhibit the translation of the messenger RNAs. They can accelerate deadenylation of the messenger RNAs leading to the acceleration of their degradation (Chen, 2004; Giraldez et al., 2006; Llave et al., 2002; Wu et al., 2006; Yekta et al., 2004). In rare cases, miRNAs can activate translation (Vasudevan et al., 2007; Wu and Belasco, 2008). miRNAs are important regulatory molecules that modulate various biological processes including cellular physiology, developmental timing, cell fate determination, apoptosis, lipid and fat metabolism, insulin secretion and progression

of various cancers (Lodish *et al*., 2008; Stadler and Ruohola-Baker, 2008; Stefani and Slack, 2008).

The functions of miRNAs in various aspects of lung biology are less studied, but are subjects of several recent investigations. Studies have suggested the important roles of miRNAs in lung cancer. It has been found that the decrease of let-7 expression is correlated to increased death rates in patients with lung cancers (Takamizawa et al., 2004). The expression of miRNAs in lung cancers has already been profiled. The results demonstrated the correlation of the miRNA expression with the prognosis of lung adenocarcinoma patients (Yanaihara et al., 2006). Studies on expression of important molecules in miRNA processing, namely members of the Argonaute (Ago) gene family, in the E11.5 lung have shown that Ago1 and Ago2 are enriched in branching regions, suggesting that miRNAs may play important roles in lung development (Lu et al., 2005). Inactivation of Dicer, a key component in miRNA processing, was found to cause the inhibition of lung epithelial branching (Harris et al., 2006). It has been reported recently that transgenic overexpression of the miR-17-92 cluster results in the promotion of proliferation and the inhibition of differentiation of epithelial progenitor cells in developing lungs (Lu et al., 2007).

Rat lung development can be divided into 5 stages (Burri, 1984; Zoetis and Hurtt, 2003). In the first 13 days, lobar division takes place. This is called the embryonic phase. Following the embryonic phase is the glandular or pseudoglandular phase (13-18 days), in which epithelial tubes of air passages are formed but have little or no lumen. In the canalicular phase (18-20 days), bronchioles are produced and a lumen can be recognized

in many tubules. With a further thinning of the interstitium and a flattening of the epithelium, alveolar ducts and air sacs are formed in the saccular phase (20 days to full term). Some epithelial cells begin to synthesize and secret pulmonary surfactant. The final stage happens after birth and is termed the alveolar phase, in which true alveoli are formed.

Each of the 5 developmental stages is coordinated by a multitude of signaling molecules and pathways (Weng et al., 2006). Some of the well-studied signaling molecules include fibroblast growth factors, transforming growth factors (TGF), retinoids, Wnt genes and Sonic hedgehog (Pongracz and Stockley, 2006; Ramasamy et al., 2007; Wang et al., 2005; White et al., 2006). However, little is known about what the role of miRNAs is in this process and how they regulate lung development by modulating these signaling pathways. In addition, the temporal and spatial expression patterns of miRNAs in rat lung development are still not known.

In this study, we used a miRNA microarray platform developed in our laboratory (Wang et al., 2007) to profile the expression of miRNAs at all stages in rat lung development. There were 21 miRNAs that were significantly changed during this process. Some of these miRNAs were selected and validated by real-time PCR. The spatial expression patterns of selected miRNAs were determined using *in situ* hybridization. We selected *miR-127* for further study based on its expression pattern. *miR-127* over-expression in an fetal lung organ culture at an earlier stage resulted in lesser and defective terminal bud formation and uneven development of the lung. These results demonstrate a critical role of *miR-127* in fetal lung development.

MATERIALS AND METHODS

Isolation of RNA from rat lung

Whole lungs were isolated from fetuses on gestational days 16, 19, and 21 (E16, E19, and E21), new born, 6-day-old, and 14-day-old (P0, P6 and P14), and 2-month-old adults (AD) rats. For each time point, there were three independent biological replicates. All procedures in this study followed the protocols approved by the Oklahoma State University Animal Care and Use committee. For the fetal lungs, pregnant Sprague-Dawley rats were sacrificed with CO_2. Fetuses were removed from the uterus and the lungs were isolated from these fetuses. For pup and adult lungs, male Sprague-Dawley rats were anesthetized before sacrifice and isolation of the lungs. Immediately after isolation, the lungs were rinsed with DMEM and then homogenized in the Lysis/Binding Buffer from the mir*Vana*™ miRNA Isolation Kit (Ambion, Austin, TX). Enriched small RNA and total RNA from the lungs were isolated with the mir*Vana*™ miRNA Isolation Kit (Ambion) according to the manufacturer's instructions.

miRNA microarray

The miRNA microarrays were performed on an in-house platform developed in our laboratory as described previously (Wang et al., 2007). There are 3 identical blocks on each slide and 6 identical probes for each miRNA in each block. The NCode miRNA Labeling System (Invitrogen, Carlsbad, CA) was used to generate labeled miRNA molecules for hybridization to the microarrays. Six hundred ng of RNA was used in each labeling reaction. One fifth of the labeled RNA was used for each hybridization. Small RNAs from the lungs at certain time points were co-hybridized with the common reference, which was pooled equally from small RNAs of all the lung samples. To each

block, one labeled samples (Alexa Fluor 3 or Alexa Fluor 5) was co-hybridized with the common reference labeled with the other dye (Alexa Fluor 5 or Alexa Fluor 3). Dye swaps were performed to eliminate dye bias. The hybridized slides were scanned with ScanArray Express (PerkinElmer Life and Analytical Sciences, Boston, MA) and the images were analyzed with GenePix 5.0 pro (Axon Instruments, Inc. Union City, CA). The data were calculated, normalized and qualified as described previously (Wang et al., 2007). The signal from each spot was normalized to the average signal of the whole block. The highest and lowest signals from the 6 identical probes in the same block were excluded from further analysis. The geometric average of the other 4 signals was considered to be the signal of that particular miRNA. The qualities of the signals were assessed with the software, Realspot. The miRNAs with an average QI (quality index) < 1 were filtered. The qualified data were then analyzed with the software SAM (Significant Analysis of Microarrays, Stanford University, http://www-stat.stanford.edu/~tibs/SAM/) (multiclass response) and any miRNAs significantly changed between any two time points were picked up ($q<0.05$). The miRNAs that passed the SAM test were clustered and viewed with Cluster (K means clustering) and TreeView software (http://rana.lbl.gov/EisenSoftware.htm).

Quantitative Real-time PCR for miRNA

Quantitative real-time PCR (qRT-PCR) was performed with the mir*Vana*™ qRT-PCR miRNA Detection Kit (Ambion, Austin, TX) (for miR-18, miR-20a, miR-29a and miR-351) or TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA) (for miR-195 and miR-351) as per the company's protocols. In brief, total RNA was isolated from rat lungs at different time points of development (E16, E19, E21, P0, P6, P14, and AD)

with the mir*Vana*™ miRNA Isolation Kit (Ambion Austin, TX). TURBO DNA-*free*™ (Ambion) was used to remove DNA contamination. Three biological replicates were performed at each time point. For qRT-PCR using mir*Vana*™ qRT-PCR miRNA Detection Kit, 50 ng RNA was used in each reverse transcription reaction with miRNA specific mir*Vana*™ RT primers. Duplicate RT reactions were performed for RNA from each biological replicate and no template controls. The reactions were incubated in 96-well plates at 37°C for 30 min, then at 95°C for 10 min. PCR Master Mix (15 µl) was added to each RT reaction. qRT-PCR was performed on an Applied Biosystems 7500 Real-Time PCR System . The reactions were incubated at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Dissociation analysis was performed after amplification to identify the characteristic peak for the specific PCR product. The C_T was determined for each miRNA. RT and PCR for U6 snRNA were also performed in each plate as an endogenous control. The comparative C_T method was used, and the relative amount of each miRNA to U6 snRNA was calculated with the equation, $2^{-(CT\ microRNA-CT\ U6)}$. For qRT-PCR with TaqMan® MicroRNA Assays, 75 ng total RNA was used as template in each RT reaction with miRNA specific RT primers. The reactions were incubated on ice for 5 min, followed by 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. For each PCR reaction, 1.33 µl RT product was used as a template. The PCR reaction was incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. All PCR reactions were run in duplicate. RT and PCR for 18 S in each sample were also performed as an endogenous control using TaqMan® Ribosomal RNA Control Reagents (Applied Biosystems). Data analysis was done as described above.

In situ hybridization for miRNA

In situ hybridization for miRNA was done using 5' DIG labeled LNA probes (Exiqon Woburn, MA). Paraffin embedded rat lung tissues were dewaxed in xylene and rehydrated in descending grades of alcohol. The slides were then washed in PBS (pH 7.5) and permeablized by incubating for 10 min in proteinase-K (Ambion, Austin, TX) for 5 min at 37^{o}C. The slides were again washed in 0.2% glycine, fixed in 4% paraformaldehyde, rinsed in PBS and pre-hybridized in hybridization buffer (50% Formamide, 5 x SSC, 0.1% Tween 20, 9.2 mM citric acid, 50 µg/ml heparin and 500 µg/ml yeast RNA, pH 6) in a humidified chamber. The 5' DIG labeled LNA probes were then added to the sections at a 20 nM concentration and incubated overnight at the hybridization temperature (21°C lower than the T_m values of the specific probes). The slides were rinsed in 2x SSC and washed 3 times for 30 min in 50% formamide, 2x SSC solution at the same hybridization temperature. This was followed by blocking with 2% sheep serum, 2 mg/ml BSA in PBST (PBS + 0.1% Tween 20) and incubation with anti-DIG-AP Fab fragments antibody (1:2000) (Roche Applied Sciences, Indianapolis, IN) overnight at 4°C in a humidified chamber. After washing in PBST and AP buffer (100 mM Tris HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl and 0.1% Tween-20), the color reaction was carried out by incubation in the color solution, BCIP/NBT solution (Roche Applied Sciences, Indianapolis, IN) with 1 mM Levamisole for 6 to 24 hours at RT. The color reaction was stopped after observing sufficient development of blue precipitate by washing with PBST. The slides were then mounted, cover slipped and observed under a Nikon E-600 microscope.

Construction of a miR-127 overexpression adenoviral vector

Rat *miR-127* with the flanking sequences (~200 base pairs at each end) was amplified from rat genomic DNA using the primers: 5'-CCTTGTCGACCTCGAGAACC TCCAG-3' and 5'-AGAATTCTTAGGCATTAAG TGGCTCCAGACCC-3' and digested by *Sal* I and *Eco* RI digestion. The digested PCR product was cloned into a modified pENTR/CMV-EGFP vector (Gou et al., 2004) between enhanced green fluorescent protein (EGFP) stop codon and SV40 polyA terminal sequences through *Xho* 1 and *Eco* R1 restriction sites. Inclusion of CMV-driven EGFP in the vector was for the purpose of monitoring transfection efficiency. The empty vector of the CMV-driven EGFP was used as a vector control. The CMV-EGFP-miRNA in the pENTR vector was moved into an adenoviral vector by the Gateway technique (Invitrogen, Carlsbad, CA). Obtained adenoviral vectors were linearized by *Pac* I and used to transfect HEK293A cells. Adenovirus was amplified by re-infecting HEK293A cells. Titer of virus was determined by making series of dilutions of viral stock, infecting HEK293A cells and counting for virus infected cells and expressed as Plaque Forming Units (PFU)/ml.

Over-expression of miR-127 in fetal lung organ culture

E14 embryos were dissected from timed pregnant Sprague-Dawley rats. Fetal lungs were isolated from each fetus by removing the surrounding tissues using 21-24 gauge needles and placed in Hank's buffered salt solution (HBSS). They were then cultured on 0.4 μm pore size culture inserts (Millipore Corporation, Billerica, MA) and placed in six well tissue culture plates for 2 days. Each well contained 1.5 ml of serum-free chemically defined BGJb medium (Fitton-Jackson modification) (Invitrogen, Carlsbad, CA) with 0.2 mg/ml ascorbic acid, 50 units/ml penicillin and 50 μg/ml streptomycin. The day of

isolation (E14) was denoted as D0 and the next 2 days of culture were denoted as D1 and D2, respectively. The adenoviruses containing EGFP and miRNA over-expression sequence or empty vector with EGFP only (Virus Control) were added to the inserts on D0 at a dose of 4.7 x 10^7 PFU/ fetal lung. As a blank control, an equal amount of DMEM media was added on the fetal lung. Excess liquid, if any, in the insert was removed using a pipette after 3 hours. Half of the initial dose of virus was added on D1 to maintain the over-expression as tissue mass increased. The lung was photographed on each day using a digital camera mounted on to a Nikon-E 600 microscope at same magnification for every lung.

Morphometric analysis of lung

The morphometric analyses of fetal lungs were done on the images taken on D0, D1 and D2 at the same magnification using MetaVue™ software (Molecular Devices Corporation, Downingtown, PA). The images were coded and analyses were performed by two blinded investigators. The number of terminal buds was counted by enlarging the images on the Metavue software and counting those buds which were at the periphery of the lung. The number of terminal buds for each lung was divided by the number of terminal buds present at the time of isolation as a normalization procedure. The terminal and internal bud sizes were measured using the software at the longest diameter for each bud. The average bud sizes were calculated by randomly selecting at least 30 internal or terminal buds from each lung and calculating the average for each lung. Data was pooled from all the lungs that received the same treatment. The data from at least 10 lungs obtained from fetuses of 3 different dams were used to measure each parameter.

Statistical Analysis

Statistical analysis of microarray data was done as described earlier (Wang et al., 2007). One way ANOVA was performed for the real time PCR study pertaining to *miR-127* over-expression in fetal lungs, followed by the Dunnett's multiple comparison test for comparison between individual treatments. For all other studies, a paired *t*-test was done between the virus control and *miR-127* over-expression group. A p value of < 0.05 was considered significant. All values are presented as means ± S.E.

RESULTS

miRNA expression profile during lung development.

To detect the miRNA expression profile during rat lung development, we used the miRNA microarray platform developed in our laboratory (Wang et al., 2007). The microarray contained probes for 227 non-redundant RNAs: 177 rat miRNAs, 5 human miRNAs, 31 mouse miRNAs and 14 other kinds of RNAs and controls. Small RNAs of rat lungs from 7 time points of lung development (E16, E19, E21, P0, P6, P14, and AD) were co-hybridized onto the slides with the common reference, which consisted of equal amounts of enriched small RNAs from each time point. Three biological replicates and dye swaps were performed for each sample. 107 miRNAs passed the quality test by *RealSpot* with an average QI>1 (Chen and Liu, 2005). The statistical analysis was performed with SAM. 21 miRNAs were shown to have significant changes between at least one pair of the 7 time points ($q<0.05$). These significantly changed miRNAs were then grouped into 4 clusters with Cluster software by K-means clustering (Fig. 7)

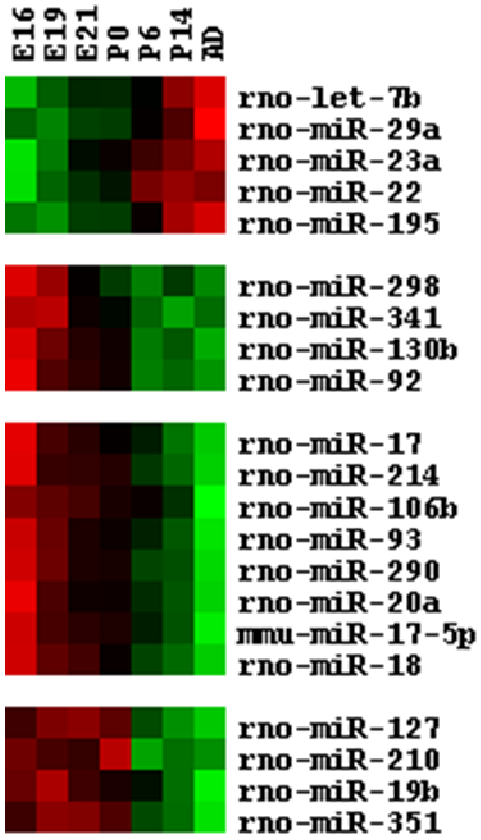

Figure 7 - Cluster analysis of miRNAs

Cluster analysis of miRNAs significantly changed during rat lung development. miRNAs from lungs on gestational days 16, 19 and 21 (E16, E19 and E21), postnatal days 0, 6 and 14 (P0, P6 and P14, or adult lungs (AD) were co-hybridized with the common reference. The normalized data were subjected to SAM test to identify miRNAs whose expression were significantly changed during this process ($q<0.05$). The identified miRNAs were grouped into 4 clusters by K-mean clustering and viewed by TreeView. Each column represents one stage and each row represents one miRNA. Each value of expression is the average of 6 replicates and is then log2 transformed. Red represents positive values, green negative values, and black zero.

The first cluster included *let-7b*, *miR-29a*, *miR-23a*, *miR-22* and *miR-195*. The second one included *miR-298*, *miR-341*, *miR-130b* and *miR-92*. The third one included *miR-17*, *miR-214*, *miR-106b*, *miR-93*, *miR-290*, *miR-20a*, *miR-17-5p* and *miR-18*. The last one consisted of *miR-127*, *miR-210*, *miR-19b* and *miR-351*.

The expression patterns of the identified miRNAs are shown in the line charts (Fig. 8). In the first cluster, the miRNA expression increased gradually from fetal to adult lungs (E16 to AD). The miRNAs in the second and third cluster decreased from E16 to AD, a large part of which markedly decreased from E16 to E19. In the fourth cluster, all the miRNAs peaked at some point between E16 and P6. For example, *miR-127* reached maximum on E21 and *miR-351* reached maximum on E19.

Figure 8

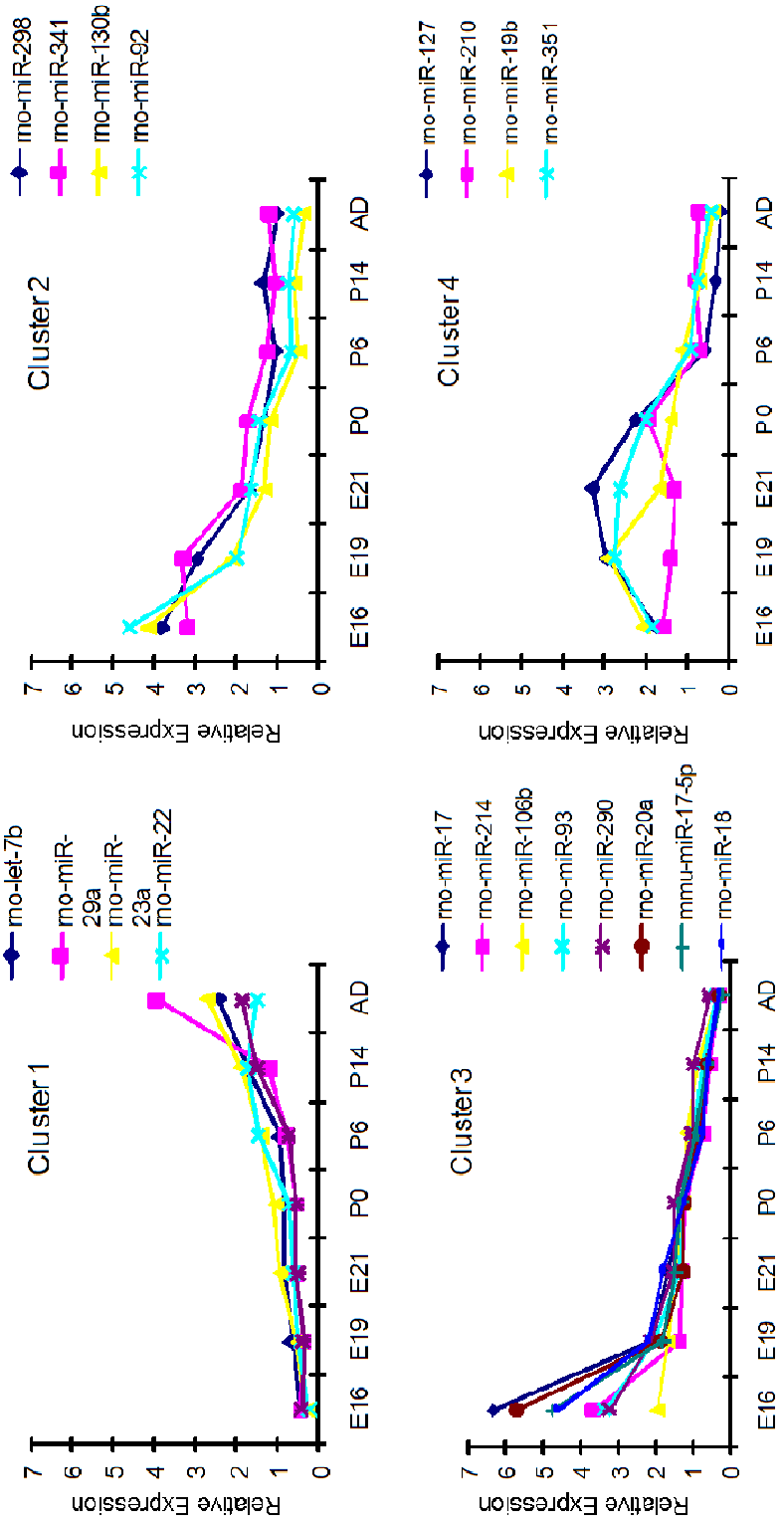
Cluster 1
mo-let-7b
mo-miR-29a
mo-miR-23a
mo-miR-22
Cluster 2
mo-miR-298
mo-miR-341
mo-miR-130b
mo-miR-92
Cluster 3
mo-miR-17
mo-miR-214
mo-miR-106b
mo-miR-93
mo-miR-290
mo-miR-20a
mmu-miR-17-5p
mo-miR-18
Cluster 4
mo-miR-127
mo-miR-210
mo-miR-19b
mo-miR-351
Relative Expression
0
1
2
3
4
5
6
7
E16
E19
E21
P0
P6
P14
AD

Figure 8 - miRNA expression patterns during rat lung development
The relative expression of miRNAs in each cluster of Fig. 7 is plotted against each stage of development. The relative expression level is the ratio of the sample signal to the common reference signal. The data shown are the means of 6 replicates.

Real-time PCR validation of the microarray results

We wanted to validate our microarray results using a more sensitive and quantitative method. qRT-PCR was done to confirm the trends of expression exhibited by miRNAs from each of the 3 clusters. *miR-29a* and *miR-195* (Fig. 9a and b) were chosen to represent the 1st cluster, while miR-18 and *miR-20a* (Fig. 9c and d) represented the 3rd cluster and *miR-127* and *miR-351* (Fig. 9e and f) represented the 4th cluster. All of these miRNAs followed the same trend of expression as seen in the microarray experiment, thus validating our microarray platform.

Figure 9

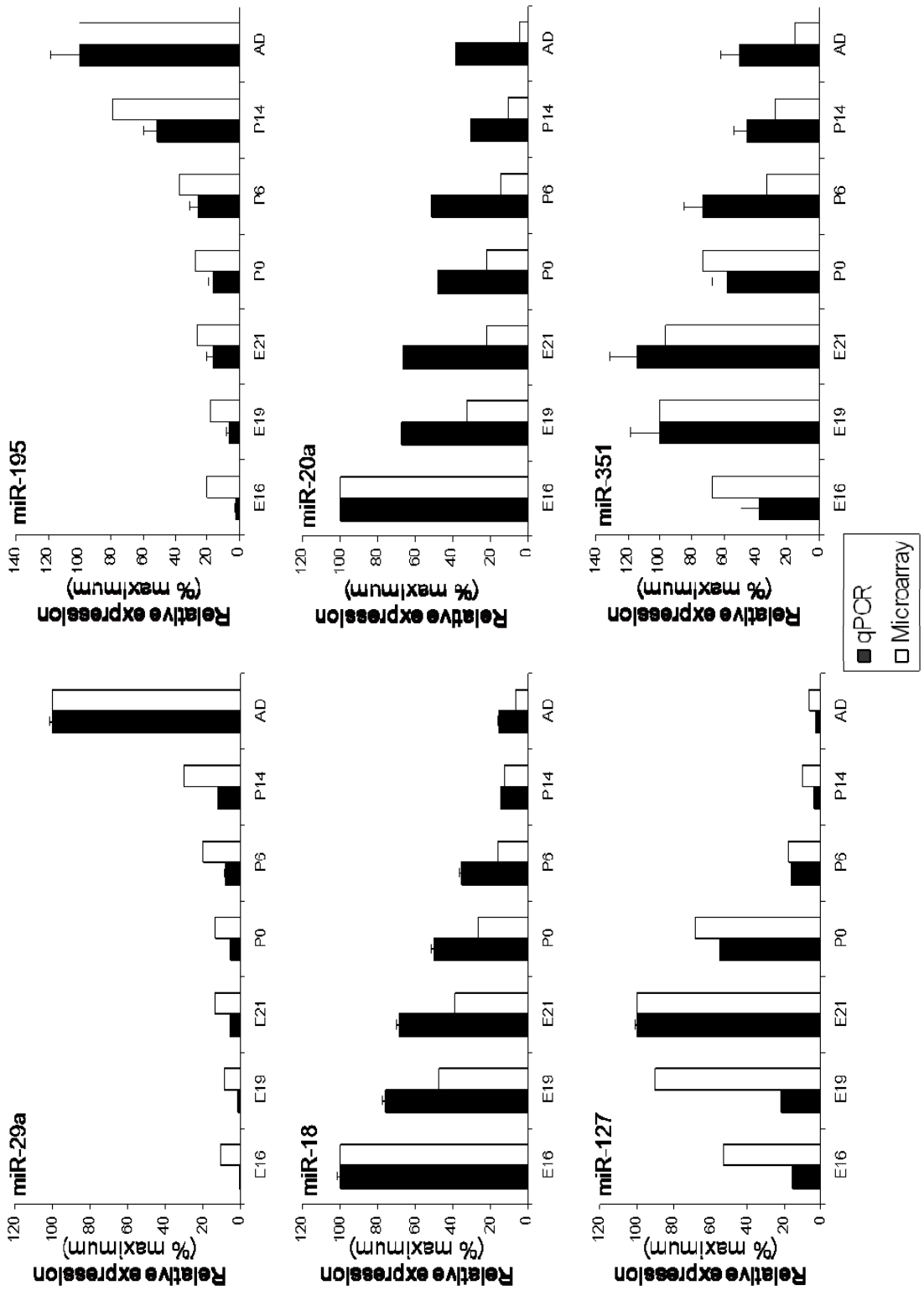
miR-29a
miR-18
miR-127
miR-195
miR-20a
miR-351
Relative expression (% maximum)
E16
E19
E21
P0
P6
P14
AD
qPCR
Microarray

Figure 9 - Validation of miRNA microarray results using quantitative real-time PCR

Total RNA was extracted from the fetal lungs with gestational days 16, 19 and 21 (E16, E19 and E21) and the lungs of postnatal days 0, 6, 14 and adult (P0, P6, P14 and AD). miRNA levels were measured by real-time PCR. The relative expression levels of *miR-29a*, *miR-195*, *miR-18*, *miR-20a*, *miR-127* and *miR-351* were expressed as percentages of maximum expression. The error bars represent standard errors. n=3 independent preparations, each assay performed in duplicates. The microarray data shown are averages from 6 replicates.

Cellular localization of miRNAs

In situ hybridization using 5' DIG labeled LNA probes was done to determine the spatial expression pattern of selected miRNAs in tissue sections from different stages of lung development. A U6 probe was used as a positive control. Positive signals were observed in nuclei of all the cells on P0 sections (Fig. 10a P0-Pos). *miR-20a* expression followed the trend of expression consistent with the microarray and qRT-PCR data (Fig. 10a). Expression was seen on E16 and not in other stages of development. The signal on E16 was confined mainly to cells of mesenchymal origin in the interstitium. A probe that contained a scrambled sequence and had no known miRNA targets was used as a negative control. No signals were detected on E16 sections (Fig. 10a, E16-Neg). The *miR-127* expression pattern also followed the trend of expression as seen in the qRT-PCR

and microarray experiments (Fig. 10b). It was highest at E19, E21 and P0 stages. E19 sections showed *miR-127* expression both in epithelial and mesenchymal cells, but more in mesenchymal cells. In E21 sections, the expression shifted more towards the epithelial regions lining the airways. P0 lungs showed much weaker signal intensity than E21 but the trend of expression was the same as on E21. Adult lung sections did not give any signals for *miR-127*. There was no signal from the lining of blood vessels (data not shown). The *miR-351* expression pattern also corroborated the qRT-PCR and microarray data (Fig. 10c). E16 sections showed *miR-351* expression both in epithelial and mesenchymal cells, but more in epithelial cells lining the future terminal airways and alveoli. Expression was highest in E19 stage where *miR-351* was seen both in epithelial cells and mesenchymal cells. The expression was strongest in the epithelial cells lining the terminal bronchioles (Fig. 10c, inbox) while it was absent in the lining of blood vessels. E21 showed the same pattern of expression though much weaker than E19. Also, the expression shifted more towards epithelial cells lining the alveoli than in mesenchymal cells. The weakening of the signal continued to the P0 stage and signal was seen mainly in epithelial cells lining the alveoli and terminal air spaces. In adult tissue section, the signal was exclusive and specific in alveolar type II cells and could not be detected in other cells. In all stages, no signal was detected from the lining of blood vessels (data not shown).

Figure 10a

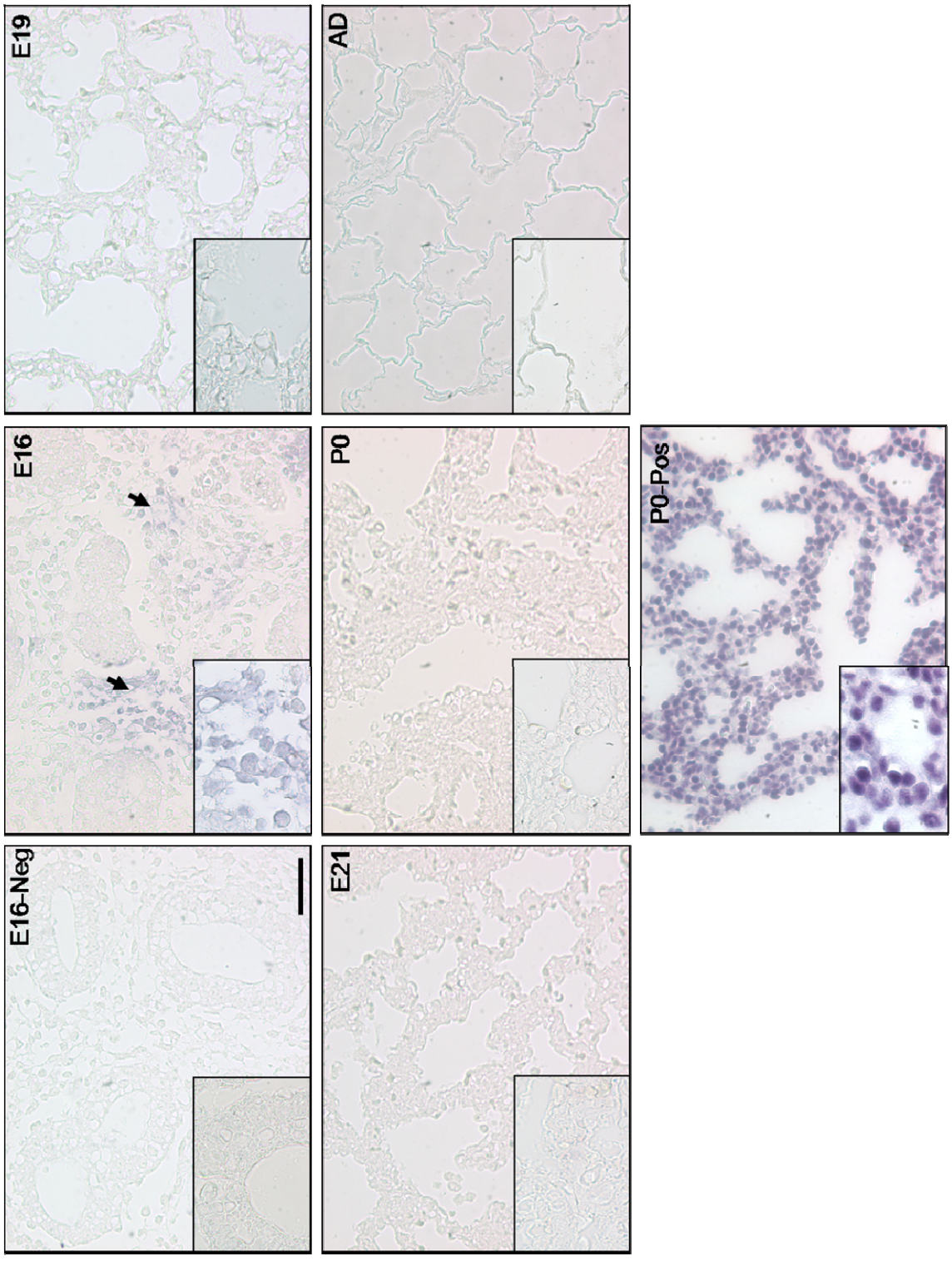

Figure 10b

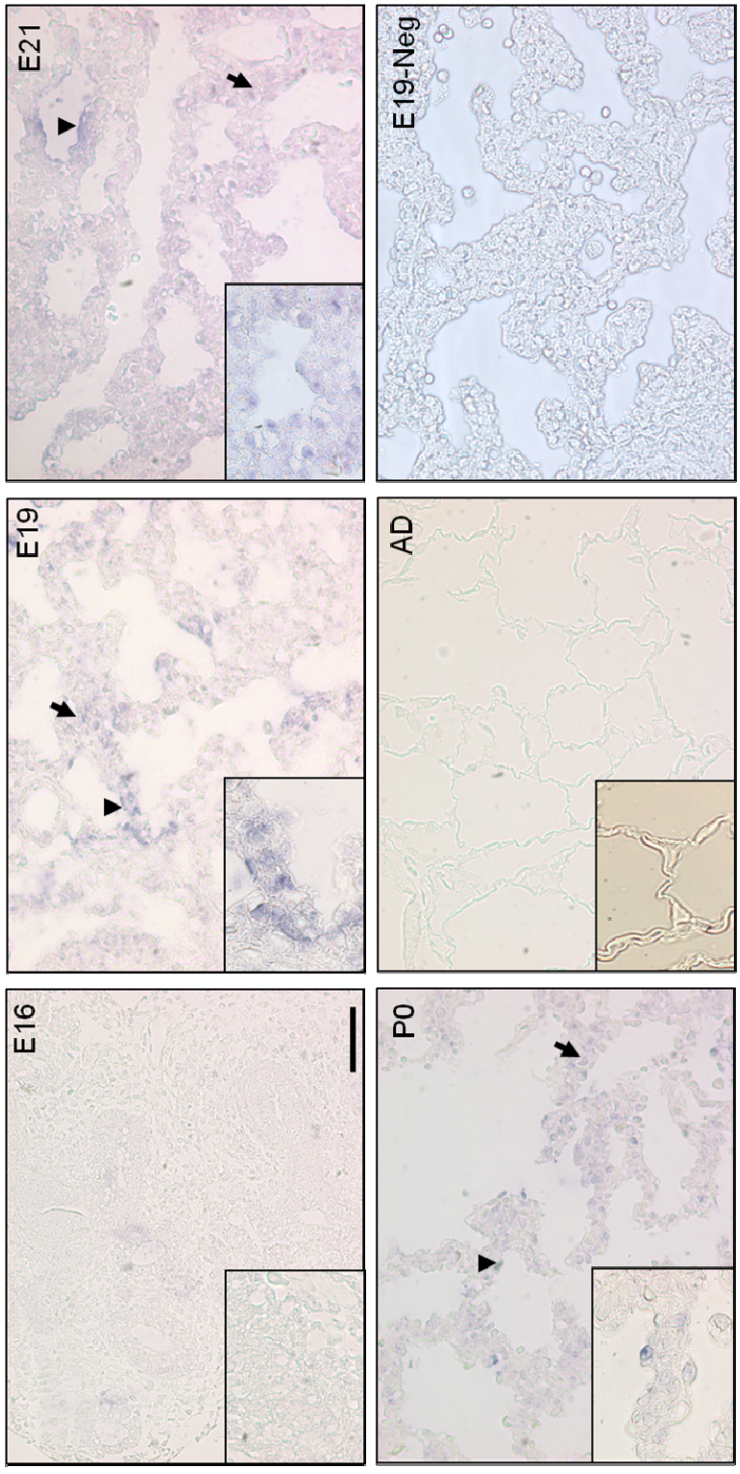

Figure 10c

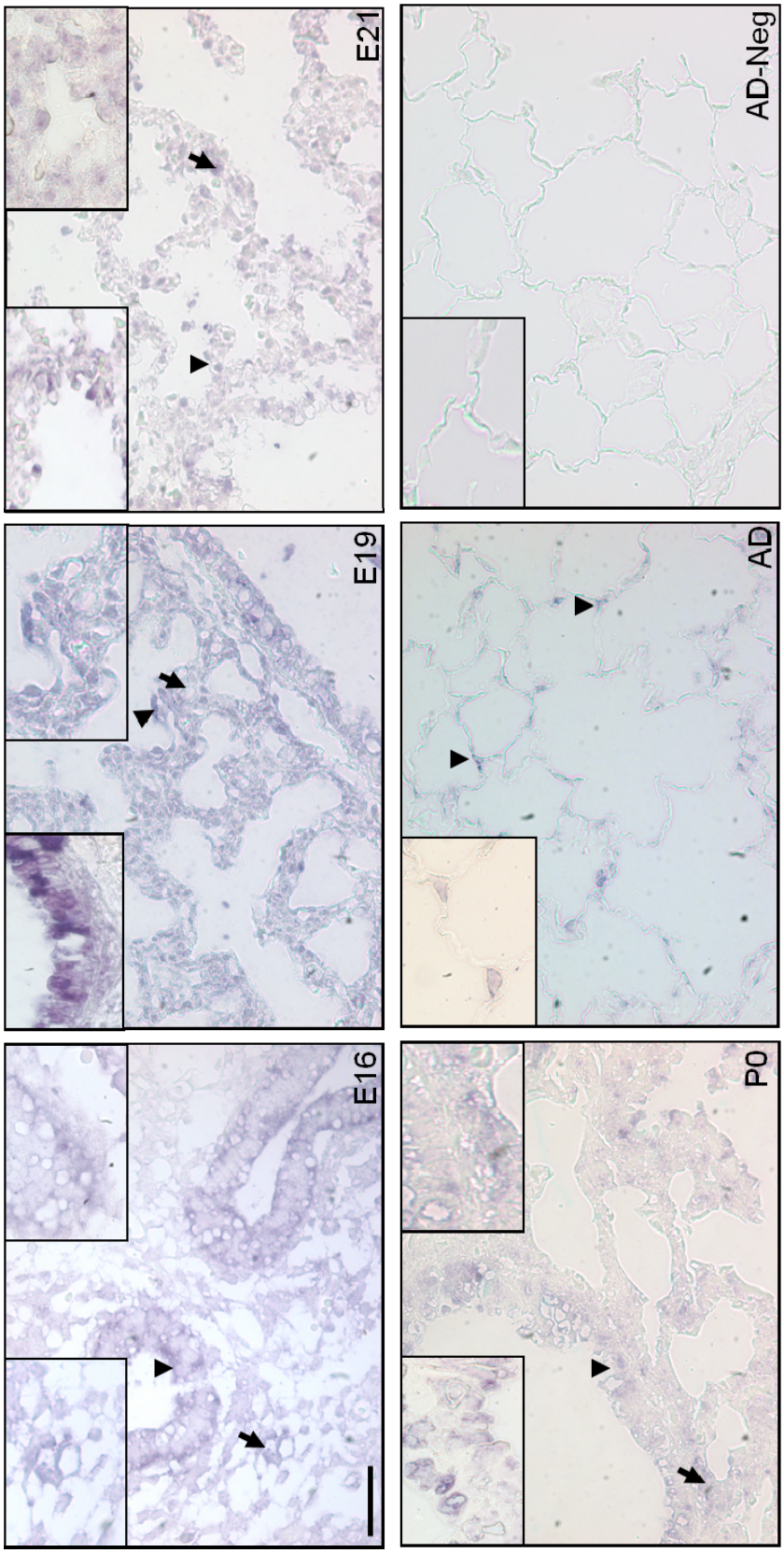

Figure 10 - *In situ* hybridization for miRNAs

In situ hybridization was carried out in dewaxed and rehydrated fetal rat lung tissue sections from gestational days 16, 19 and 21 (E16, E19 and E21) and the lungs of postnatal day 0 and adult (P0 and AD). The sections were hybridized with 5' DIG labeled LNA probes against *miR-20* (a), *miR-127* (b) and *miR-351* (c). A probe with scrambled sequence unrelated to known miRNAs was used as a negative control (Neg) and a probe for U6 was used as a positive control (Pos). Positive signals were visualized as dark blue/purple color. Arrow heads denote signals from epithelial cells while the arrows denote signals from mesenchymal cells. Scale bar: 40 μm.

Effect of miR-127 over-expression on fetal lung development.

miR-127 was selected for further functional studies because of its interesting expression trend. *miR-127* was expressed at E19, E21 and P0, the period immediately before birth and the period directly after birth. We decided to over-express *miR-127* at an earlier stage of development (E14) in an *in vitro* fetal lung culture model to see whether it causes any changes of fetal lung development. An adenoviral vector that over-expressed *miR-127* was used to transduce E14 fetal lungs cultured for 2 days as described in Materials and Methods. *miR-127* over-expression and its effect on lung branching morphogenesis were visualized on each day (Fig. 11a). The dose of the virus was standardized by examining the intensities and even distribution of GFP along the whole lung. Since there was a significant increase in the lung tissue mass as a function of culture time, we added half the initial dose of virus on D1 and this gave a constant and more even GFP

expression than a single treatment on D0. The same treatment regimen was followed for both virus control and blank control. The over-expression was confirmed using real-time PCR (Fig. 11b). It was seen that there was a significant increase in *miR-127* over-expression on D1 and D2. An increase in the endogenous expression of *miR-127* was also noted as the culture progressed.

Figure 11

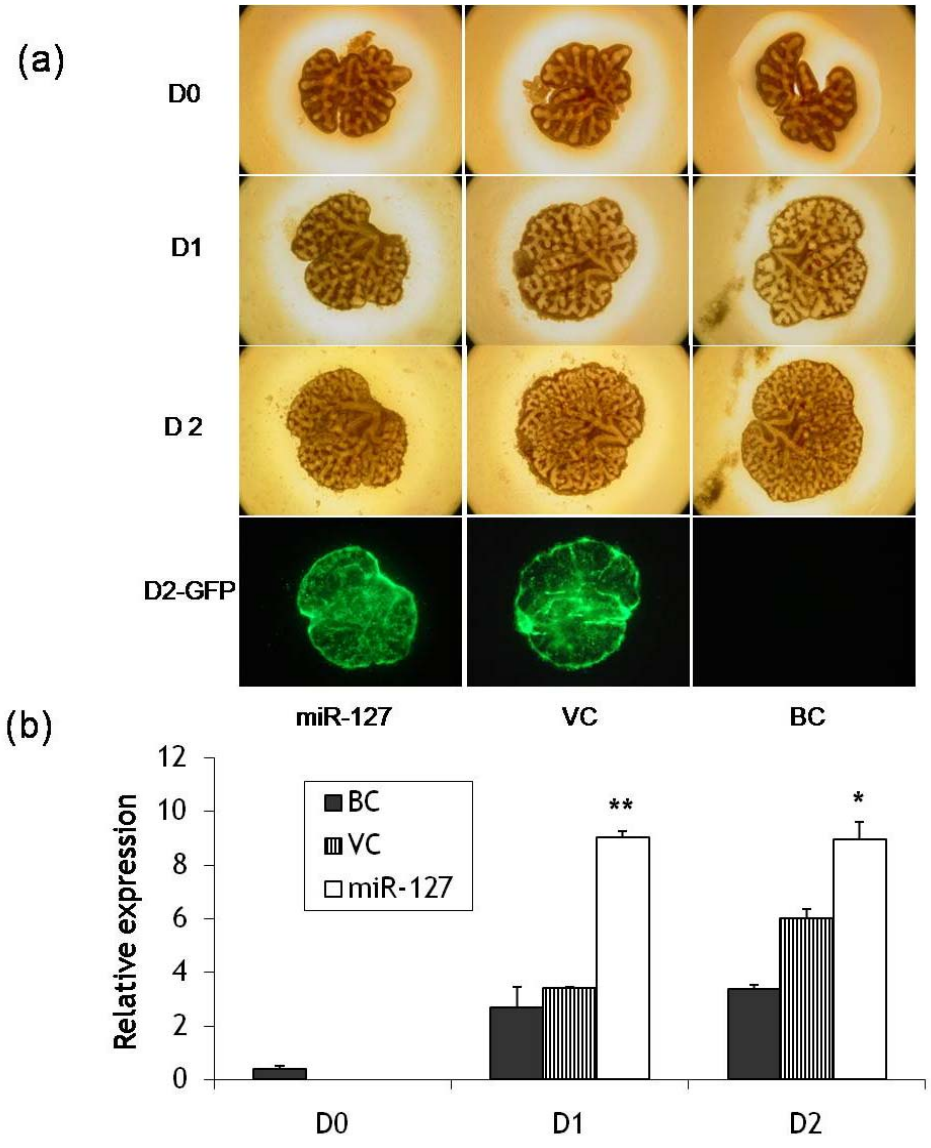

Figure 11 - *miR-127* over-expression in fetal lung culture

(a) E14 fetal lungs were cultured in an insert. *miR-127* over-expression adenovirus or virus control (VC) were added to the culture on day 0 (D0) and culture continued for 2 days (D1 and D2). The blank control (BC) was treated with medium alone. The images and GFP fluorescence were taken at D0-D2. Each treatment was carried out in at least 10 lungs from 3 different mothers in 3 separate experiments.

(b) qRT-PCR to quantify the over-expression of *miR-127*. Total RNAs from D1 and D2 of the fetal lung culture were isolated. *miR-127* levels were measured by qRT-PCR. The error bars represent standard error. n=3 independent preparations, each assay performed in duplicates. *P<0.05 v.s. VC, **P≤0.02 v.s. VC.

miR-127 over-expression resulted in a larger terminal bud size when compared to blank control (BC) and virus control (VC) (Fig. 12a and b). Variability in the sizes of terminal buds was also plotted on a graph by arranging the various terminal bud sizes from at least 250 terminal buds from 10 fetal lungs per treatment in ascending order of their individual sizes. The sizes of the terminal buds from the lungs treated with *miR-127* seemed to fluctuate unevenly between almost 10 to 180 relative units while those treated with virus control or blank control showed a size variation that fluctuated in a much narrower range (20-80 relative units) (Fig 12 C). The average internal bud size was higher for *miR-127* as in the case of terminal buds when compared to controls (Fig. 12d). We define internal buds as those buds which were not at the periphery of the developing lung. They were enclosed and not tubular and budded out from the secondary tubular formations in the developing lung. The terminal bud count, another important indicator of proper lung

branching, showed that *miR-127* over-expression decreased the number of terminal buds. Fig 12e) Taken together, *miR-127* over-expression resulted in larger yet lesser numbers of terminal buds which showed a high amount of variability between bud sizes and this trend was reflected in internal buds too. These results clearly demonstrate that *miR-127* if over-expressed at an early stage causes defective lung branching morphogenesis.

Figure 12a,b,c

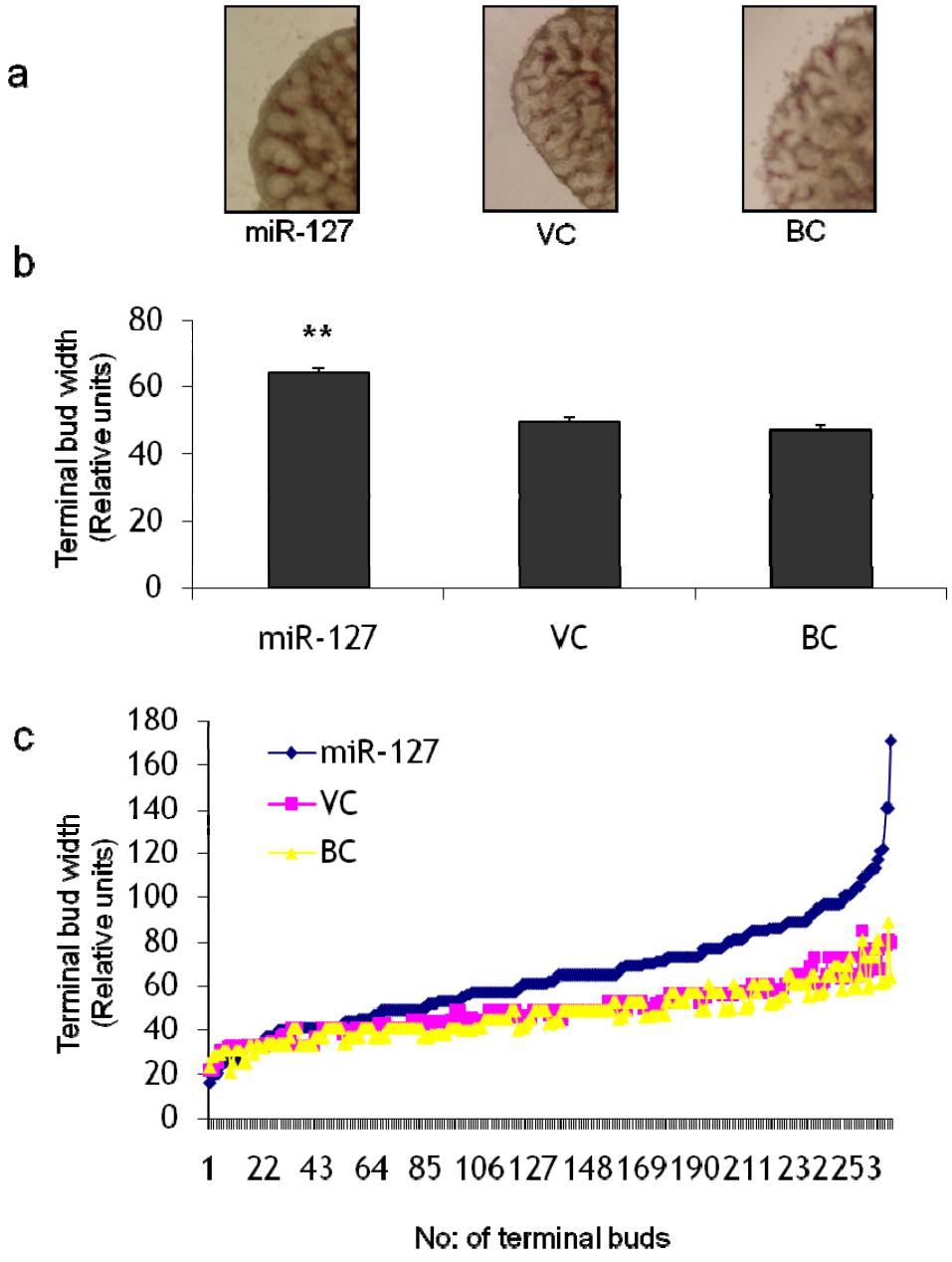

Figure 12d,e

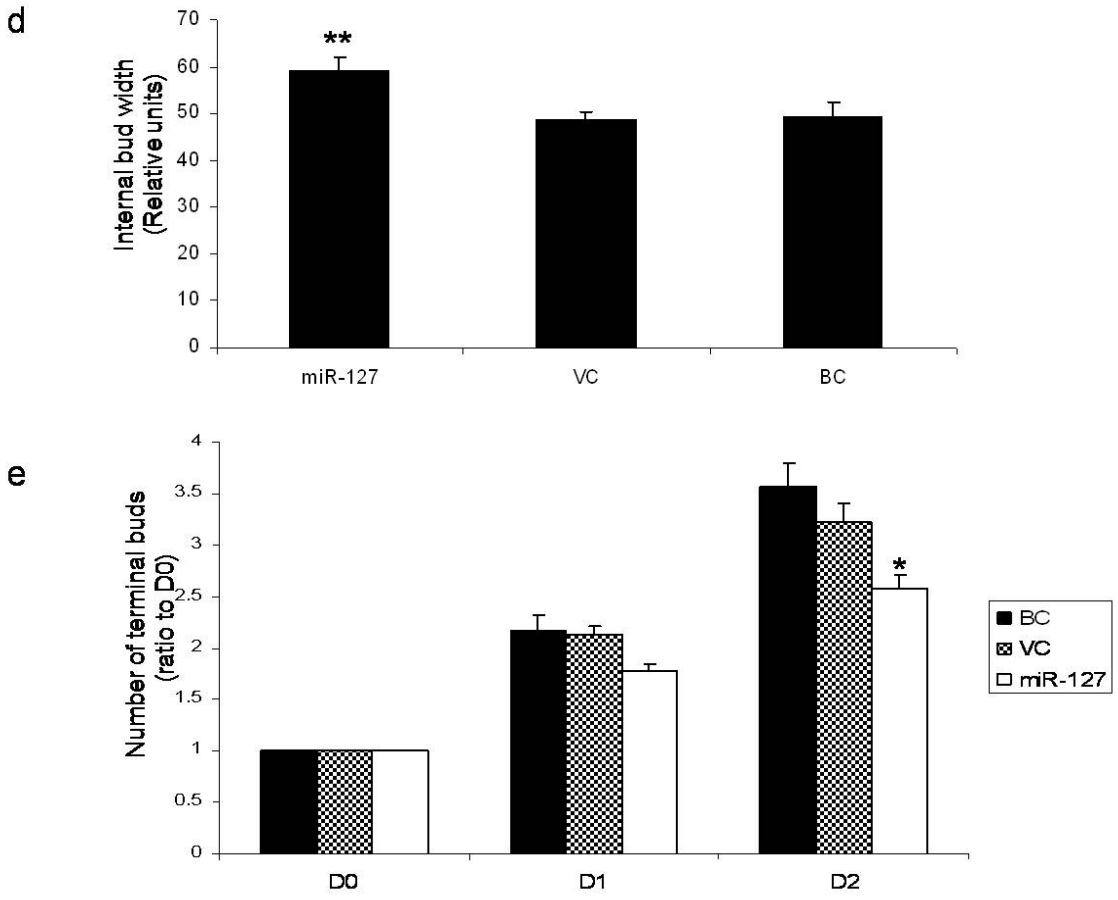
d
Internal bud width (Relative units)
70
60
50
40
30
20
10
0
**
miR-127
VC
BC
e
Number of terminal buds (ratio to D0)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
D0
D1
D2
BC
VC
miR-127

Figure 12 - Effect of *miR-127* over-expression on fetal lung development.

E14 fetal lungs cultured in inserts were treated with *miR-127* over-expression adenovirus or viral control (VC) or blank control (BC) for 2 days. Images were taken at the end of culture. (a) An enlarged image from Fig. 5a. (b) Terminal bud width was measured using Metavue software. The width of each bud was measured at its longest diameter and expressed in relative units (c) Variability in the terminal bud width. The terminal bud width values were arranged in ascending order in each treatment and plotted against the number of buds to demonstrate the variability in the terminal bud size. The data was obtained from more than 250 terminal buds from at least 10 fetuses obtained from 3 mothers. (d) Average internal bud width was calculated using metavue software and the same measuring parameter for terminal buds. The value was expressed in relative units. (e) The number of terminal buds formed at the end of day 2 in *miR-127* over-expressed lungs was compared with VC and BC after normalizing with the terminal buds on day 0. The number of terminal buds was counted blindly by at least 2 different individuals and the relative number at the end of day 2 was expressed as a ratio to number of terminal buds on day 0. At least 25 terminal or random internal buds from each lung were used for the respective analyses. The data is from at least 10 lungs obtained from 3 different mothers (n=3). The error bars represent standard error. *$P<0.05$ v.s. VC, **$P\leq0.02$ v.s. VC.

Target Prediction of Identified miRNAs

The target genes of the identified miRNAs were predicted by TargetScan (http://genes.mit.edu /targetscan.test/ucsc.html) and PicTar (http://pictar.bio.nyu.edu/). Gene ontology (GO) information (http://www.geneontology.org/) on gene functions revealed 24% and 18% of the target genes are related to cell growth/maintenance and signal transduction, respectively. Assuming that up- or down-regulation of miRNAs results in down- or up-regulation of their target genes, we further used GenMAPP (www.genmapp.org) and KEGG Pathway database (http://www.genome. ad.jp/kegg/ pathway.html) to identify biological pathways regulated by miRNAs. Two major pathways that are potentially involved in lung development, TGF-β and Wnt/β-catenin pathways, were identified. Sixty five components in the TGF-β pathway were targeted by the miRNAs on our array (KEGG); of them, 37 were targeted by the 21 miRNAs changed during lung development. The numbers of the components in the TGF-β pathway targeted by each cluster are also listed in Table 2.

Table 2 miRNAs targeting different pathways

	Components in β-catenin Signaling	Components in TGF β Signaling
Total miRNAs	81	65
21 miRNAs	40	37
Cluster 1	28	24
Cluster 2	9	14
Cluster 3	12	17
Cluster 4	11	13

Similar analysis was done with the Wnt/β-catenin pathway. 40 components in the Wnt/β-catenin pathway were targeted by the identified 21 miRNAs. Further studies on the regulation of these pathways by these miRNAs will help to uncover the functions of miRNAs in lung development.

DISCUSSION

In the present work, we described the miRNA expression profile during lung development and identified four clusters of miRNAs that showed specific trends of expression. Expression levels of 21 miRNAs were found to be significantly changed during the course of lung development. The miRNA microarray results were validated by qRT-PCR analysis and *in situ* hybridization of the selected miRNAs. The over-expression of *miR-127* in a fetal lung organ culture system caused defective lung development characterized by decreased terminal bud counts and varied bud sizes.

miRNAs have rapidly emerged as one of the key regulatory molecules that control various biological processes ranging from development to disease. Various miRNAs have been implicated in regulating developmental timing and controlling left/right neuronal asymmetry in *Caenorhabditis elagans* (Johnston and Hobert, 2003; Reinhart et al., 2000), insulin secretion (Poy et al., 2004), lipid metabolism (Esau et al., 2006), modulating proliferation and apoptosis (Brennecke et al., 2003; Cheng et al., 2005), stem cell division (Hatfield et al., 2005; Shcherbata et al., 2006) and B cell differentiation (Xiao et al., 2007). Involvement of miRNAs in progression of various cancers have been extensively investigated (Blenkiron et al., 2007; He et al., 2005; Ma et al., 2007; Subramanian et al., 2007; Takamizawa et al., 2004; Yanaihara et al., 2006). But studies on their role in the physiology of the lung have been very limited. Some important proteins involved in miRNA processing such as Ago1, Ago2, and Dicer have been shown to be important to lung morphogenesis (Harris et al., 2006; Lu et al., 2005). These discoveries suggest the importance of miRNAs in the lung. Our previous study has shown that two miRNAs, namely, *miR-195* and *miR-200c*, are specifically expressed in the lung (Wang et al., 2007). It has been shown that the expression levels of some miRNAs are changed after lipopolysaccharide-induced inflammation and *miR-146a* can regulate the inflammatory response in lung alveolar epithelial cells (Moschos et al., 2007; Perry et al., 2008).

The first cluster we identified included *miR-29a* and *miR-195*. Their expression remained low during all stages of fetal lung development and was high in adult lung. *miR-29a* showed a similar trend of expression during development of the brain, i.e. low in embryonic brain tissue and high in adult cortex and striatum (Landgraf et al., 2007).

Over-expression of the *miR-29* family in lung cancer cell lines has been shown to inhibit tumorigenicity both *in vitro* and *in vivo* (Fabbri et al., 2007). *miR-195*, on the other hand, has been identified as a key regulator of cardiac growth and function. The over-expression of *miR-195* in cardiomyocytes led to abnormal cardiac remodeling and heart failure (van Rooij et al., 2006). Its low expression during lung development may be an important factor that helps in the controlled proliferation and differentiation of cells in the lung. This cluster also contained let-7b, a member of let-7 family known to regulate developmental timing in drosophila (Pasquinelli et al., 2000).

The second and third clusters contained miRNAs whose expression decreased as the development progressed. Interestingly, this cluster contained *miR-17-5p*, *miR-18* and *miR-20a*, all of which are encoded by the *miR-17-92* cluster, a conserved gene that encodes 7 miRNAs. A recent study in mouse embryonic lung development has shown a similar trend of expression for these 3 miRNAs from E11.5 to adult lungs (Lu et al., 2007). Our *In situ* hybridization of *miR-20a* indicated its expression in the mesenchymal region only at E16. The expression rapidly disappeared as the development progressed. Analysis of predicted targets of miR-17-92 cluster showed that almost 58% of their predicted targets were transcription factors, regulators of nucleotide or nucleic acid metabolism or cellular protein metabolism, all of which are key features in driving the developmental process in the right direction.

The *miR-17-92* cluster has been found to be over-expressed in lung cancers and has been demonstrated to promote proliferation and inhibit differentiation of lung epithelial progenitor cells (Hayashita et al., 2005; Lu et al., 2008; Lu et al., 2007; Matsubara et al.,

2007). This cluster has also been demonstrated to influence the translation of E2F family of transcription factors which are important in regulation of cell cycle and apoptosis (O'Donnell et al., 2005). A recent study has shown that *miR-20a* has an important role in the regulation of E2F2 and E2F3 expression (Sylvestre et al., 2007). The same study found that E2F1, E2F2 and E2F3 could directly bind to the promoter of *miR-17-92* cluster and act as an auto regulatory feed loop mechanism. *miR-20a*, also seemed to have an anti-apoptotic role in this study where its over expression decreased apoptosis in a prostrate cancer cell line and its inhibition caused increased cell death. Another group found that E2F3 was the primary E2F family member that bound to the promoter of *miR-17-92*. They have proposed that the *miR-17-92* is also pro-proliferative as it shifts the transcriptional balance towards the proliferative E2F3 network than the pro-apoptotic E2F1 network (Woods et al., 2007). The anti-apoptotic role of *miR-20a* and *miR-17-5p* was further confirmed in another study where their inhibition caused increased apoptosis in lung cancer cells (Matsubara et al., 2007). These studies have concentrated on the effect of miRNAs on cancer cell lines. Their role in affecting the expression of E2F factors in normal cells of a developing organ have not been studied. Since controlled cell death, proliferation and differentiation go hand in hand in the development of any organ systems and since these miRNAs seem to regulate these processes, we believe that they have a critical role in regulating lung development as well. This view is strengthened by another study where the members of *miR-17-92* family have also been implicated in the promotion of adipocyte differentiation by negatively regulating Rb2/p130, the retinoblastoma genes which also interact with E2F transcription factors (Wang et al., 2008).

The fourth cluster contained *miR-127* and *miR-351*, which showed the highest expression just before and after birth in the sacculo-alveolar stage. Many dramatic events including differentiation of alveolar epithelial type I and type II cells, the initiation of formation of alveoli and progressive decrease in the interstitial tissue occur in these stages (Burri, 1984; Roth-Kleiner and Post, 2005).

In situ hybridization showed that both *miR-127* and *miR-351* tend to shift from the mesenchymal compartment of the developing lung to the epithelial cells, which may indicate a role for these miRNAs in the cellular re-organization process and differentiation of alveolar epithelial cells or mesenchymal to epithelial transition.

miR-127 is embedded in a CpG island and remains methylated in most tissues except sperm. It shows an imprinted expression in the mouse (Seitz et al., 2004; Seitz et al., 2003). The functional studies on *miR-127* so far has identified it as a potential tumor suppressor whose expression goes down in cancer cell lines and in a significant number of primary tumors (Saito et al., 2006). When treated with chromatin modifying drugs, *miR-127* expression was up-regulated, and this, in turn, inhibited the expression of its target, a protooncogene, BCL6. Modulation in the *miR-127* expression pattern in the context of organ development has not yet been reported. The over-expression of *miR-127* in E14 fetal lung cultures significantly affected the normal branching and terminal bud formation, indicating its role in fetal lung development.

Taken together, our results have demonstrated the reliability of a miRNA microarray platform to identify the miRNA profile during fetal lung development. We have also confirmed the expression profile and localization of selected miRNAs and have

demonstrated that *miR-127* over-expression results in defective fetal lung development. Since miRNAs are believed to have multiple targets and because there are many signaling pathways that are involved in the lung development process, it is likely that the miRNAs regulate multiple mechanisms of control of lung development.

REFERENCES

1. Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281-97.

2. Blenkiron, C., Goldstein, L. D., Thorne, N. P., Spiteri, I., Chin, S. F., Dunning, M. J., Barbosa-Morais, N. L., Teschendorff, A. E., Green, A. R., Ellis, I. O. et al. (2007). MicroRNA expression profiling of human breast cancer identifies new markers of tumour subtype. Genome Biol 8, R214.

3. Brennecke, J., Hipfner, D. R., Stark, A., Russell, R. B. and Cohen, S. M. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. Cell 113, 25-36.

4. Burri, P. H. (1984). Fetal and postnatal development of the lung. Annu Rev Physiol 46, 617-28.

5. Chen, X. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science 303, 2022-5.

6. Chen, Z. and Liu, L. (2005). RealSpot: software validating results from DNA microarray data analysis with spot images. Physiol Genomics 21, 284-91.

7. Cheng, A. M., Byrom, M. W., Shelton, J. and Ford, L. P. (2005). Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Res 33, 1290-7.

8. Esau, C., Davis, S., Murray, S. F., Yu, X. X., Pandey, S. K., Pear, M., Watts, L., Booten, S. L., Graham, M., McKay, R. et al. (2006). miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab 3, 87-98.

9. Fabbri, M., Garzon, R., Cimmino, A., Liu, Z., Zanesi, N., Callegari, E., Liu, S., Alder, H., Costinean, S., Fernandez-Cymering, C. et al. (2007). MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci U S A 104, 15805-10.

10. Filipowicz, W., Bhattacharyya, S. N. and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9, 102-14.

11. Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K., Enright, A. J. and Schier, A. F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science 312, 75-9.

12. Gou, D., Narasaraju, T., Chintagari, N. R., Jin, N., Wang, P. and Liu, L. (2004). Gene silencing in alveolar type II cells using cell-specific promoter *in vitro* and in vivo. Nucleic Acids Res 32, e134.

13. Harris, K. S., Zhang, Z., McManus, M. T., Harfe, B. D. and Sun, X. (2006). Dicer function is essential for lung epithelium morphogenesis. Proc Natl Acad Sci U S A 103, 2208-13.

14. Hatfield, S. D., Shcherbata, H. R., Fischer, K. A., Nakahara, K., Carthew, R. W. and Ruohola-Baker, H. (2005). Stem cell division is regulated by the microRNA pathway. Nature 435, 974-8.

15. Hayashita, Y., Osada, H., Tatematsu, Y., Yamada, H., Yanagisawa, K., Tomida, S., Yatabe, Y., Kawahara, K., Sekido, Y. and Takahashi, T. (2005). A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res 65, 9628-32.

16. He, L. and Hannon, G. J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5, 522-31.

17. He, L., Thomson, J. M., Hemann, M. T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S. W., Hannon, G. J. et al. (2005). A microRNA polycistron as a potential human oncogene. Nature 435, 828-33.

18. Johnston, R. J. and Hobert, O. (2003). A microRNA controlling left/right neuronal asymmetry in Caenorhabditis elegans. Nature 426, 845-9.

19. Khvorova, A., Reynolds, A. and Jayasena, S. D. (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell 115, 209-16.

20. Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A. O., Landthaler, M. et al. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129, 1401-14.

21. Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature 425, 415-9.

22. Lee, Y., Jeon, K., Lee, J. T., Kim, S. and Kim, V. N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. Embo J 21, 4663-70.

23. Llave, C., Xie, Z., Kasschau, K. D. and Carrington, J. C. (2002). Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 297, 2053-6.

24. Lodish, H. F., Zhou, B., Liu, G. and Chen, C. Z. (2008). Micromanagement of the immune system by microRNAs. Nat Rev Immunol 8, 120-30.

25. Lu, J., Qian, J., Chen, F., Tang, X., Li, C. and Cardoso, W. V. (2005). Differential expression of components of the microRNA machinery during mouse organogenesis. Biochem Biophys Res Commun 334, 319-23.

26. Lu, Y., Okubo, T., Rawlins, E. and Hogan, B. L. (2008). Epithelial Progenitor Cells of the Embryonic Lung and the Role of MicroRNAs in Their Proliferation. Proc Am Thorac Soc 5, 300-4.

27. Lu, Y., Thomson, J. M., Wong, H. Y., Hammond, S. M. and Hogan, B. L. (2007). Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. Dev Biol 310, 442-53.

28. Ma, L., Teruya-Feldstein, J. and Weinberg, R. A. (2007). Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 449, 682-8.

29. Matsubara, H., Takeuchi, T., Nishikawa, E., Yanagisawa, K., Hayashita, Y., Ebi, H., Yamada, H., Suzuki, M., Nagino, M., Nimura, Y. et al. (2007). Apoptosis induction by antisense oligonucleotides against miR-17-5p and miR-20a in lung cancers overexpressing miR-17-92. Oncogene.

30. Moschos, S. A., Williams, A. E., Perry, M. M., Birrell, M. A., Belvisi, M. G. and Lindsay, M. A. (2007). Expression profiling in vivo demonstrates rapid changes in lung microRNA levels following lipopolysaccharide-induced inflammation but not in the anti-inflammatory action of glucocorticoids. BMC Genomics 8, 240.

31. O'Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V. and Mendell, J. T. (2005). c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435, 839-43.

32. Pasquinelli, A. E., Reinhart, B. J., Slack, F., Martindale, M. Q., Kuroda, M. I., Maller, B., Hayward, D. C., Ball, E. E., Degnan, B., Muller, P. et al. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 408, 86-9.

33. Perry, M. M., Moschos, S. A., Williams, A. E., Shepherd, N. J., Larner-Svensson, H. M. and Lindsay, M. A. (2008). Rapid Changes in MicroRNA-146a Expression Negatively Regulate the IL-1{beta}-Induced Inflammatory Response in Human Lung Alveolar Epithelial Cells. J Immunol 180, 5689-98.

34. Pongracz, J. E. and Stockley, R. A. (2006). Wnt signalling in lung development and diseases. Respir Res 7, 15.

35. Poy, M. N., Eliasson, L., Krutzfeldt, J., Kuwajima, S., Ma, X., Macdonald, P. E., Pfeffer, S., Tuschl, T., Rajewsky, N., Rorsman, P. et al. (2004). A pancreatic islet-specific microRNA regulates insulin secretion. Nature 432, 226-30.

36. Ramasamy, S. K., Mailleux, A. A., Gupte, V. V., Mata, F., Sala, F. G., Veltmaat, J. M., Del Moral, P. M., De Langhe, S., Parsa, S., Kelly, L. K. et al. (2007). Fgf10 dosage is critical for the amplification of epithelial cell progenitors and for the formation of multiple mesenchymal lineages during lung development. Dev Biol 307, 237-47.

37. Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R. and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403, 901-6.

38. Roth-Kleiner, M. and Post, M. (2005). Similarities and dissimilarities of branching and septation during lung development. Pediatr Pulmonol 40, 113-34.

39. Saito, Y., Liang, G., Egger, G., Friedman, J. M., Chuang, J. C., Coetzee, G. A. and Jones, P. A. (2006). Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell 9, 435-43.

40. Seitz, H., Royo, H., Lin, S. P., Youngson, N., Ferguson-Smith, A. C. and Cavaille, J. (2004). Imprinted small RNA genes. Biol Chem 385, 905-11.

41. Seitz, H., Youngson, N., Lin, S. P., Dalbert, S., Paulsen, M., Bachellerie, J. P., Ferguson-Smith, A. C. and Cavaille, J. (2003). Imprinted microRNA genes transcribed antisense to a reciprocally imprinted retrotransposon-like gene. Nat Genet 34, 261-2.

42. Shcherbata, H. R., Hatfield, S., Ward, E. J., Reynolds, S., Fischer, K. A. and Ruohola-Baker, H. (2006). The MicroRNA pathway plays a regulatory role in stem cell division. Cell Cycle 5, 172-5.

43. Stadler, B. M. and Ruohola-Baker, H. (2008). Small RNAs: keeping stem cells in line. Cell 132, 563-6.

44. Stefani, G. and Slack, F. J. (2008). Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol 9, 219-30.

45. Subramanian, S., Lui, W. O., Lee, C. H., Espinosa, I., Nielsen, T. O., Heinrich, M. C., Corless, C. L., Fire, A. Z. and van de Rijn, M. (2007). MicroRNA expression signature of human sarcomas. Oncogene.

46. Sylvestre, Y., De Guire, V., Querido, E., Mukhopadhyay, U. K., Bourdeau, V., Major, F., Ferbeyre, G. and Chartrand, P. (2007). An E2F/miR-20a autoregulatory feedback loop. J Biol Chem 282, 2135-43.

47. Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., Harano, T., Yatabe, Y., Nagino, M., Nimura, Y. et al. (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64, 3753-6.

48. van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., McAnally, J., Gerard, R. D., Richardson, J. A. and Olson, E. N. (2006). A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci U S A 103, 18255-60.

49. Vasudevan, S., Tong, Y. and Steitz, J. A. (2007). Switching from repression to activation: microRNAs can up-regulate translation. Science 318, 1931-4.

50. Wang, Q., Li, Y. C., Wang, J., Kong, J., Qi, Y., Quigg, R. J. and Li, X. (2008). miR-17-92 cluster accelerates adipocyte differentiation by negatively regulating tumor-suppressor Rb2/p130. Proc Natl Acad Sci U S A 105, 2889-94.

51. Wang, X., Inoue, S., Gu, J., Miyoshi, E., Noda, K., Li, W., Mizuno-Horikawa, Y., Nakano, M., Asahi, M., Takahashi, M. et al. (2005). Dysregulation of TGF-beta1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice. Proc Natl Acad Sci U S A 102, 15791-6.

52. Wang, Y., Weng, T., Gou, D., Chen, Z., Chintagari, N. R. and Liu, L. (2007). Identification of rat lung-specific microRNAs by micoRNA microarray: valuable discoveries for the facilitation of lung research. BMC Genomics 8, 29.

53. Weng, T., Chen, Z., Jin, N., Gao, L. and Liu, L. (2006). Gene expression profiling identifies regulatory pathways involved in the late stage of rat fetal lung development. Am J Physiol Lung Cell Mol Physiol 291, L1027-37.

54. White, A. C., Xu, J., Yin, Y., Smith, C., Schmid, G. and Ornitz, D. M. (2006). FGF9 and SHH signaling coordinate lung growth and development through regulation of distinct mesenchymal domains. Development 133, 1507-17.

55. Woods, K., Thomson, J. M. and Hammond, S. M. (2007). Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. J Biol Chem 282, 2130-4.

56. Wu, L. and Belasco, J. G. (2008). Let me count the ways: mechanisms of gene regulation by miRNAs and siRNAs. Mol Cell 29, 1-7.

57. Wu, L., Fan, J. and Belasco, J. G. (2006). MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci U S A 103, 4034-9.

58. Xiao, C., Calado, D. P., Galler, G., Thai, T. H., Patterson, H. C., Wang, J., Rajewsky, N., Bender, T. P. and Rajewsky, K. (2007). MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell 131, 146-59.

59. Yanaihara, N., Caplen, N., Bowman, E., Seike, M., Kumamoto, K., Yi, M., Stephens, R. M., Okamoto, A., Yokota, J., Tanaka, T. et al. (2006). Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9, 189-98.

60. Yekta, S., Shih, I. H. and Bartel, D. P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. Science 304, 594-6.

61. Yi, R., Qin, Y., Macara, I. G. and Cullen, B. R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17, 3011-6.

62. Zoetis, T. and Hurtt, M. E. (2003). Species comparison of lung development. Birth Defects Res B Dev Reprod Toxicol 68, 121-4.

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Contribution of co-authors

Yang Wang did the miRNA microarray and rtPCR for miR-195 and miR-351. Honghao Zhang made the mir-127 over expression vector. Tingting Weng helped with the organ culture and in morphometric analysis. Pradyumna Baviskar helped with the organ extraction and organ culture. Deming Gou developed the miRNA overexpression library.

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CHAPTER IV

MicroRNA EXPRESSION PROFILE IN BRONCHOPULMONARY DYSPLASIA

ABSTRACT

MicroRNAs (miRNAs) are small RNAs ~21-24 nucleotides in length. They are endogenous in origin and regulate the expression of proteins post transcriptionally. miRNA expression profile in a disease provides valuable insights into the pathogenesis of that disease and possible therapeutic strategies. Bronchopulmonary dysplasia (BPD) is a chronic lung disease manifested in premature infants and has multifactorial etiology. BPD can be attributed to the dysregulation of normal lung development due to defects in signaling and regulatory components involved. In this study we determined the miRNA profile in BPD using miRNA microarray. In a rat model that exhibited the pathology for 'new' BPD, we found that five miRNAs, miR-342, miR-335, miR-150, miR-126* and miR-151* were downregulated and three miRNAs miR-21, miR-141 and miR-34a upregulated in the diseased lungs. The expression of these miRNAs was confirmed using quantitative real-time PCR and their possible functional significance have been discussed. Identification of miRNAs involved is the first step towards deciphering the fine control mechanisms involved in the spatial and temporal regulation of proteins that contribute to the pathogenesis of BPD.

Keywords: Bronchopulmonary dysplasia, microRNA, Preterm infants, new BPD pathology, miRNA microarray.

INTRODUCTION

Spatially and temporally controlled cascades of signaling through multiple pathways and transcription factors are important in the normal development of any organ systems. The dysregulation of one or more of these factors contribute to the pathogenesis of various diseases. The recent discovery of small non-coding RNAs called microRNAs (miRNAs) has added a new dimension into regulatory mechanisms that control development and disease. These ~21-24 nucleotide long RNAs can inhibit protein expression at translational level (Lee *et al.*, 1993; Bartel, 2004). They exert their regulatory control over signaling cascades that govern multiple biological processes in a wide array of organisms (Pasquinelli *et al.*, 2000; Mattick, 2003). The regulation/dysregulation of multiple proteins by microRNAs and thereby the pathways involved might be a contributing factor to the pathogenesis of various diseases.

miRNAs are transcribed as primary miRNAs (pri-miRNAs) which are hundreds of nucleotides long. An RNase III enzyme called drosha within the nucleus processes the pri-miRNA into ~70 nucleotide precursor miRNA (pre-miRNA)(Lee *et al.*, 2003). The pre-miRNA has a stem-loop structure and has a typical 5' phosphate and ~2 nucleotide overhang. A Ran-dependent nuclear transport protein named exportin-5 (Exp5) mediates the transport of pre-miRNA into the cytoplasm (Yi *et al.*, 2003; Lund *et al.*, 2004). Another RNase III enzyme, dicer, processes pre-miRNAs to mature ~21-24 nucleotide duplexes (Lee *et al.*, 2003; Yi *et al.*, 2003). The mature miRNAs are recruited into nucleoprotein complexes called RNA- induced silencing complexes (RISC) (Schwarz *et al.*, 2003). The mechanism by which these complexes regulate the expression of the target protein may vary depending upon the complementarity between the miRNA and

target regions on the mRNA. A higher complementarity is believed to degrade the mRNA (common in plants) and lower complementarity leads to translational suppression (common in animals) (Hutvagner & Zamore, 2002). Another mechanism of action has been recently proposed where miRNA binding leads to faster deadenylation and thereby degradation of target mRNA (Wu *et al.*, 2006).

Bronchopulmonary dysplasia (BPD) is the chronic lung disease of infancy. Multiple factors contribute to the manifestation of this disease. Immaturity of lung due to pre-term birth, complications due to positive pressure ventilation and a high concentration of oxygen leading to oxidant injury, barotraumas, volutrauma and treatment using pro-inflammatory mediators are considered to be major contributing factors to this disease (Northway *et al.*, 1967; Groneck & Speer, 1995; Van Marter *et al.*, 2000; Jobe & Ikegami, 2001; Turunen *et al.*, 2006). New treatment strategies like exogenous surfactant administration and advances in critical care management of premature babies have changed the course of disease progression and related pathology (Coalson, 2003). The 'old' BPD pathology manifest due to high concentration of oxygen and ventilator induced injury on an immature and surfactant deficient lung (Coalson, 2003, 2006). The lesions included fibrosis, cellular hyperplasia, vascular lesions and various degrees of air way injury and inflammation (Bonikos *et al.*, 1976; Kunig *et al.*, 2005). The 'new' BPD pathology is less severe and manifests as a result of an immature lung forced to undergo gaseous exchange without completing the proper development process. The pathology, here, is characterized by decreased fibrosis, large and simplified alveoli, capillary defects, negligible airway epithelial lesions, variable interstitial fibro proliferation and smooth muscle hyperplasia (Husain *et al.*, 1998; Coalson, 2003). Alveolar simplification and

enlargement of alveoli are the major and consistent changes in 'new' BPD pathology. Insight into the developmental process of the lung, especially interdependence of alveolarization and microvascular development, may be paramount in understanding the pathophysiology of BPD (Jobe & Bancalari, 2001). The expression profile and role of individual miRNAs in the development of various diseases are subjects of many current investigations. They have already been implicated in the pathogenesis of various diseases like cancer (He *et al.*, 2005a; Yanaihara *et al.*, 2006; Blenkiron & Miska, 2007). miRNA expression have also been demonstrated to be very important in regulating the physiology of development of various tissues, cell types and metabolic processes (Brennecke *et al.*, 2003; Cheng *et al.*, 2005; Esau *et al.*, 2006; van Rooij *et al.*, 2007; Zhao *et al.*, 2007). In the lung, dicer knockout has already been shown to impair lung development implicating the role of miRNA in controlling lung morphogenesis (Lu *et al.*, 2005; Harris *et al.*, 2006). Another study have shown that miR-17-92 cluster might be important in the differentiation of epithelial progenitor cells in developing lung (Lu *et al.*, 2007)

In this study, we have profiled miRNAs in BPD on a microarray platform. We found five miRNAs that were significantly downregulated and 4 miRNAs that were upregulated in BPD. The expression profile of eight of these miRNAs was confirmed using quantitative real time PCR. This would give us insight into the target proteins whose expression changes might be important in the pathogenesis of this disease and possibly, new tools for rational therapy.

MATERIALS AND METHODS

Animals, treatment and sample collection

All animal procedures were carried out according to the protocol approved by the Animal Care and use Committee at Oklahoma State University. Timed pregnant Sprague-Dawley rats were bred in house. Only pups from two mother rats which gave birth within 0-3 hour interval were used in each set of experiment so as to ensure the same stage of development. The pups from each litter were marked and then divided equally one day after birth (P1, P0 being the day of birth). Half of the pups from one mother were mixed with half of the litter of another. Each of these mixed pup group was assigned to the two mothers. The mothers were then switched between litters each day so that they were trained to feed foster pups. From P3, one mother with the mixed litter was exposed to ~95% oxygen in a sealed plexi-glass chamber (90 x 45 x 45 cm). Thereafter the mothers were interchanged every 24 hours till P13. The time points and oxygen concentration were selected as per previously reported studies where the structural changes resembled the 'new' BPD pathology (Veness-Meehan *et al.*, 2000; Veness-Meehan *et al.*, 2002) The oxygen concentration was continuously measured using an oxygen sensor (Vacu-Med, Ventura, CA). The oxygen flow rate was maintained at 4 liters/min and the chamber was cleaned everyday. Soda lime was used to remove excess CO_2. On P14, the animals were anesthetized, and midline sternotomy was performed on each animal to expose and remove lung for further studies.

Sample collection

RNA: A total of 8 lung samples were used for this purpose, 4 BPD lung and 4 normal control lungs. It was made sure that each of the 4 lung per treatment came from pups born

to 4 separate mothers. The lungs were washed in DMEM and homogenized in the Lysis/Biniding Buffer from mir*Vana*^{tm} miRNA isolation kit (Ambion, Austin, TX). Total RNA and enriched small RNA was isolated using mirVana^{tm} miRNA isolation kit (Ambion) according to the manufacturer's instructions.

Fixation of lung tissue and sectioning: After midline sternotomy, lungs were perfused with ice cold PBS through right ventricle. The trachea was cannulated, and 4% paraformaldehyde was instilled into the lung under constant pressure (30 cm H_2O for 25 minutes). The inflation pressure was chosen based on previous studies (Hayatdavoudi *et al.*, 1980; Bachofen *et al.*, 1982). The trachea was then ligated at this constant distending pressure and lungs were removed and immersed in 4% paraformaldehyde for 24 hours. Paraffin embedded sections of 4 μm thickness on glass slides were made for further morphometric analysis and staining.

Histological staining and morphometric analysis

Morphometric analysis was performed on hematoxylin and eosin stained slides (Ashour *et al.*, 2006). Two parameters were measured to quantify interalveolar distance. One was mean alveolar diameter (MAD) where the longest distance between walls of single alveoli was measured using MetaVue software (Molecular devices, Sunnyvale, CA). At least 20 alveoli / field were measured and at least 8 fields were counted per lung section and expressed in relative units. The other was mean alveolar intercept (MLI) measurement. The measurement was done as described by other group (Ashour *et al.*, 2006) with some modifications. Briefly, the H & E stained sections of both BPD and control lung were used to capture images using a mounted digital camera under 10 X

objective of a Nicon Eclipse E600 microscope. Similar fields were selected which were devoid of many bronchi and bigger blood vessels. Using the MetaVue software, the digital images where enhanced to the same magnification and five lines were drawn across each image: two connecting opposite vertices, 2 bisecting the opposite sides and one at a random position. The MLI was then calculated by dividing the length of each line by the total number of alveolar intercepts for that line. Twenty five lines were used per lung to calculate the MLI and there were at least 4 lungs per treatment.

For alveolar secondary crests analysis, modified Hart's elastin staining was done. The resorcin-fuchsin stock solution, tartarazine and the protocol were generous gifts from Dr. Barry Starcher at University of Texas Health Center at Irving. Briefly, the slides were de-paraffinized in xylene and rehydrated in descending grades of alcohol. They were then placed in the working solution made by diluting the resorcin-fuchsin stock using 70% alcohol and concHCl. The staining was done overnight. Counter staining was done using tartarazine solution made using water and acetic acid. Elastin was stained purple to black and tartarazine provided a yellow background. For quantification, the number of alveolar secondary crests per 20 X field was counted and at least 5 such fields were counted per slide. There were 4 lungs per treatment.

Microarray

miRNA expression profiling was performed on an in-house miRNA microarray platform as previously described (Wang et al., 2007). For each sample, 600 ng enriched small RNA was labeled with the NCode miRNA Labeling System (Invitrogen, Carlsbad, CA) and purified with the MinElute PCR Purification Kit (Qiagen, Valencia, CA). Labeled

RNA recovered from 120 ng small RNA was used in each hybridization. Equal amounts of small RNAs of all the samples were pooled together as a common reference. To each block, one sample labeled with Alexa Fluor 3 or Alexa Fluor 5 was co-hybridized with the common reference labeled with the other dye (Alexa Fluor 5 or Alexa Fluor 3). Dye swaps were performed. After hybridization, the slides were scanned with ScanArray Express (PerkinElmer Life and Analytical Sciences, Boston, MA) and the images were analyzed with GenePix 5.0 pro (Axon Instruments, Inc. Union City, CA). The signals were qualified with the software, Realspot (Chen & Liu, 2005). MiRNAs with an average QI (quality index) < 1 were excluded from further analysis. The miRNAs that passed the quality test were then analyzed with the software SAM (Significant Analysis of Microarrays, Stanford University, http://www-stat.stanford.edu/~tibs/SAM/).

Quantitative real time PCR

The qrtPCR was performed following the protocol described previously with some modifications (Shi & Chiang, 2005). Total RNA was treated with TURBO DNA-*free*™ (Ambion) to remove DNA contamination. The Poly(A) tail was added to total RNA (1 µg) by *E. coli* Poly(A) Polymerase (Ambion) at 37°C for 1 h. The first strand cDNA was generated with M-MLV Reverse Transcriptase (Invitrogen) and Poly T adaptor (5'-GCGAGCACAGAAT TAATACGACTCACTATAGGTTTTTTTTTTTTVN-3', where V= A, G or C, N= A, T, G or C). The first strand cDNA was diluted 50 times. The diluted template cDNA (10 µl) was mixed with 12.5 µl qPCR™ Mastermix Plus for SYBR® Green I – Low Rox (Eurogentec, San Digeo, CA), the universal reverse primer and the forward primer specific for each miRNA to a final volume of 25 µl. The list of primers is given in Table 1. The reactions were incubated at 95°C for 10 min, followed by 40 cycles

of 95°C for 15 s and 60°C for 60 s. All PCR reactions were carried out in duplicate. U2 was also amplified as an internal reference. The relative expression of each miRNA was calculated with the equation, $2^{-(CTmiRNA-CTU2)}$

Table 3 Primers used for real time PCR

miRNA	Forward Primer
rno-miR-21	5'-TAGCTTATCAGACTGATGTTGA-3'
rno-miR-34a	5'-TGGCAGTGTCTTAGCTGGTTGTT-3'
rno-miR-126*	5'-CATTATTACTTTTGGTACGCGAA-3'
rno-miR-141	5'-TAACACTGTCTGGTAAAGATGG-3'
rno-miR-150	5'-TCTCCCAACCCTTGTACCAGTG-3'
rno-miR-151*	5'-TCGAGGAGCTCACAGTCTAGTA-3'
rno-miR-290	5'-CTCAAACTATGGGGGCACTTTTT-3'
rno-miR-335	5'-TCAAGAGCAATAACGAAAAATGT-3'
rno-miR-342	5'-TCTCACACAGAAATCGCACCCGTCA-3'
U2	5'-GTTGGAATAGGAGCTTGCTCCGTCC-3'
Reverse primer	5'-GCGAGCACAGAATTAATACGAC-3'

Statistical Analysis

For microarray, statistical analysis was done as described earlier. For all other experiments, paired t-test was performed. A p value of < 0.05 was considered significant. All values are presented as means ± S.E.

RESULTS

Survival, lung histology, morphometrical analysis, fibrin deposition and alveolar secondary crest formation

In both oxygen-exposed and control pups, the survival rate was similar (100%) till P7. From P7 mortality was observed in the oxygen-exposed group. By the end of treatment

(P13) the survival rate of the exposed group was 44% while that of control was 100%. This is consistent with the previously reported survival rates in hyperoxia exposure studies in neonatal rats (Wagenaar *et al.*, 2004).

From H&E stained sections, microscopic lesions in the lung specimens are consistently present in tissues from treated (BPD) animals and ranged from moderate to marked in severity (Fig.13a). In lungs from animals with BPD, alveolar septa remain simplified with absence of alveolar branching. Alveolar lumens were markedly enlarged, compared to those of control animals, as a result of the decrease in alveolar septal complexity (Fig. 13a). An unequivocal and consistent difference in the cellularity or thickness of alveolar septa was not discernible. The alveolar size change was quantified by measuring the mean alveolar diameter (MAD), which is the mean of the longest distance between the walls of alveoli and by mean linear intercept (MLI) measurement which is a more extensive representation of alveolar septal development (Fig.13b). MAD measurements showed almost 50% increase in the longest diameter in BPD lung while MLI values for BPD was ~40 % more than the control lung (Fig. 13b).

Figure 13a

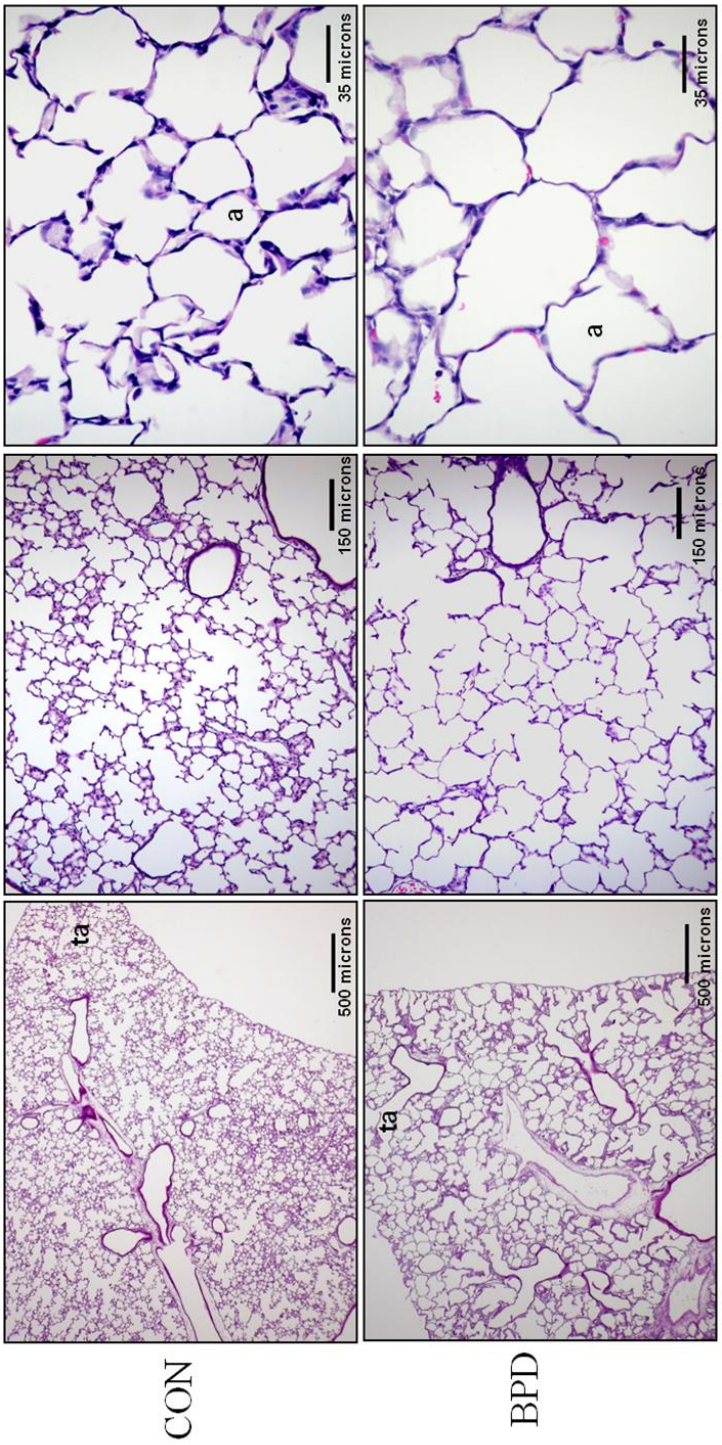

CON
BPD
ta
ta
500 microns
500 microns
150 microns
150 microns
a
a
35 microns
35 microns

Figure 13b

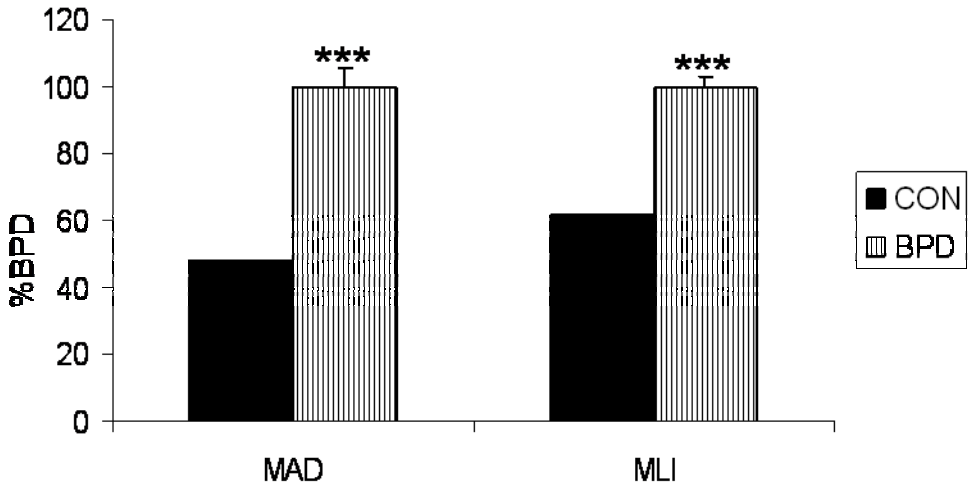


Figure 13a - H&E staining of BPD rat lung sections

BPD was induced in new born rats by exposing them to ~95% oxygen from P3 to P13. The control pups from the same litter were kept side by side in atmospheric O_2 concentration for the same period. At the end of P13 the lung were isolated after proper fixation using 4% paraformaldehyde, sectioned and H&E stained.The upper panels represent the sections from control rat lungs (CON) and the lower panels contain sections from BPD lungs. a=alveolus, ta= terminal airway.

13b - Mean alveolar diameter (MAD) and mean linear intercept measurements (MLI) of BPD and control rat pups

H&E stained rat lung sections from BPD and control rats were taken and the mean diameter of individual alveoli was measured using MetaVue soft ware. MLI, representing the distance between inter-alveolar walls, which gives a more extensive measurement of alveolar sizes, was also measured in BPD and control rat lung sections. For MAD, at least 20 alveoli from 8 different fields were measured per lung section in relative units and expressed as %BPD. For MLI, at least 5 different fields per section were analyzed and given in relative units as %BPD. The data was from lung sections of 4 different rats per treatment. Value was expressed as mean ± SE. *** P< .005 vs Con. Paired t-test.

Harts's stain for elastin revealed significantly fewer secondary crests, which were distinctive because of their abundant elastic fibers (the black arrows), in BPD tissues than in control tissues (Fig. 2a). In most regions of the control lung, ~ 40 secondary crests

were recognized per single 20 X field. In BPD lungs, secondary crests were less distinct because they contained fewer elastic fibers. Their presence was significantly less and their prevalence was regionally variable, averaging approximately 12 per 20X field. The amount of elastic fibers in the alveolar septae of BPD lungs was subjectively less than that seen in alveolar septae of control lungs. Trichrome stains revealed an equivocal and subtle increase in collagen within the alveolar septa of BPD lungs when compared to control tissues (data not shown). There was no inflammatory infiltrates or signs of prominent fibrosis. All these results demonstrated that the lesions in this model are consistent with that described for 'new' BPD pathology.

Figure 14

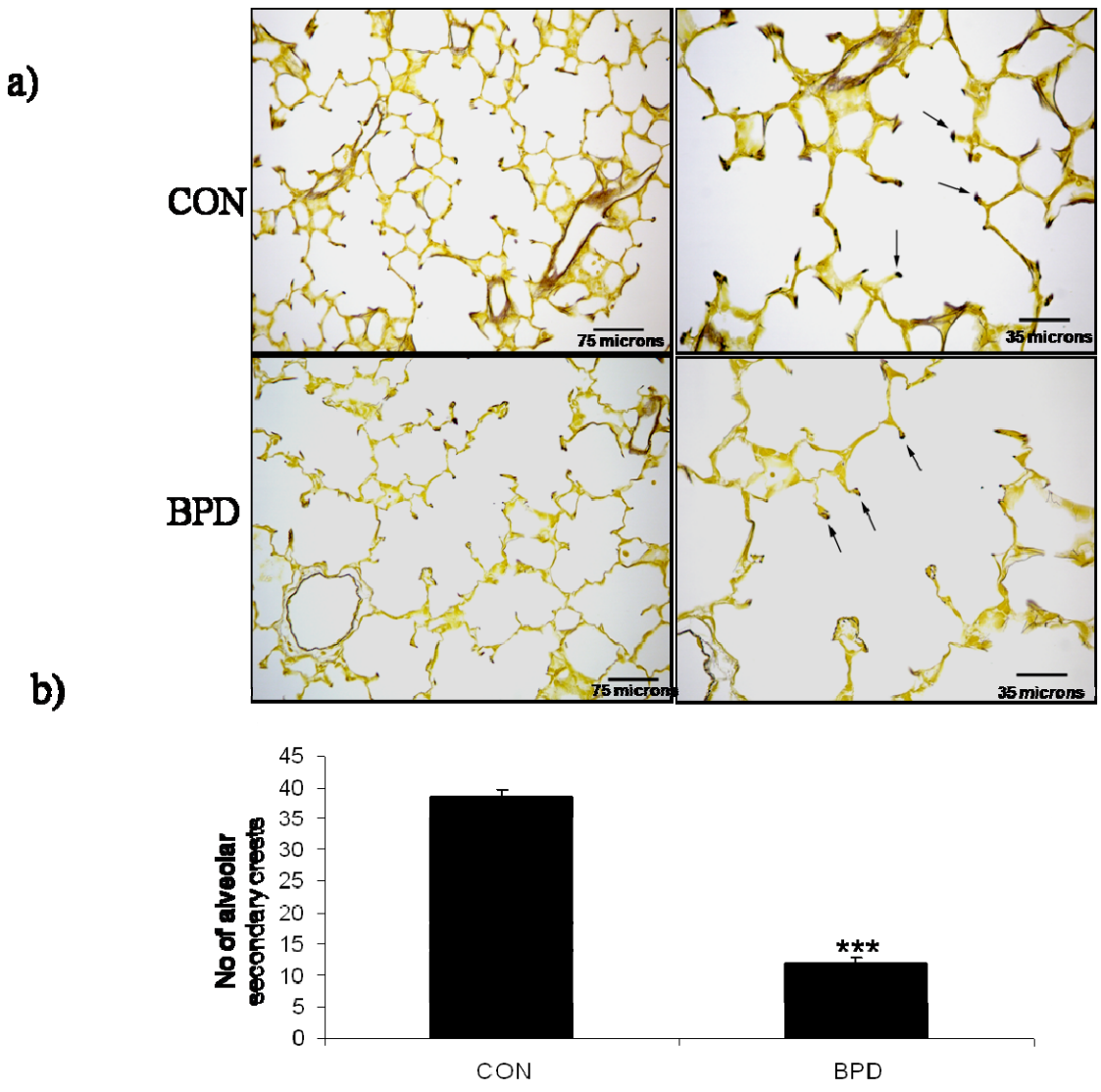

a)
CON
BPD
75 microns
35 microns
75 microns
35 microns
b)
No of alveolar secondary crests
45
40
35
30
25
20
15
10
5
0
***
CON
BPD

Figure 14a - Evaluation of the formation of alveolar secondary crests

Alveolar secondary crest formation was evaluated using the Harts's elastin staining. Elastin which is abundant in the secondary crests can be visualized as purple stained areas in the sections (arrows). The upper and lower panel shows elastin staining in control (CON) and BPD lung, respectively.

14b - Quantification of secondary crests

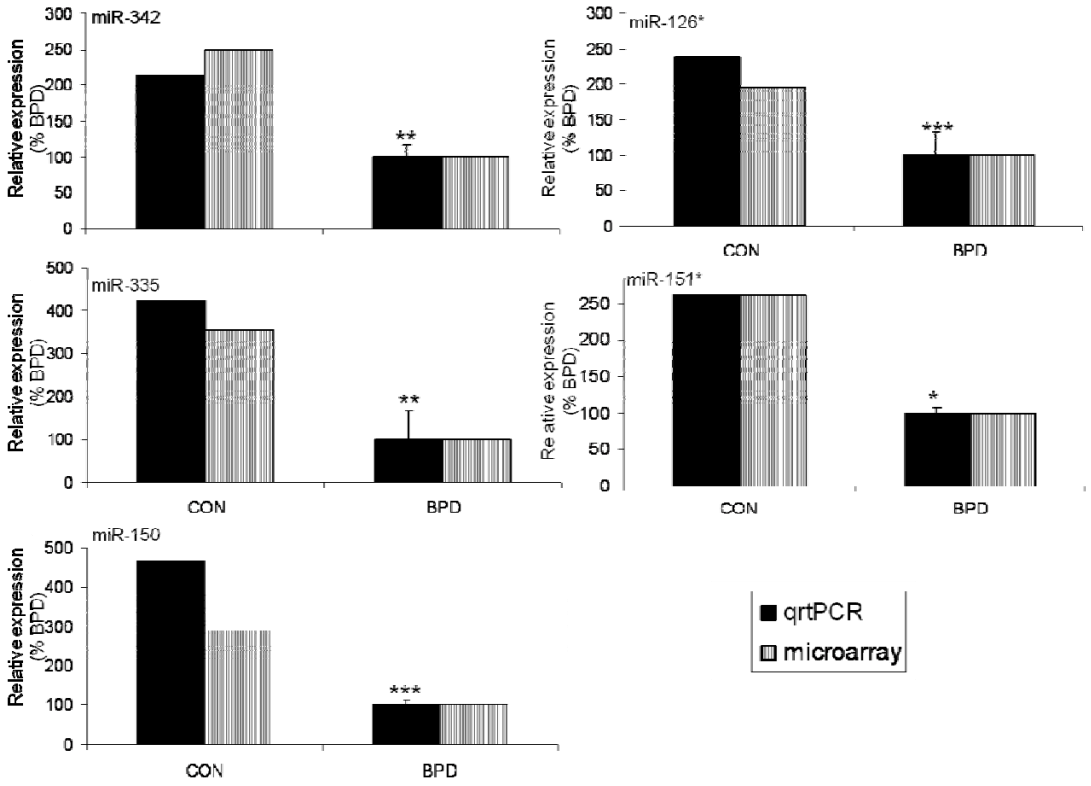
The number of secondary crests per 20X filed were counted. At least 5 different fields per section were counted. The data was from lung sections of 4 different rats per treatment. Value was expressed as mean ± SE. *** = P < .005 v.s Con. Paired t-test.

Microarray and quantitative real time PCR verification of miRNAs differentially expressed in BPD

To detect the miRNA expression profile in BPD, we used the miRNA microarray platform used in our laboratory (Wang *et al.*, 2007). The microarray contained probes for 227 non-redundant RNAs: 177 rat miRNAs, 5 human miRNAs, 31 mouse miRNAs and 14 other kinds of RNAs and controls. miRNAs miR-342, miR-335, miR-150, miR-126* and miR-157* were significantly down regulated in the BPD lung while miR-21, miR-141, miR-290 and miR-34a were upregulated. To further confirm this result, quantitative rtPCR was done and all the results were verified except for miR-290. The expression levels of downregulated (Fig. 15a) and the upregulated miRNA (Fig. 15b) in both microarray and qrtPCR, were given as a percentage to BPD.

Figure 15

a.


miR-342
Relative expression (% BPD)
300
250
200
150
100
50
0
**
CON
BPD
miR-126*
***
miR-335
400
300
**
miR-151*
Re ative expression
250
200
150
100
50
*
miR-150
500
400
300
200
100
***
qrtPCR
microarray

b

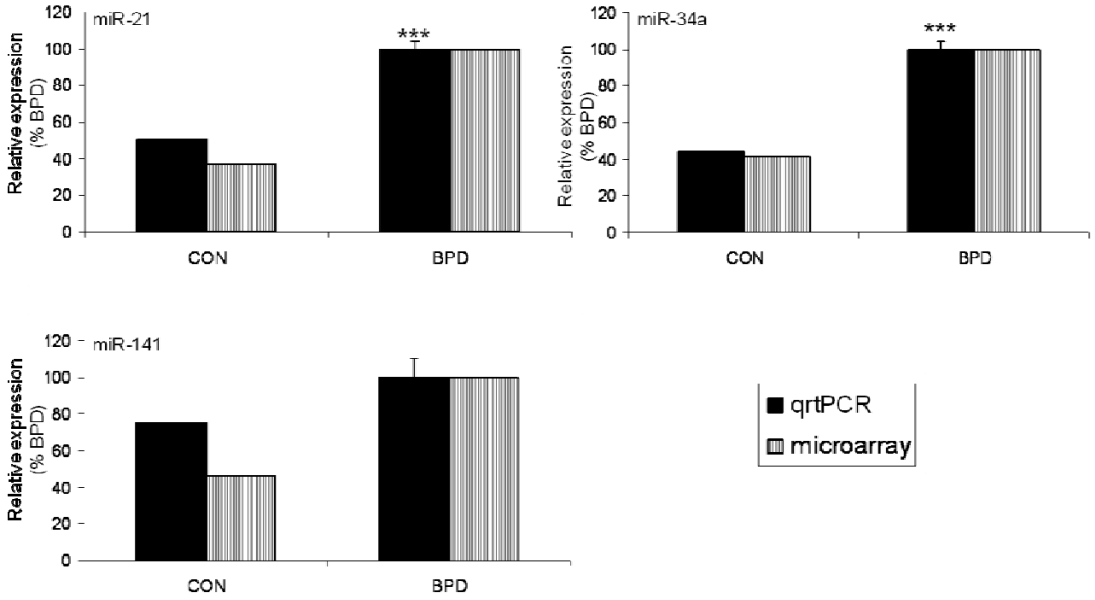

miR-21
Relative expression (% BPD)
120
100
80
60
40
20
0
***
CON
BPD
miR-34a
miR-141
qrtPCR
microarray

Figure 15 - miRNA expression pattern in BPD

Microarray and qrtPCR were done to determine the expression of various miRNAs that significantly changed in BPD lung. BPD and control rat lungs were collected, RNA isolated and used for microarray and qrtPCR. The results were expressed as percentage of BPD.

a) Downregulated miRNAs in BPD

miR-342, miR-335, miR-150, miR-126* and miR-151*.

b) Upregulated miRNAs in BPD

miR-21, miR-141 and miR-34a. The data was from samples of 4 different rat lungs per treatment. Value was expressed as mean ± SE. *** P value < 0.005 vs Con, ** P value < 0.01 vs Con and * P value < 0.05. Paired t-test.

DISCUSSION

In this study, we have identified miRNAs that were significantly changed in BPD using a microarray platform. Five miRNAs, namely, miR-342, miR-335, miR-150, miR-126* and miR-151* were down regulated in BPD. miRNAs miR-21, miR-141 and miR-34a and miR-290 were found to be up regulated in BPD. The results were further confirmed using qrtPCR. All miRNAs except miR-290 showed consistent expression trend when compared to microarray expression profile thus validating both our platform and data.

We found Neonatal rats exposed to ~95% oxygen from P3 to P13 showed lesions consistent with that described for ‘new’ BPD. Decreased in number, large and simplified alveoli with fewer secondary alveolar crests have been the hall mark of new BPD with

other lesions like elastin formation, fibrosis, inflammation and airway epithelial proliferation varying from moderate to absent (Coalson, 2003; Bourbon *et al.*, 2005). The new and milder form of BPD manifests in more immature infants and the primary cause for pathogenesis shifted from oxidative injury and mechanical ventilation to the immaturity of the developing lung that is forced to undergo gaseous exchange. The molecular mechanisms that contribute to both manifestation and progression of this disease are poorly understood. It has been shown that disruption of normal angiogenesis and improper development of capillaries in relation to the alveolar septa might be a major contributing factor towards manifestation of BPD (Jakkula *et al.*, 2000; Kunig *et al.*, 2005). Abnormal signaling mediated by vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF) and insulin like growth factors (IGF) are thought to contribute to BPD (Veness-Meehan *et al.*, 1997; Weinstein *et al.*, 1998; Jakkula *et al.*, 2000; Kasahara *et al.*, 2000; Afshar *et al.*, 2003; Das & Ravi, 2004; Wagenaar *et al.*, 2004). All these pathways are major players in controlling proper lung development. Since impaired alveolar formation and subsequent defective development of the respiratory zone is an essential feature of BPD, understanding the molecular mechanisms that control proper septation and alveolarization can provide greater insights into pathogenesis of BPD. The advent of miRNA as one of the major regulatory components of biological process necessitates the study of its role in BPD pathogenesis. This, in turn, can bring new insights to the finer mechanisms of BPD pathogenesis.

The role of miRNAs in controlling various biological processes ranging from organ development to virus invasion is subject of recent investigations. Their importance in various developmental processes is established including embryonic cell differentiation,

embryo development, neuronal development, cardiac development, regulation of immunity and adipocyte differentiation and lung morphogenesis is established (Esau *et al.*, 2004; Kanellopoulou *et al.*, 2005; Lu *et al.*, 2005; Harris *et al.*, 2006; Hobert, 2006; Kloosterman & Plasterk, 2006; Rodriguez *et al.*, 2007; van Rooij *et al.*, 2007; Zhao *et al.*, 2007). Their role in modulating various forms of cancer and infectious diseases mediated by viruses have also been studied (Takamizawa *et al.*, 2004; Eis *et al.*, 2005; He *et al.*, 2005b; Yanaihara *et al.*, 2006; Blenkiron & Miska, 2007; Triboulet *et al.*, 2007).

In the present study, the most interesting miRNAs that showed differential expression between BPD and control lung were miR-21 and miR-335. miR-21 was up regulated in BPD while miR-335 was down regulated. It is known that miR-335 is a potent metastasis suppressor, a proliferation inhibitor and an apoptosis promoter (Sathyan *et al.*, 2007; Tavazoie *et al.*, 2008). miR-21, on the other hand, is a potent proliferation promoter, tumor promoter and inhibitor of apoptosis (Chan *et al.*, 2005; Meng *et al.*, 2006; Si *et al.*, 2007; Zhu *et al.*, 2007). It has been shown that knockdown of miR-21 can lead to the activation of caspases and cause increased cell death (Chan *et al.*, 2005). More interestingly, it has also been demonstrated that miR-335 knockdown prevented cell death caused by miR-21 suppression and that miR-335 might be a functional antagonist of miR-21 (Sathyan *et al.*, 2007). The opposite trends of expressions showed by these potentially antagonizing miRNAs in BPD might have some functional significance. Proper development of any organ involves controlled proliferation and apoptosis. Down regulation of miR-335 and concurrent up regulation of miR-21 is suggestive of a pro-proliferative and anti-apoptotic signaling environment in BPD lung. There are ~ 170 predicted targets for miR-335 and ~186 predicted targets for miR-21 as per the

TargetScan 4.2 algorithim (Lewis *et al.*, 2003). But till date, only one target has been experimentally verified for miR-335 and two for miR-21. Sox-4, a transcription factor important in regulation of embryonic development and determination of cell fate is verified as a target for miR-335 (Kamachi *et al.*, 2000; Tavazoie *et al.*, 2008). The verified targets for miR-21 are tropomyocin and BCL-2, both tumor suppressor genes (Si *et al.*, 2007; Zhu *et al.*, 2007). These studies were performed in cancer cell lines. However since development involves controlled cell proliferation and cell death and a developmental disease like BPD might involve dysregulaton of these processes, the differential expression of these two miRNAs might have functional significance in BPD pathogenesis.

Other predicted targets for miR-335 that might have functional significance in BPD includes dishevelled associated activator of morphogenesis 1(DAAM1), Smad nuclear interacting protein 1 (SNIP1), ubiquitin-conjugating enzyme E2H (UBC8), eukaryotic translation termination factor 1 (ETF), RAS p21 protein activator 1 (RASA1), mitogen-activated protein kinase kinase kinase 2 (MAP3K2), insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2), fibroblast growth factor receptor substrate 2 (FRS2), homeobox B3 (HOXB3), integrin beta 8 (ITGB8) and early growth response 3 (EGR3) (http://www.targetscan.org/cgi-bin/targetscan/vert_42/targetscan.cgi?species=Human&gid=&mir_c=&mir_sc=miR-335&mir_nc=&mirg=). miR-21 is predicted to target sprouty homolog 1 (SPRY1), fibroblast growth factor receptor substrate 2 (FRS2), Smad 7 and programmed cell death 4 (PDCD4) (http://www.targetscan.org/cgi-bin/vert_42/targetscan.cgi?mirg=rno-miR-21). It is interesting to note that all these targets are components of pathways that regulate

development by controlling proliferation, differentiation, cell migration or apoptosis. Of these targets, ITGB8, a target of miR-335 is known to play an important role in airway epithelial cell proliferation and cell matrix interactions by modulating transforming growth factor beta pathway (Fjellbirkeland *et al.*, 2003). In tumor systems, It is also known to play an important role in mediating epithelial-mesenchymal transformations in unison with EGFR signaling pathways (Yan & Shao, 2006). So down regulation of miR-335 in BPD might lead to dysregulation of these processes contributing to pathogenesis. For miR-21, the predicted target SPRY1 might be of great significance as it is an antagonizer of FGF signaling and also modulate EGF signaling (Hacohen *et al.*, 1998; Egan *et al.*, 2002). Both these signaling pathways are important in lung development.

miR-342 is another miRNA that is down regulated in BPD. Just like miR-335, it is also shown to be pro-apoptotic tumor suppressor whose down regulation induces progression of colorectal cancer (Grady *et al.*, 2008). miR-342 is located in the introns of the host gene EVL, a member of Ena/Vasp protein family important in controlling cytoskeleton remodeling, cell polarity and cell migration which is coordinately down regulated with miR-342 (Grady *et al.*, 2008). Recently it has been shown to be a member of a large cluster of imprinted miRNAs expressed only from the maternal chromosome. The the cluster is present in a locus that is traditionally known to have genes important in development regulation (Seitz *et al.*, 2004). Other important predicted targets for miR-342 includes bone morphogenetic protein receptor, type II (BMPR2), nuclear respiratory factor 1(NRF1), homeobox B8 (HoxB8) and insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3). BMPR2 is known to have important role in modulating

morphogenesis while NRF1 is a transcription factor that regulates some key metabolic genes important in cell growth and regulation of respiration.

miR-126* is also down regulated in BPD and it has been known that miR-126* is located on a region that is deleted in lung cancer (Yanaihara *et al.*, 2006). The gene for miR-126* was down regulated in almost all types of lung cancer. miR-126*, therefore, might have an anti-proliferative.

miR-150, another down regulated miRNA in BPD, have predicted targets like vascular endothelial zinc finger 1 (VEZF1) implicated in vascular development. Since improper development of vasculature is a contributing factor to BPD, this might be of functional significance. Other important targets for miR-150 include insulin-like growth factor and mitogen-activated protein kinase kinase kinase 12, which contribute to multiple signaling processes important in development. miR-151*, which is also down-regulated in BPD, has the predicated targets insulin-like 3 precursor and LIM homeobox 9. these genes are important in development of multiple organs (Hunter & Rhodes, 2005).

It is both interesting and contrasting to note that one of the upregulated miRNAs in BPD, miR-34a, is a tumor suppresser. It is anti-proliferative, pro-apoptotic and can induce cell cycle arrest by down regulating CCND1 and CDK6 (Chang *et al.*, 2007; Tazawa *et al.*, 2007). miR-141, another up regulated miRNA, is a member of miRNA-200 family which have 5 members. Their down regulation is shown to be essential for epithelial-mesenchymal transitions, which are important in organ development and early tumor metastasis (Gregory *et al.*, 2008). The upregulation of this family can inhibit epithelial-mesenchymal transition by targeting transcriptional repressors of E-cadherin namely

ZEB1 and ZEB2 (Gregory *et al.*, 2008; Korpal *et al.*, 2008). Since miR-141 expression is significantly up regulated in BPD, this might have some effect on the differentiation process involved in lung development too and might contribute to the pathogenesis.

As we see the functional implications of the targets of both up regulated and down regulated miRNAs in BPD, there is no clear cut demarcation that one set have targets that down regulates one process while the other have targets that exclusively antagonizes the same process. Both up regulated and down regulated miRNAs have potential targets that can modulate a variety of processes. The regulatory mechanisms involving miRNAs seems to be much more complex and can fill the whole spectrum ranging from functional antagonism to a significant degree of agonism. However, the present study has given us insights into the miRNAs that might be important players in modulating BPD pathogenesis. To decipher, the role of each individual miRNA in relation to others is the subject of our current investigations.

REFERENCES

1. Afshar S, Gibson LL, Yuhanna IS, Sherman TS, Kerecman JD, Grubb PH, Yoder BA, McCurnin DC & Shaul PW. (2003). Pulmonary NO synthase expression is attenuated in a fetal baboon model of chronic lung disease. *Am J Physiol Lung Cell Mol Physiol* **284,** L749-758.

2. Ashour K, Shan L, Lee JH, Schlicher W, Wada K, Wada E & Sunday ME. (2006). Bombesin inhibits alveolarization and promotes pulmonary fibrosis in newborn mice. *Am J Respir Crit Care Med* **173,** 1377-1385.

3. Bachofen H, Ammann A, Wangensteen D & Weibel ER. (1982). Perfusion fixation of lungs for structure-function analysis: credits and limitations. *J Appl Physiol* **53,** 528-533.

4. Bartel DP. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116,** 281-297.

5. Blenkiron C & Miska EA. (2007). miRNAs in cancer: approaches, aetiology, diagnostics and therapy. *Hum Mol Genet* **16 Spec No 1,** R106-113.

6. Bonikos DS, Bensch KG, Northway WH, Jr. & Edwards DK. (1976). Bronchopulmonary dysplasia: the pulmonary pathologic sequel of necrotizing bronchiolitis and pulmonary fibrosis. *Hum Pathol* **7,** 643-666.

7. Bourbon J, Boucherat O, Chailley-Heu B & Delacourt C. (2005). Control mechanisms of lung alveolar development and their disorders in bronchopulmonary dysplasia. *Pediatr Res* **57,** 38R-46R.

8. Brennecke J, Hipfner DR, Stark A, Russell RB & Cohen SM. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell* **113,** 25-36.

9. Chan JA, Krichevsky AM & Kosik KS. (2005). MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* **65,** 6029-6033.

10. Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Ferlito M, Lowenstein CJ, Arking DE, Beer MA, Maitra A & Mendell JT. (2007). Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* **26,** 745-752.

11. Chen Z & Liu L. (2005). RealSpot: software validating results from DNA microarray data analysis with spot images. *Physiol Genomics* **21,** 284-291.

12. Cheng AM, Byrom MW, Shelton J & Ford LP. (2005). Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. *Nucleic Acids Res* **33,** 1290-1297.

13. Coalson JJ. (2003). Pathology of new bronchopulmonary dysplasia. *Semin Neonatol* **8,** 73-81.

14. Coalson JJ. (2006). Pathology of bronchopulmonary dysplasia. *Semin Perinatol* **30,** 179-184.

15. Das KC & Ravi D. (2004). Altered expression of cyclins and cdks in premature infant baboon model of bronchopulmonary dysplasia. *Antioxid Redox Signal* **6,** 117-127.

16. Egan JE, Hall AB, Yatsula BA & Bar-Sagi D. (2002). The bimodal regulation of epidermal growth factor signaling by human Sprouty proteins. *Proc Natl Acad Sci U S A* **99,** 6041-6046.

17. Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, Lund E & Dahlberg JE. (2005). Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci U S A* **102,** 3627-3632.

18. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, Subramaniam A, Propp S, Lollo BA, Freier S, Bennett CF, Bhanot S & Monia BP. (2006). miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* **3,** 87-98.

19. Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, Ravichandran LV, Sun Y, Koo S, Perera RJ, Jain R, Dean NM, Freier SM, Bennett CF, Lollo B & Griffey R. (2004). MicroRNA-143 regulates adipocyte differentiation. *J Biol Chem* **279,** 52361-52365.

20. Fjellbirkeland L, Cambier S, Broaddus VC, Hill A, Brunetta P, Dolganov G, Jablons D & Nishimura SL. (2003). Integrin alphavbeta8-mediated activation of transforming growth factor-beta inhibits human airway epithelial proliferation in intact bronchial tissue. *Am J Pathol* **163,** 533-542.

21. Grady WM, Parkin RK, Mitchell PS, Lee JH, Kim YH, Tsuchiya KD, Washington MK, Paraskeva C, Willson JK, Kaz AM, Kroh EM, Allen A, Fritz BR, Markowitz SD & Tewari M. (2008). Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVL in colorectal cancer. *Oncogene*.

22. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y & Goodall GJ. (2008). The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* **10,** 593-601.

23. Groneck P & Speer CP. (1995). Inflammatory mediators and bronchopulmonary dysplasia. *Arch Dis Child Fetal Neonatal Ed* **73,** F1-3.

24. Hacohen N, Kramer S, Sutherland D, Hiromi Y & Krasnow MA. (1998). sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell* **92,** 253-263.

25. Harris KS, Zhang Z, McManus MT, Harfe BD & Sun X. (2006). Dicer function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci U S A* **103,** 2208-2213.

26. Hayatdavoudi G, Crapo JD, Miller FJ & O'Neil JJ. (1980). Factors determining degree of inflation in intratracheally fixed rat lungs. *J Appl Physiol* **48,** 389-393.

27. He H, Jazdzewski K, Li W, Liyanarachchi S, Nagy R, Volinia S, Calin GA, Liu CG, Franssila K, Suster S, Kloos RT, Croce CM & de la Chapelle A. (2005a). The role of microRNA genes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A* **102,** 19075-19080.

28. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ & Hammond SM. (2005b). A microRNA polycistron as a potential human oncogene. *Nature* **435,** 828-833.

29. Hobert O. (2006). Architecture of a microRNA-controlled gene regulatory network that diversifies neuronal cell fates. *Cold Spring Harb Symp Quant Biol* **71,** 181-188.

30. Hunter CS & Rhodes SJ. (2005). LIM-homeodomain genes in mammalian development and human disease. *Mol Biol Rep* **32,** 67-77.

31. Husain AN, Siddiqui NH & Stocker JT. (1998). Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. *Hum Pathol* **29,** 710-717.

32. Hutvagner G & Zamore PD. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297,** 2056-2060.

33. Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF & Abman SH. (2000). Inhibition of angiogenesis decreases alveolarization in the developing rat lung. *Am J Physiol Lung Cell Mol Physiol* **279,** L600-607.

34. Jobe AH & Bancalari E. (2001). Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* **163,** 1723-1729.

35. Jobe AH & Ikegami M. (2001). Prevention of bronchopulmonary dysplasia. *Curr Opin Pediatr* **13,** 124-129.

36. Kamachi Y, Uchikawa M & Kondoh H. (2000). Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet* **16,** 182-187.

37. Kanellopoulou C, Muljo SA, Kung AL, Ganesan S, Drapkin R, Jenuwein T, Livingston DM & Rajewsky K. (2005). Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev* **19,** 489-501.

38. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J & Voelkel NF. (2000). Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* **106,** 1311-1319.

39. Kloosterman WP & Plasterk RH. (2006). The diverse functions of microRNAs in animal development and disease. *Dev Cell* **11,** 441-450.

40. Korpal M, Lee ES, Hu G & Kang Y. (2008). The miR-200 Family Inhibits Epithelial-Mesenchymal Transition and Cancer Cell Migration by Direct Targeting of E-cadherin Transcriptional Repressors ZEB1 and ZEB2. *J Biol Chem* **283,** 14910-14914.

41. Kunig AM, Balasubramaniam V, Markham NE, Morgan D, Montgomery G, Grover TR & Abman SH. (2005). Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats. *Am J Physiol Lung Cell Mol Physiol* **289,** L529-535.

42. Lee RC, Feinbaum RL & Ambros V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75,** 843-854.

43. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S & Kim VN. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425,** 415-419.

44. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP & Burge CB. (2003). Prediction of mammalian microRNA targets. *Cell* **115,** 787-798.

45. Lu J, Qian J, Chen F, Tang X, Li C & Cardoso WV. (2005). Differential expression of components of the microRNA machinery during mouse organogenesis. *Biochem Biophys Res Commun* **334,** 319-323.

46. Lu Y, Thomson JM, Wong HY, Hammond SM & Hogan BL. (2007). Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. *Dev Biol* **310,** 442-453.

47. Lund E, Guttinger S, Calado A, Dahlberg JE & Kutay U. (2004). Nuclear export of microRNA precursors. *Science* **303,** 95-98.

48. Mattick JS. (2003). Challenging the dogma: the hidden layer of non-protein-coding RNAs in complex organisms. *Bioessays* **25,** 930-939.

49. Meng F, Henson R, Lang M, Wehbe H, Maheshwari S, Mendell JT, Jiang J, Schmittgen TD & Patel T. (2006). Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology* **130,** 2113-2129.

50. Northway WH, Jr., Rosan RC & Porter DY. (1967). Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. *N Engl J Med* **276,** 357-368.

51. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E & Ruvkun G. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* **408,** 86-89.

52. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, van Dongen S, Grocock RJ, Das PP, Miska EA, Vetrie D, Okkenhaug K, Enright AJ, Dougan G, Turner M & Bradley A. (2007). Requirement of bic/microRNA-155 for normal immune function. *Science* **316,** 608-611.

53. Sathyan P, Golden HB & Miranda RC. (2007). Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: evidence from an ex vivo model of the fetal cerebral cortical neuroepithelium. *J Neurosci* **27,** 8546-8557.

54. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N & Zamore PD. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115,** 199-208.

55. Seitz H, Royo H, Bortolin ML, Lin SP, Ferguson-Smith AC & Cavaille J. (2004). A large imprinted microRNA gene cluster at the mouse Dlk1-Gtl2 domain. *Genome Res* **14,** 1741-1748.

56. Shi R & Chiang VL. (2005). Facile means for quantifying microRNA expression by real-time PCR. *Biotechniques* **39,** 519-525.

57. Si ML, Zhu S, Wu H, Lu Z, Wu F & Mo YY. (2007). miR-21-mediated tumor growth. *Oncogene* **26,** 2799-2803.

58. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T & Takahashi T. (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* **64,** 3753-3756.

59. Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, Gerald WL & Massague J. (2008). Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* **451,** 147-152.

60. Tazawa H, Tsuchiya N, Izumiya M & Nakagama H. (2007). Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc Natl Acad Sci U S A* **104,** 15472-15477.

61. Triboulet R, Mari B, Lin YL, Chable-Bessia C, Bennasser Y, Lebrigand K, Cardinaud B, Maurin T, Barbry P, Baillat V, Reynes J, Corbeau P, Jeang KT & Benkirane M. (2007). Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* **315,** 1579-1582.

62. Turunen R, Nupponen I, Siitonen S, Repo H & Andersson S. (2006). Onset of mechanical ventilation is associated with rapid activation of circulating phagocytes in preterm infants. *Pediatrics* **117,** 448-454.

63. Van Marter LJ, Allred EN, Pagano M, Sanocka U, Parad R, Moore M, Susser M, Paneth N & Leviton A. (2000). Do clinical markers of barotrauma and oxygen toxicity explain interhospital variation in rates of chronic lung disease? The Neonatology Committee for the Developmental Network. *Pediatrics* **105,** 1194-1201.

64. van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J & Olson EN. (2007). Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **316,** 575-579.

65. Veness-Meehan KA, Bottone FG, Jr. & Stiles AD. (2000). Effects of retinoic acid on airspace development and lung collagen in hyperoxia-exposed newborn rats. *Pediatr Res* **48,** 434-444.

66. Veness-Meehan KA, Moats-Staats BM, Price WA & Stiles AD. (1997). Re-emergence of a fetal pattern of insulin-like growth factor expression during hyperoxic rat lung injury. *Am J Respir Cell Mol Biol* **16,** 538-548.

67. Veness-Meehan KA, Pierce RA, Moats-Staats BM & Stiles AD. (2002). Retinoic acid attenuates O2-induced inhibition of lung septation. *Am J Physiol Lung Cell Mol Physiol* **283,** L971-980.

68. Wagenaar GT, ter Horst SA, van Gastelen MA, Leijser LM, Mauad T, van der Velden PA, de Heer E, Hiemstra PS, Poorthuis BJ & Walther FJ. (2004). Gene expression profile and histopathology of experimental bronchopulmonary dysplasia induced by prolonged oxidative stress. *Free Radic Biol Med* **36,** 782-801.

69. Wang Y, Weng T, Gou D, Chen Z, Chintagari NR & Liu L. (2007). Identification of rat lung-specific microRNAs by micoRNA microarray: valuable discoveries for the facilitation of lung research. *BMC Genomics* **8,** 29.

70. Weinstein M, Xu X, Ohyama K & Deng CX. (1998). FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. *Development* **125,** 3615-3623.

71. Wu L, Fan J & Belasco JG. (2006). MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci U S A* **103,** 4034-4039.

72. Yan W & Shao R. (2006). Transduction of a mesenchyme-specific gene periostin into 293T cells induces cell invasive activity through epithelial-mesenchymal transformation. *J Biol Chem* **281,** 19700-19708.

73. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM & Harris CC. (2006). Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* **9,** 189-198.

74. Yi R, Qin Y, Macara IG & Cullen BR. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17,** 3011-3016.

75. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ & Srivastava D. (2007). Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell* **129,** 303-317.

76. Zhu S, Si ML, Wu H & Mo YY. (2007). MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem* **282,** 14328-14336.

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Contribution of co-authors

Yang Wang did the miRNA microarray for BPD. Melanie Breshears helped with evaluating the histopathology of BPD.

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CHAPTER V

HEMOGLOBIN IS EXPRESSED IN ALVEOLAR EPITHELIAL TYPE II CELLS

ABSTRACT

Hemoglobin is the main oxygen carrying heme protein in erythrocytes. In an effort to study the differential gene expression of alveolar epithelial type I and type II cells using DNA microarray technique, we found that the mRNAs of hemoglobin α- and β-chains were expressed in type II cells, but not in type I cells. The microarray data were confirmed by RT-PCR. The mRNA expression of both chains decreased when type II cells trans-differentiated into type I-like cells. Immunocyto/histochemistry revealed that hemoglobin protein was specifically localized in type II cells of a lung cell mixture and rat lung tissue. The endogenous synthesis of hemoglobin in alveolar epithelial cells suggest that hemoglobin may have unidentified functions other than oxygen transport in the lung.

Key words: Hemoglobin, Trans-differentiation, Alveolar Epithelial Cells.

INTRODUCTION

Alveolar epithelium of the lung is primarily composed of two kinds of cells: type I cells (AEC I) and type II cells (AEC II). The AEC I are large squamous cells while AEC II are cuboidal in shape (Crandall and Matthay, 2001). Even though the number of AEC II is roughly twice as much as type I cells, AEC II occupy only a small percentage of alveolar surface area (<5%) and are mainly limited to the corners of alveoli. These cells

are also functionally different. AEC I are the main epithelial component of the thin air–blood barrier. AEC II have important secretory capacities, such as the secretion of the lung surfactant, and are the progenitor cells for the terminally differentiated AEC I (Fehrenbach, 2001; Warburton and Bellusci, 2004). Because of the difference in the functions of these two cell types, there are a large number of genes and proteins differentially expressed in these cells and many of them have been used as markers for the specific cell type (Dobbs et al., 1985; Kalina et al., 1992; Padro et al., 1990).

Hemoglobin is one of the most abundant heme-binding proteins and consists of two α- and two β-globin polypeptides, which form a heterotetramer with a heme moiety in each monomer. The primary function of hemoglobin has been attributed to oxygen binding and transport in the blood circulation. Other heme binding proteins like cytochromes, extracellular peroxidases, and cytoglobin, neuroglobin, and myoglobin have roles in catalysis, oxygen consumption, cytoprotection and oxygen sensing (Brunori, 2001; Burmester and Hankeln, 2004; Edgar and Polak, 2001; Hardison, 1998). In this paper, we report, for the first time, the expression of hemoglobin in AEC II of the lung. This finding is novel in the way that no other known cell population, other than erythrocytes and their progenitors, was reported to express this protein under normal physiological conditions. Further functional studies in this direction may uncover unidentified roles of hemoglobin in the alveolar epithelium.

MATERIALS AND METHODS

Isolation and culture of AEC II

AEC II were isolated from pathogen free male Sprague-Dawley rats (250-275g) by the improved method as previously described by us (Chen et al., 2004). In brief, the perfused lungs were lavaged and digested with elastase (3 units/ml, Worthington Biochemical Corporation, Lakewood, NJ). The cell mixture was filtered through 160- and 37-µm nylon mesh once, and 15-µm nylon mesh twice, and plated on rat IgG-coated plates to remove macrophages. The unattached cells from IgG plates were further incubated with anti-Leukocyte Common Antigen (anti-LC, 40 µg/ml) (Accurate, Westbury, NY), rat IgG (70 µg/ml), and anti-T1 α antibodies for 30 minutes at 40C. The cells were then incubated for 20 min with anti-rat and anti-mouse IgG-conjugated magnetic beads (Dynal Biotech, Lake success, NY). A magnetic field was applied to remove the cells attached to the magnetic beads. The purity of AEC II was >96 as determined by modified Papanicolaou staining and the viability was >98% Freshly isolated cells were directly used for DNA microarray analysis and RT-PCR. AEC II were also seeded onto 35 mm tissue culture-treated plastic dishes at a density of 1.3 x 10^6 cells per dishes in Minimum Essential Medium (MEM) with 10 % fetal bovine serum and cultured for 1 to 7 days. In this culture system, the trans-differentiation of AEC II to AEC I-like cells becomes evident from day 3 onward and was nearly complete by day 5.

Isolation of AEC I

AEC I was isolated according to our recently published protocol (Chen et al., 2004). The perfused lungs were digested three times with 4.5 U/ ml elastase at 37°C for 10 minutes. After being filtered through 160- and 37-µm nylon mesh, the resulting cell mixture was

panned on a rat IgG-coated bacteriological Petri dish for 30 min at 37°C. The cells were then incubated with rat IgG (40 μg/ml) and anti-LC (40 μg/ml), followed by the incubation with sheep anti-rat IgG Dynabeads (100 μl/rat) and goat anti-mouse IgG Dynabeads (100 μl/rat) at 4°C for 20 min. The beads were removed and the cells were further incubated with rabbit anti-rat T1α antibodies (40 μg/ml) at 4°C for 40 min. AEC I were collected by the incubation with goat anti-rabbit IgG BioMag beads. The purity of AEC I was >90% and the viability was >95%.

Isolation of lung cell mixture

The procedure was the same as for the isolation of AEC I. The cell mixture was collected after filtering and cytospinned onto microscopic glass slides. The cells were fixed with 4% paraformaldehyde and used for immunocytochemistry.

Microarray analysis

The total RNAs from isolated AEC II and AEC I were extracted using TRI reagents (Molecular Research Center, Cincinnati, OH) and used for cDNA synthesis and 2-step microarray hybridization with 3DNA 50 Expression kit (Genisphere Inc., Hatfield, PA). Total RNA was reverse-transcribed with Cy3- or Alexa 647-specific primers. The cDNA, thus obtained, was purified with the Microcom YM-30 columns (Millipore, Billerica, MA) and mixed with 2x formamide hybridization buffer (50% formamide, 6x SSC, 0.2% SDS) for hybridization at 42ºC for 48 hours. The slides were washed and incubated with Cy3- and Alexa 647-specific capture reagents at 42ºC for 2 hours. The slide was scanned by a laser confocal scanner, ScanArray Express (PerkinElmer Life and Analytical Sciences, Boston, MA). Hybridization images were analyzed using GenePix pro 4

software (Axon Instruments, Inc. Union City, CA). Data analysis were performed using our in-house software Realspot (Chen and Liu, 2005) and SAM package. The details of microarray experiments and the whole data set will be published elsewhere .

Reverse Transcription- Polymerase Chain Reaction

PCR primers were as follows:

β chain of hemoglobin,

Forward, 5'-TGTGACAAGCTGCATGTGGAT-3',

Reverse, 5'-TGACCATTGCACAAAGACAAGA-3',

α-chain of hemoglobin,

Forward, 5'- CCACTCTGAGCGACCTGCAT-3',

Reverse, 5'- GGTGCTCAC AGAGGCAAGGA-3',

18S RNA,

Forward, 5'- TCCCAGTAAGTGCGGGTCATA-3',

Reverse, 5'-CGAGGGCCTCACTAAACCATC-3'.

One microgram of total RNA was reverse-transcribed into cDNA using Super script II (Invitrogen, Carlsbad, CA). The PCR was performed using the following conditions: 96º for 5 minutes, followed by denaturation at 94ºfor 30 seconds, annealing at 60º for 30 seconds, and extension at 72 º for 30 seconds. The PCR product was then visualized by agrose gel electrophoresis.

Immunocytochemistry and Immunohistochemistry

Paraffin embedded tissue sections of perfused rat lung were dewaxed using xylene, rehydrated using descending grades of ethanol, and washed in phosphate buffer saline

(pH 7.4). Antigen retrieval was done by boiling the slides for 15 minutes in 20 mM citrate buffer (pH 6.0). The subsequent steps were the same for both immunocytochemistry and immunohistochemistry. Briefly, the fixed tissues or cytospinned cells were permeabilized with 0.4% Triton X-100 for 20 minutes and blocked for one hour in 10% fetal bovine serum .The slides were then incubated with mouse anti-LB-180 (1:200 dilution) (Covance, Richmond, CA) and rabbit anti-rat hemoglobin (1:300 dilution) (Biogenesis, Poole, England) antibodies for overnight at 40C. The slides were then washed and incubated with Alexa 568-conjugated anti-rabbit and Alexa 488-conjugated anti-mouse secondary antibodies (Jackson Immuno Res, PA). Slides were then washed and mounted on an antifade medium (5% n-propyl gallate and 80% glycerol in PBS) and viewed through a Nikon Eclipse E600 fluorescence microscope.

RESULTS

Hemoglobin was highly expressed in AEC II and not in AEC I

Using our in-house 10K rat gene DNA microarray, we performed gene profiling of freshly isolated AEC I and AEC II. Two of the genes with the highest fold change between AEC II and AEC I were α-chain (NM_013096) and ß-chain (NM_033234) of hemoglobin. The ratios between AEC II and AEC I were 13.8 ± 1.2 and 7.4 ± 1.2, respectively (means ± SE, n=30) (Fig. 1A). Two other hemoglobin probes (X56327 and M32509) in the 10K set also showed a similar change (9.3 ± 1.2 and 9.1 ± 1.2). The M32509 probe corresponds to the ß -chain mRNA 3' end and has a sequence similarity of 96 % to the 3' end of NM_033234. The X56327 probe represents epsilon 2 globin gene. Three related heme proteins, cytoglobin (Burmester et al., 2002a; Pesce et al.,

2002a), neuroglobin (Burmester and Hankeln, 2004c; Pesce et al., 2002b), and myoglobin (Brunori, 2001b) were not expressed in both cell types in our microarray data set. RT-PCR analysis showed that the mRNAs of hemoglobin α- and ß-chains were highly expressed in AEC II, but was not detectable in AEC I (Fig. 16B), validating the microarray data.

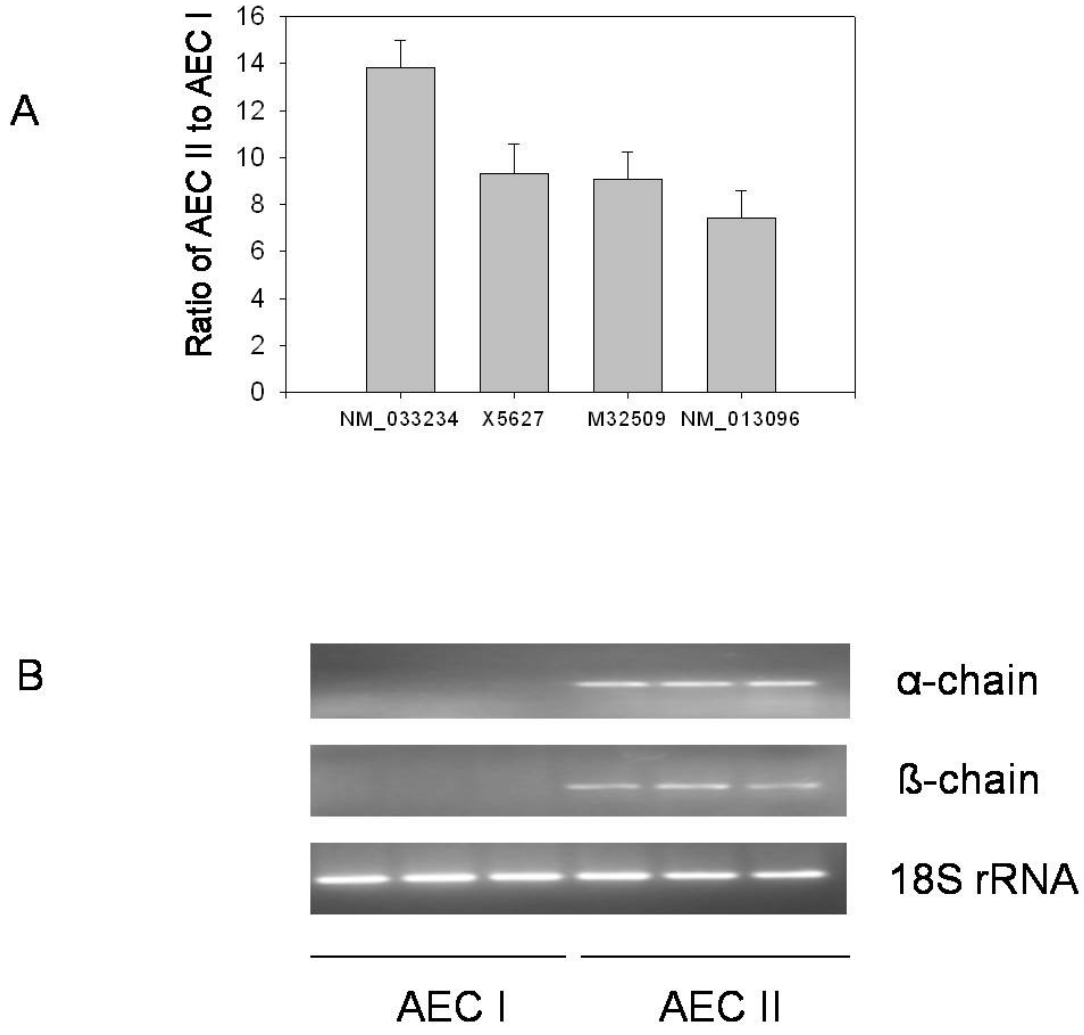


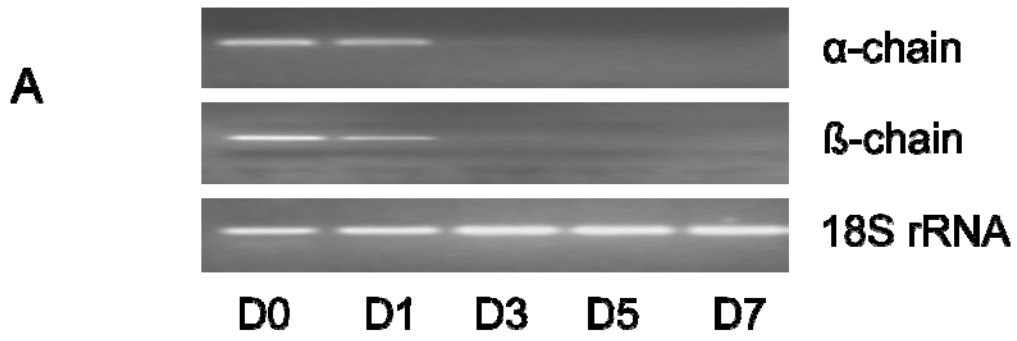
Figure 16 - The mRNA expression of hemoglobin in alveolar epithelial cells.

(A) DNA microarray. The ratios of AEC II and AEC I were from 10K rat gene DNA microarray hybridizations and are presented as means ±SE (n=30, 5 biological and 6 technical replications). Two probes from the β chains of hemoglobin (NM_033234, and M32509), one from epsilon 2 (X56327), and one from α chain (NM_013096) were shown. (B) RT-PCR. The total RNA from 3 independent AEC II and AEC I preparations were reversed transcribed to cDNA. The α- and β-chain of hemoglobin PCR-amplified for 30 cycles. 18S rRNA was used as a control.

The mRNA expression of hemoglobin decreased as AEC II trans-differentiate to AEC I-like cells

Although the purity of AEC II was >96%, the possibility still exists regarding contaminations in our cell preparations. We further examined the mRNA expression of hemoglobin during the trans-differentiation of AEC II to AEC I-like cells. When being cultured on tissue culture treated plastic dishes, AEC II gradually converted to AEC I-like cells. The latter had a similar cell shape as AEC I and express AEC I markers, but lacked AEC II markers. By day 2, the trans-differentiation began and was completed by day 5 to day 7, depending on culture conditions. The cells were collected on Day 0, 1, 3, and 5 and total RNA extracted. RT-PCR analysis revealed that both chains of hemoglobin were expressed on day 0 and gradually decreased as the trans-differentiation initiated (Fig. 17).

Figure 17


A
α-chain
ß-chain
18S rRNA
D0
D1
D3
D5
D7

B

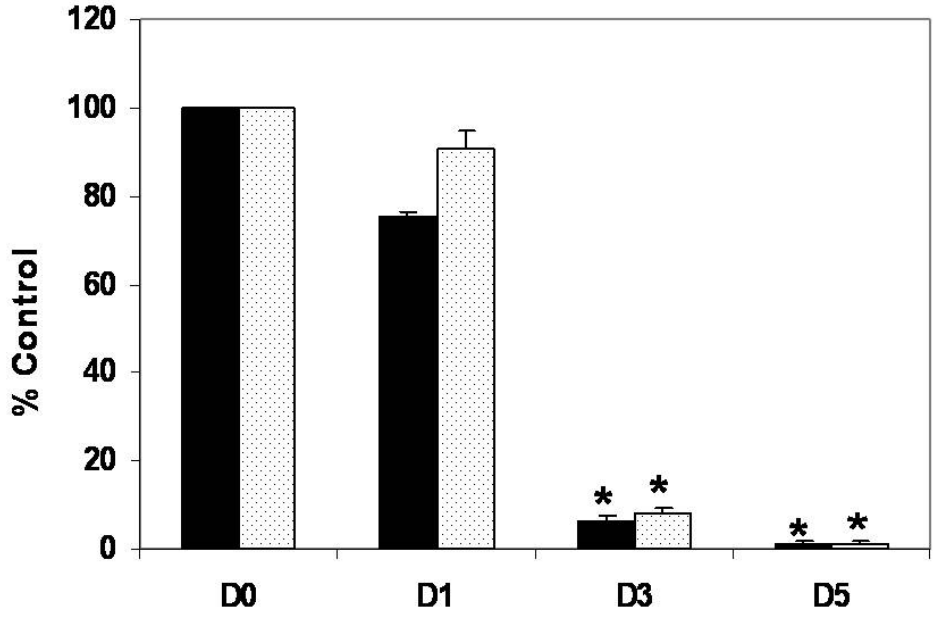

120
100
80
60
40
20
0
% Control
*
*
*
*
D0
D1
D3
D5

Figure 17 - The mRNA expression of hemoglobin during the trans-differentiation of AEC II into AEC I-like cells

Freshly isolated AEC II were seeded on plastic dishes in MEM medium containing 10 % FBS and cultured for 0 (D0), 1 day (D1), 3 days (D3) , 5 days (D5), and 7 days (D7). Total mRNAs was extracted from cultured cells and RT-PCR was performed to detect α-, and β-chains of hemoglobin. 18S rRNA was used as a control. Panel A, representative gels; and panel B, quantitation. The results were expressed as a percentage of control (D0). Data shown are means ± SE. *P<0.05 (n=3 independent cell preparations). Black bar: α-chain; dotted bar: β-chain

Hemoglobin is specially expressed in AEC II of a lung mixture cell population

To further examine the expression of hemoglobin at the protein level in AEC II, we isolated a population of mixed cells from the rat lung as described in the Materials and Methods, and cytospinned onto microscopic glass slides. This mixture of cells consisted of AEC I, AEC II, Clara cells, ciliated airway epithelial cells, fibroblasts, macrophages, and lymphocytes. LB-180, an AEC II marker, was used to identify AEC II in the mixture. The double labeling with anti-hemoglobin and anti-LB-180 antibodies revealed the co-localization of hemoglobin with LB-180 in AEC II, but not in other cells (Fig. 18a-d). The omission of primary antibodies did not generate any signals (Fig. 18e-h).

Figure 18

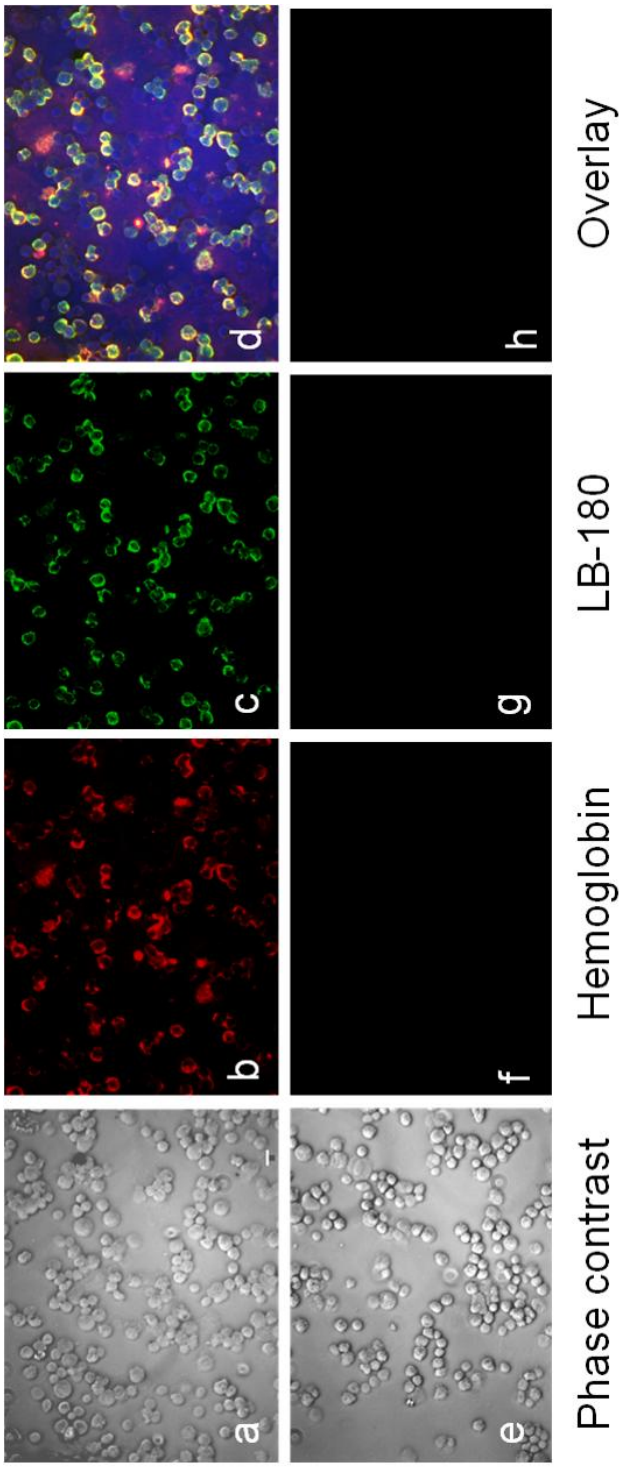


Figure 18 - Double-labeling of a lung cell mixture for LB-180 (an AEC II marker) and hemoglobin

A lung cell mixture was cytospinned onto microscopic glass slides. The slides were incubated with rabbit anti-hemoglobin and mouse anti-LB-180 antibodies, followed by incubation with Alexa 568-conjugated anti-mouse and Alexa 488-conjugated anti-rabbit IgG (Panels a-d). The negative control without primary antibodies was shown in Panels e-h. Scale bar: 5 μm.

Hemoglobin is localized in AEC II of the lung

The gene and protein expression could be potentially affected by cell isolation procedures. To avoid this problem, we directly immunostained rat lung tissue. The rat lung was perfused to remove red blood cells before fixation and sectioning. Once again, LB-180 was used as an AEC II marker. The results revealed that hemoglobin was localized in the corners of the alveoli, which were occupied by AEC II. The hemoglobin staining pattern was the same as LB-180 (Fig. 19 a-h). The control without primary antibodies did not have signals (Fig. 19i-l). The result indicated that hemoglobin protein was localized only in AEC II, but not in AEC I and other lung cell types in the normal rat lung. The endothelial cells did not show any staining for hemoglobin while the airway epithelium showed faint positive staining (data not shown).

Figure 19

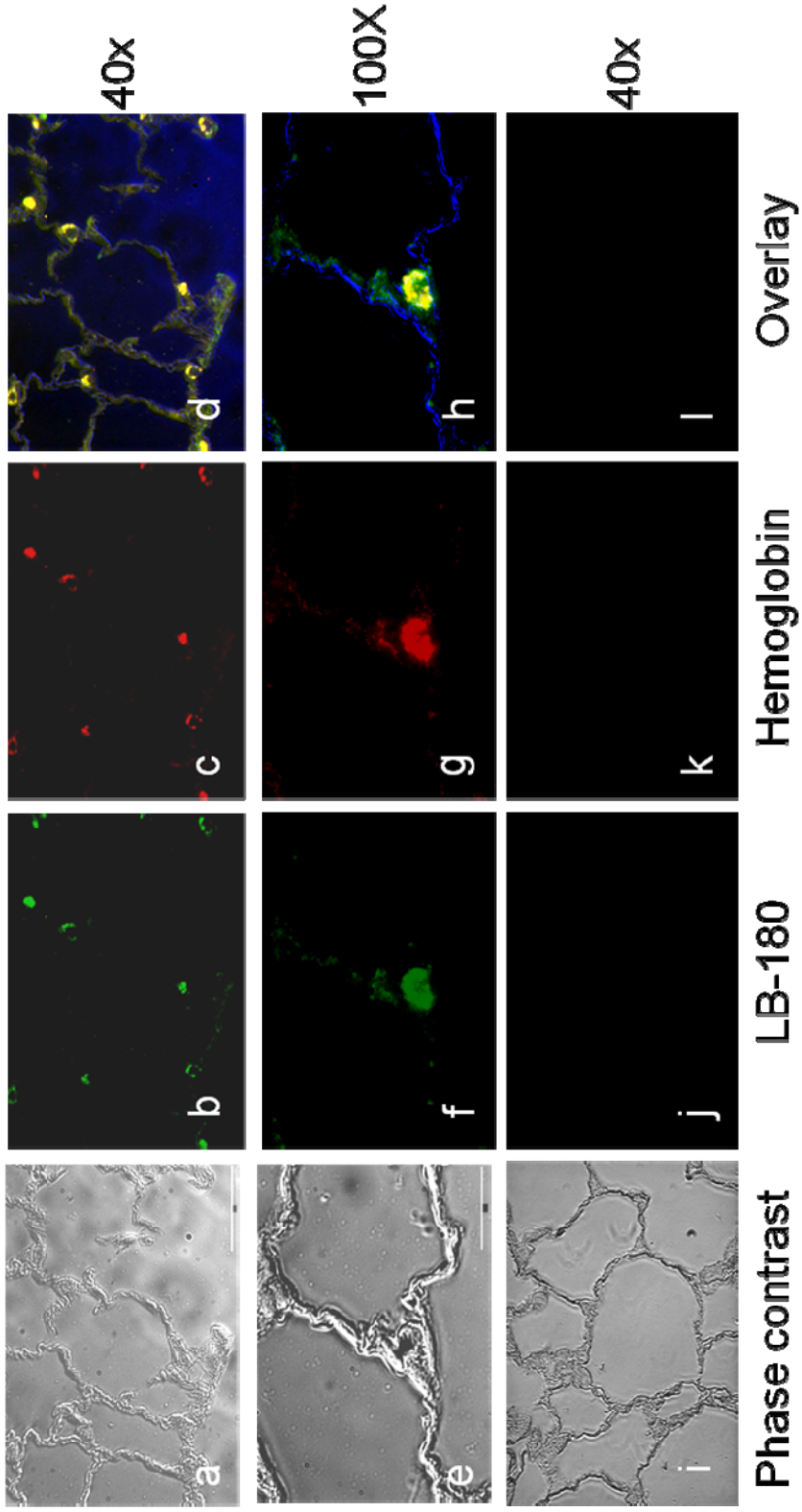

40x
100X
40x
Phase contrast
LB-180
Hemoglobin
Overlay
a
b
c
d
e
f
g
h
i
j
k
l

Figure 19 - Double-labeling of rat lung tissue sections for LB-180 (AEC II marker) and hemoglobin.

Rat lung tissue sections were incubated with rabbit anti-rat hemoglobin and mouse anti-LB -180 antibodies, followed by incubation with Alexa 568-conjugated anti-mouse or Alexa 488-conjugated anti-rabbit IgG (Panels a-h). The negative control was without primary antibodies (Panels i-l).

Scale bars: 20 μm for x40 and 8 μm for 100x

DISCUSSION

Aside from its most important and well-known function, oxygen transport, hemoglobin also has a variety of other functions. These functions include being a molecular heat transducer by virtue of its oxygenation-deoxygenation cycle, the alteration of red blood cell metabolism, hemoglobin oxidation, enzymatic activities, and drug interactions (Hardison, 1998). Since oxygen can be a damaging agent to the cells exposed to it, the oxygen binding hemoglobins may have a role in the protection of cells from the damage (Hardison, 1998). Hemoglobin is also thought to be involved in the protection of cells against nitrosative stress (Gardner et al., 1998; Hausladen et al., 1998). Recent studies have shown that hemoglobin can bind and release NO as a redox reaction, thus functioning in close resemblance to cytochromes (Gow and Stamler, 1998). Neuroglobin and cytoglobin, two other members of the vertebrate globin family, also have protective functions including oxygen sensing and scavenging (Burmester et al., 2002; Burmester and Hankeln, 2004; Pesce et al., 2002). Neuroglobin is predominantly expressed in the nervous system while cytoglobin is present in almost all tissues. Even though their amino

acid and gene sequences are distinct from each other, their functional structure has striking similarities. The related globins in fungi and bacteria are involved in electron transport and protection from oxidative stress (Hardison, 1998).

A long standing notion that hemoglobin gene can only be expressed in the cells of erythroid lineage has been challenged by a recent study, in which the treatment with lipopolysaccharide and interferon- led to the activation of the β globin gene in murine macrophages (Liu et al., 1999). In the current study, we provide evidence that hemoglobin was specially expressed in AEC II of the lung. Since AEC II are in close proximity to environmental air, blood circulation, and facing the conditions of altered gaseous environment around it, our finding may have an important functional significance. We speculated the following possible functions of hemoglobin in the lung: (i) facilitate oxygen transport across the air-blood barrier; (ii) behave as an oxygen sensor; and (iii) function as oxygen or nitric oxide scavenger and thus protect alveolar epithelium from oxidative/nitrosative stress. Further studies are needed to uncover un-identified functions of hemoglobin in the lung.

REFERENCES

1. Brunori, M. (2001). Nitric oxide, cytochrome-c oxidase and myoglobin. Trends Biochem. Sci. 26, 21-23.
2. Burmester, T., Ebner, B., Weich, B. and Hankeln, T. (2002). Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. Mol. Biol. Evol. 19, 416-421.
3. Burmester, T. and Hankeln, T. (2004). Neuroglobin: a respiratory protein of the nervous system. News Physiol Sci. 19:110-3., 110-113.

4. Chen, J. W., Chen, Z., Narasaraju, T., Jin, N. and Liu, L. (2004). Isolation of hightly pure alveolar epithelial type I and type II cells from rat lungs. Lab Invest 84, 727-735.

5. Chen, Z. and Liu, L. (2005). RealSpot: Software validating results from DNA microarray data analysis with spot images. Physiol Genomics DOI 10:1152/physiologenomics. 00236.2004.

6. Crandall, E. D. and Matthay, M. A. (2001). Alveolar epithelial transport. Basic science to clinical medicine. Am J Respir. Crit. Care Med. 163, 1021-1029.

7. Dobbs, L. G., Williams, M. C. and Brandt, A. E. (1985). Changes in biochemical characteristics and pattern of lectin binding of alveolar type II cells with time in culture. Biochim. Biophys. Acta 846, 155-166.

8. Edgar, A. J. and Polak, J. M. (2001). Flotillin-1: gene structure: cDNA cloning from human lung and the identification of alternative polyadenylation signals. Int. J. Biochem. Cell Biol. 33, 53-64.

9. Fehrenbach, H. (2001). Alveolar epithelial type II cell: defender of the alveolus revisited. Respir Res 2, 33-46.

10. Gardner, P. R., Gardner, A. M., Martin, L. A. and Salzman, A. L. (1998). Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. Proc. Natl. Acad. Sci. U. S. A 95, 10378-10383.

11. Gow, A. J. and Stamler, J. S. (1998). Reactions between nitric oxide and haemoglobin under physiological conditions. Nature 391, 169-173.

12. Hardison, R. (1998). Hemoglobins from bacteria to man: evolution of different patterns of gene expression. J. Exp. Biol. 201, 1099-1117.

13. Hausladen, A., Gow, A. J. and Stamler, J. S. (1998). Nitrosative stress: metabolic pathway involving the flavohemoglobin. Proc. Natl. Acad. Sci. U. S. A 95, 14100-14105.

14. Kalina, M., Mason, R. J. and Shannon, J. M. (1992). Surfactant protein C is expressed in alveolar type II cells but not in Clara cells of rat lung. Am J Respir. Cell Mol Biol 6, 594-600.

15. Liu, L., Zeng, M. and Stamler, J. S. (1999). Hemoglobin induction in mouse macrophages. Proc. Natl. Acad. Sci. U. S. A 96, 6643-6647.

16. Padro, T., van den Hoogen, C. M. and Emeis, J. J. (1990). Distribution of tissue-type plasminogen activator (activity and antigen) in rat tissues. Blood Coagul. Fibrinolysis 1, 601-608.

17. Pesce, A., Bolognesi, M., Bocedi, A., Ascenzi, P., Dewilde, S., Moens, L., Hankeln, T. and Burmester, T. (2002). Neuroglobin and cytoglobin. Fresh blood for the vertebrate globin family. EMBO Rep. 3, 1146-1151.

18. Warburton, D. and Bellusci, S. (2004). The molecular genetics of lung morphogenesis and injury repair. Paediatr. Respir. Rev. 5 Suppl A:S283-7., S283-S287.

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Contribution of co-authors

Haifeng Chen did the RTPCR for alpha and beta chains for hemoglobin. Zhongming Chen did the microarray for differentially expressed genes between AEC I and II

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CHAPTER VI

SUMMARY AND CONCLUSION

The first aim of the present study was to understand the role of TGF β1 and its down stream signaling components in alveolar epithelial trans-differentiation, a phenomenon seen in acute lung injuries. The second aim was to determine the expression profile and possible roles of microRNAs in fetal lung development and in a chronic lung disease of development called Bronchopulmonary dysplasia (BPD).

The expression of TGF β1 and the components of its signaling pathway was studied in an in vitro trans-differention model. Trans-differentiation was monitored by using cell type specific markers, LB-180 for AEC II and P2X7 for AEC I. We for the first time demonstrated that the in vitro trans-differentiation model was bi-phasic with an initial proliferation phase and a later differentiation phase. Even though TGF β1 and Smad 4 were highly expressed in normal AEC II cells, isolation and culturing initially reduced the expression of TGF β1. The initial proliferation phase had low expression of TGF β1 and its down stream effectors Smad 2 and Smad 3 including their phosphorylated forms. The expression of anti-proliferative $p15^{Ink4b}$ and $p21^{kip1}$ were also downregulated during the proliferative phase with a concurrent upregulation of pro-proliferative CDKs 2, 4 and 6. This expression profile was completely reversed during the differentiation phase. There was increased secretion of TGF β1 into the media during the differentiation phase indicating that the active signaling is autocrine in nature. We demonstrated that the

concurrent increase of both phosphorylated and unphosphorylated Smads 2 &3, $p15^{Ink4b}$ and $p21^{kip1}$ and decrease of CDKs 2, 4 and 6 were facilitated the transition from proliferation phase to differentiation phase. The addition of TGF β1 in the pre-proliferative phase blocked the trans-differentiation process. Moreover the addition of TGF β1after the proliferation phase did not have any effect. This demonstrated that the initial proliferation phase was necessary for the differentiation to take place. This view was further strengthened by the fact that blocking of TGF β1 by the antibody completely stopped the trans-differentiation process. To prove that TGF β1 is essential for trans-differentiation and the signaling is Smad-dependent, we knocked down the expression of common partner Smad 4 which is essential for Smad-mediated TGF β1 signaling. Silencing of Smad 4 resulted in blocking of trans-differentiation process. These results demonstrated that the trans-differentiation of AEC II to AEC I was mediated by autrocrine signaling through Smad-mediated TGF β1 pathway. The effect was elicited by altering the expression of proteins that controlled G1 to S phase entry in cell cycle.

Since miRNAs are important regulators of development and disease, the later part of this study has focused on the profiling of miRNAs during lung development and in a chronic lung disease named BPD. A miRNA microarray platform was used to profile miRNAs that are differentially expressed during fetal lung development. 21 microRNAs that showed significant changes during fetal lung development and were grouped into 4 distinct clusters based on their expression pattern. Using rtPCR, the expression pattern of 6 miRNAs that were representative of these clusters were verified. *In situ* hybridization demonstrated that miR-127 expression gradually shifted from mesenchymal cells to epithelial cells as development progressed. miR-127 was chosen for further functional

studies. Using an *in vitro* lung culture system, we overexpressed miR-127 in the developing lung. This significantly decreased the terminal bud count, increased terminal and internal bud sizes and caused unevenness in bud sizes, indicating improper development. These findings suggest that miR-127 may have an important role in fetal lung development.

For the BPD studies, we first established a rat model of the disease where the pathology corresponded to that described for 'new' BPD. Using the samples from this model miRNA microarray was done to see the miRNAs that showed significant change in BPD. miR-342, miR-335, miR-150, miR-126* and miR-151* were down regulated and miR-21, miR-141, miR-290 and miR-34a were up regulated in BPD. The expression profiles of eight of these miRNAs were confirmed using quantitative real-time PCR and their possible functional significance have been discussed.

In an effort to study the differential gene expression of alveolar epithelial type I and type II cells using DNA microarray technique, we found that hemoglobin was present in AEC II and not in AECI. Hemogobin is the main oxygen carrying component of erythrocytes. The presence of both α- and β-chains of hemoglobin was confirmed in type II cells, but not in type I cells. Their expression decreased as AEC II trans-differentiated to AECI. The protein form was also found to be specifically localized in AEC II both in adult lung and in a mixed cell population. This is the first study to our knowledge that has identified hemoglobin in a cell type other than erythrocytes. This study also points to the possibility that hemoglobin may have functions other than oxygen transport in the lung.

ADDENDUM

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Chapter II

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VITA

Manoj Bhaskaran

Candidate for the Degree of Candidate for the Degree of Doctor of Philosophy

Thesis: ALVEOLAR EPITHELIAL TRANS-DIFFERENTIATION, LUNG DEVELOPMENT AND DISEASE – ROLE OF TGF β1 AND MicroRNAs

Major Field: Veterinary Biomedical Sciences

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Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: ALVEOLAR EPITHELIAL TRANS-DIFFERENTIATION, LUNG DEVELOPMENT AND DISEASE – ROLE OF TGF β1 AND MicroRNAs

Pages in Study: 171 Candidate for the Degree of Doctor of Philosophy

Major Field: Veterinary Biomedical Sciences

Scope and Method of Study:
The present study was undertaken to understand the role of TGF β1 and its down-stream signaling components in alveolar epithelial trans-differentiation, a phenomenon seen in acute lung injuries. The second aim was to elucidate the expression profile and possible roles of microRNAs in fetal lung development and in a chronic lung disease of development called Bronchopulmonary dysplasia (BPD). An *in vitro* model for trans-differentiation of alveolar epithelial cells was used and expression of TGF β1 and multiple components of its pathway and the effect of blocking the pathway on the process were looked into. The microRNA expression profile during different time points in fetal lung development was characterized and confirmed. Effect of miR-127 over expression on lung branching and morphology in an *in vitro* organ culture system was visualized and quantified using morphometric analysis. The BPD model in neonatal rat was established and microRNAs that significantly changed during disease and their expression pattern was confirmed. We have used a number of techniques like immunohisto/cytochemistry, western blotting, ELISA, real time PCR, adeno virus-mediated RNA interference and over expression, microarray and lung morphometry to address these biological questions.

Findings and Conclusions:
1. The trans-differentiation of alveolar epithelial type 2 cells to type 1 was modulated by autocrine signaling through TGF β1 pathway and was Smad mediated.
2. TGF β1 elicited its effect by altering the expression levels of cell cycle proteins, CDKs2, 4 and 6 and $p15^{Ink4b}$ and $p21^{kip1.}$
3. Blocking the signaling through TGF β1 blocked the trans-differentiation process.
4. 21 microRNAs were identified that showed significant changes during fetal lung development and grouped into 4 distinct clusters based on their expression pattern.
5. miR-127 over expression significantly affected lung branching and morphogenesis implicating its role in fetal lung development.
6. A neonatal rat model for BPD was established.
7. 5 down-regulated and 4 up-regulated microRNAs were identified in BPD, providing an insight into the pathogenesis of BPD.

ADVISER'S APPROVAL: DR. LIN LIU _____